Concise Book of MEDICAL LABORATORY TECHNOLOGY

Methods & Interpretations

Second Edition

Ramnik Sood

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Methods and Interpretations

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2nd Edition

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Preface to the Second Edition

I authored an exhaustive book entitled "Medical Laboratory Technology – Methods and Interpretations" that hit the stands in mid-eighties in the previous century and is now running in its 6th edition. This was the first such book in the subcontinent and was much appreciated and used by technologists and pathologists alike. The book has seen testimonies in courts and has been appreciated in the West too. However, in our subcontinent, I was requested by many technologists that they wanted a little younger sister of the book popularly known as MLT authored by me.

And so was born the *Concise Book of Medical Laboratory Technology: Methods and Interpretations*. The book essentially covers everything presented in MLT-6 but in an abridged/shortened and easy-to-digest format. The book is presented in a flowing noninterrupted format and not in a cumbersome experiment-wise cascading flow. There is no break in the style that runs smoothly and is easier to absorb and assimilate. As experiments are a part of any course, more stress has been laid out in the book to understand the intricacies of relevant theories and even troubleshooting all experiments that you would conduct during the course of your study. As I have written multiple modules for many Universities in India, I did not have to think for too long to devise a style and format for this book. You will find everything from ESR to PCR and you will find foam test for bile pigments as also complete automation in urinalysis. You will find basic biochemistry as also detailed cytogenetics. So whatever be your course or query, you will find it within the covers of this short but sweet book now going into its second edition.

The book is designed for you not to mug but to understand and elicit the answers from the book of all questions in your mind. I am aware that this book is used by most of your teachers and tutors too.

Nothing wrong that you are holding now. It will help you all your life!

Ramnik Sood

Preface to the First Edition

The first book on Medical Laboratory Technology from the house of Jaypee's came out in 1985 and has been the best seller in its class till date. The title "Medical Laboratory Technology – Methods and Interpretations" has seen 6 editions. The latest one hit the stands in January this year and was released in two volumes. It is a four-color book and has over 1670 pages. The reader base of all editions of our vastly popular title "MLT" has been upcoming laboratorians, undergraduate and postgraduate medical students. It was requested by a few institutes to produce a little smaller version of the two-volume set that would be suitable for the upcoming Laboratory Technology students. So here it is! It is exhaustive yet precise and concise too.

The book will help you to appreciate things as they appear in real life under the microscope and otherwise. All **current technologies** find a mention within the covers of this book. Gone are the days when we had to prepare reagents first thing in the morning (or the days usage); therefore, the current trend of consuming ready-to-use reagents/kits is followed here to make your job and understanding simpler. So, what is available in the market for all investigations, is what is presented here. The Tulip Group has very kindly given us the rights to reproduce the text related to all their kits and reagents in this book. Latest **instruments** are not forgotten too.

Most important—

Parasitology section is presented in ample detail as it is relevant to all the developing nations.

Quality control/assurance is mentioned in appropriate details. Working is not enough. Working properly and producing nothing but the most accurate reports are the order of the day. This book will not fail you there. Follow the recommendations to the hilt and you would be running the most accurate laboratory available anywhere.

Should problems arise! The book has **"Troubleshooting"** section for every possible test mentioned inside. If this happens – then what! If that happens – then what! You will find all answers.

From ESR to PCR – you will find everything.

The book is based on most syllabi as applicable to most institutes in India and elsewhere internationally.

All necessary care has been taken to weed out any discrepancies/typographical errors at the time of going to press. However, if anything has remained inadvertently, the publishers/author do not take any responsibility for the same in any manner whatsoever.

Learning can be enjoyable experience, flip a few pages to experience that.

Ramnik Sood

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CHAPTER 1

Laboratory

INTRODUCTION

The definition of health includes a state of complete and perfect physical, mental, social and spiritual well-being and not just the absence of disease or infirmity and good health is a fundamental right of every living human being on earth. However, modern world, though, has to an extent eliminated infectious diseases. But the focus has now shifted to lifestyle diseases. Pollution of every nature too has taken its toll. About half a century back, the predominant diseases used to be infective ones but now you may find individuals in mid-twenties waiting for their turn for open heart surgeries. Also, modern medicine has increased the longevity of life accompanied by attendant geriatric diseases like Alzheimer's disease and malignancies. The polluted and toxic world has not spared the fetuses in utero and neonates. A new face of disease has emerged, diseases like HIV-AIDS and severe acute respiratory syndrome (SARS), are new entrants in the long list of infective diseases. We may have eradicated smallpox but tuberculosis and malaria have raised their heads with a vengeance. So, do what you might. Some forms of disease, mild or severe will strike every human being living. On getting sick, the patient first comes in contact with a clinician—medical or surgical. The clinician gives a patient hearing (if the patient is conscious) to his problems and symptoms and also takes note of various signs, which he sees or elicits. Sometimes, he may immediately arrive at a diagnosis and may under emergency circumstances institute treatment at first instances. In most cases, however, he will have a differential diagnosis in mind and to arrive at a specific diagnosis he usually orders for a battery of tests.

Various means of diagnosis are available.

1. *Most important*: Clinical laboratory tests which include any tissue or fluid obtained from the body.
2. *Imaging sciences*: X-rays, ultrasound, color Doppler, computerized axial tomography (CAT) scan, magnetic resonance imaging (MRI) scan and the latest positron emission tomography (PET) scan.
3. *Electrical signal processing techniques:* ECG, EMG, EEG and nerve transmission techniques, etc.
4. *Direct visualization techniques*: With the availability of fiberoptic-based technologies, the clinician is now capable of passing small tubes (called scopes) through natural passage ways of the human body (without actually surgically opening up the part), e.g. gastroscopy, cystoscopy, etc. These techniques, eventually culminate in taking small tissue samples (biopsies) which are sent to histopathology laboratories.

So, whenever, any sample from a human body is taken (either voided naturally or obtained by the clinician or the laboratorian), it is referred to the clinical laboratory for investigation. On receipt of a report from the laboratorian, the clinician, then, makes up his mind and starts a unidirectional or specific treatment against the disease thus diagnosed. It would not be wrong to designate medical laboratory personnel as the backbone of the clinicians. But, for these technologists, the clinicians would forever grope in the dark. Gone are the days when diabetes mellitus was presented with the classical triad of symptoms—increased thirst, hunger and urination; likewise, typhoid seldom presents with a step-ladder pattern fever. Blood testing is absolutely mandatory, to know that they exist, their severity and eventually, after treatment; to know that they are under control or cured. Investigations are diagnostic as well as prognostic tools.

Clinical laboratory investigations nowadays are being utilized as future predictors. On getting warning signals, one can take necessary corrective measures (lifestyle and/or dietary) and can prevent diseases from striking or at least deferring or postponing their arrival.

HELP! AND YOU DID AND YOU ALWAYS WILL. When a clinician is lost, you shall show him the way in the best possible way, you lead him to a diagnosis and let him do his job thereafter. He may come back to you later to determine that his efforts have been fruitful.

The following pages within the covers of this book will show you the right path on how to be an excellent laboratorian. Do your best in serving mankind. As you yourself may be a patient tomorrow. This book shall also serve you well by providing interpretation of the results obtained by you. This book shall be true to its title "Concise Book of Medical Laboratory Technology: Methods and Interpretations".

While *physiology* is the study of essentially normal structures and functions of a body, *pathology* deals with the study of a diseased organ or system of the body, its abnormal functions, their mode of origin, their progress to recovery or otherwise. All these studies come under the ambit of a clinical pathology laboratory. A clinical laboratory has further sub-branches such as: hematology, biochemistry, seroimmunology, microbiology, cytogenetics, histopathology, cytopathology, blood banking and last but not least—clinical microscopy.

A clinical laboratory can be manned by a qualified doctor specializing in clinical pathology, biochemistry, immunology, blood banking, histopathology, cytopathology, hematology, microbiology or cytogenetics. The pathologist is usually assisted by laboratory technicians or technologists (they are also qualified for the job) and lastly the cleaning and documentation staff. Only by collective efforts of the individuals mentioned above, a proper report can be generated. Be grateful to the clinician for having faith in you and give back nothing except an accurate and correct timely report. A delayed report may at times be too late. The patient may have lost his life by then. A timely correct report is the essence of running a good laboratory.

The cycle of health-disease with all intermediaries is given in Figure 1.1. Just as there are primary, secondary and tertiary health centers, there are also the primary, secondary and tertiary laboratories too. In India, there are no specific guidelines as to what or how much they can do and overlapping can occur. A superior laboratory may perform all functions of an inferior laboratory too.

Primary Laboratory

In rural setups, for instance, a primary laboratory may provide only the basic investigations. These investigations are simple to perform and do not involve expensive machinery usage. Such laboratories are also attached to

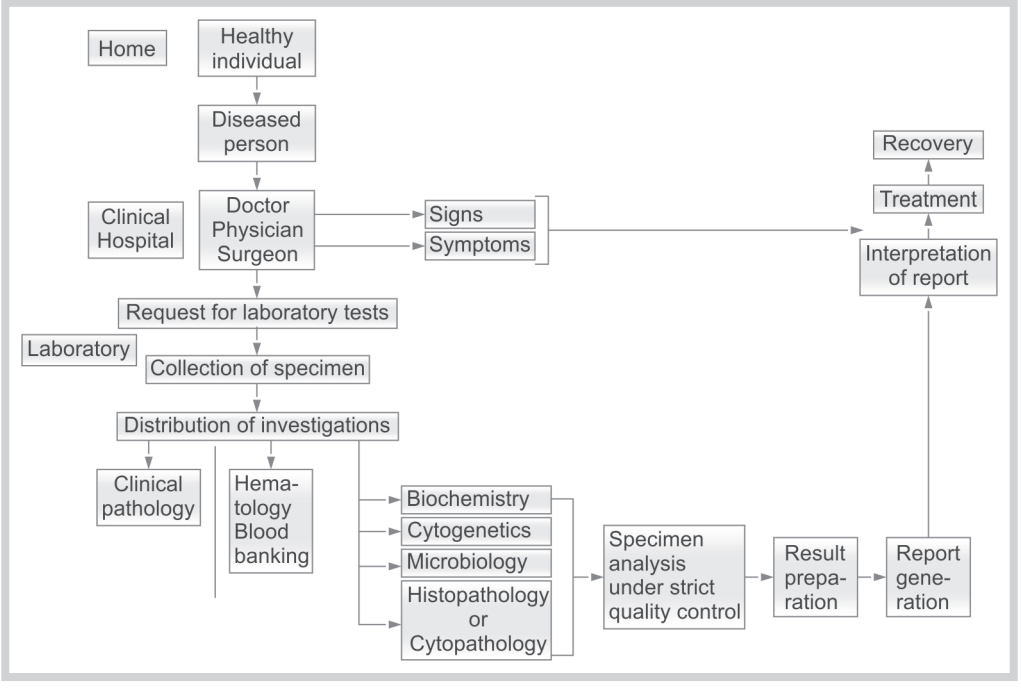


FIG. 1.1: Health-disease-health cycle

physician chambers nowadays, so that clinicians may obtain basic inputs right in their own premises. These primary laboratories may provide the following simple investigations:

- Hemograms (hemoglobin estimation, total and differential counts, erythrocyte sedimentation rate and packed cell volume with basic peripheral smear study including the reporting of hemoparasites)
- Routine and microscopic studies of urine and stool. Routine examination also entails chemical examination either by laborious and time-consuming old chemical methods or by new generation dipstick tests. These may include tests for glucose, bilirubin, ketones, hemoglobin, leukocytes, pH, nitrites, protein, urobilinogen and specific gravity in case of urine. For stool samples, reducing substances, pH and occult blood may be performed. Basic spot/latex/device tests (e.g. pregnancy test) may be conducted.

Secondary Laboratory

These are laboratories that assist a clinician to confirm a clinical suspicion or establish a diagnosis. Therapy and prognosis monitoring can also be provided from these laboratories. Such laboratories are staffed by qualified personnel who are trained and experienced to perform the tests. They also have a perfect knowledge of the equipment and machines they use. They should be aware of quality control essentials and be well versed with interpretational aspects of the reports generated by their laboratories. In addition to what has been mentioned under primary laboratories, secondary laboratories also perform:

- Routine immunohematological tests.
- Routine examination of all body fluids, e.g. semen, cerebrospinal fluid (CSF), sputum, etc.
- Routine bacteriologic studies including stains, cultures and antibiograms. Routine mycological investigations would include—primary cultures, isolation and identification techniques along with microscopic evaluation.
- Routine immunoserological tests. These can include tests like Widal, STS, ELISA or strip or device tests HIV I and II, hepatitis B and hepatitis C, etc.
- Routine biochemistry investigation and organ profile tests, e.g. lipid, cardiac, liver and renal profiles.
- Under hematology, these laboratories may also provide RBC indices, platelet, reticulocyte count and absolute eosinophil counts. They can also classify anemias and should be able to indicate hematologic malignancies. When headed by a pathologist, they should be in a position to report bone marrow smears/preparation too.

Tertiary Laboratory

These kinds of laboratories should be able to perform all kinds of sophisticated and delicate/precise investigations. The tertiary laboratories can branch out in very special fields and not cater to all aspects of specialized tests. Besides doing all investigations that are conducted in secondary laboratories, they also carry out the following:

- Specialized hematological (e.g. leukemia type), coagulation profiles and immunohematological investigations. They are equipped with 18 parameter cell counters with differentials and flow cytometry
- Complete biochemical assays, commonly referred to as SMA-12, SMA 27, etc. Also included are elemental assays, e.g. zinc, magnesium, iron, total iron binding capacity (TIBC), lithium, etc. special enzymes like HBDH, lipase and isoenzymes, etc.
- Complete immunology based assays for hormones, cancer markers, hepatitis markers, rheumatic/autoimmunity etiology-based profiles, TORCH profiles, rare infectious diseases (e.g. brucellosis leptospirosis, cysticercosis, echinococcosis, etc.)
- All microbiological processes, e.g. cultures—aerobic, anaerobic, fungal, tubercular, etc. with antibiograms.

The techniques for these investigations may vary. They may be ELISA, chemiluminescence, turbidimetry, PCR, etc. These laboratories are totally automated and have sizable workload. Furthermore, they also undertake all histopathology (simple H and E, special staining techniques, immunohistochemistry methods) and cytopathology processing and reportings. They may also undertake cytogenetic investigations, e.g. chromosomal analysis. The dissemination of reports from these laboratories is in keeping with recent trends in telecommunications, e.g. fax, e-mail, etc.

In the United States of America, these laboratories though classified differently (with a few differences) are covered under the Clinical Laboratory Improvement Act (CLIA) of 1988.

LABORATORY SET-UP

Unless the laboratory is hygienic and provides necessary physical and operative comfort, it would be wrong to expect perfect results. To get perfect results, one has to provide a perfect set-up for people to work in.

Laboratory Building and Space

Ample working space is absolutely essential. For smaller laboratories up to 25 square meters (Fig. 1.2), the working platforms can be arranged along the walls while the central area is kept free for movement.

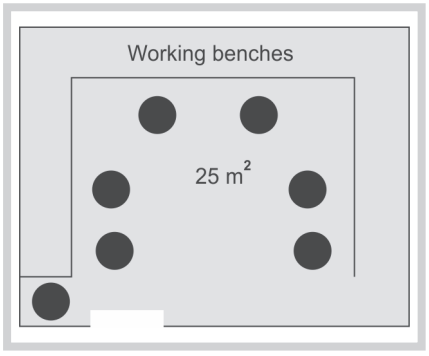


FIG. 1.2: A typical small laboratory

For larger areas, partitions can be made which would create separate spaces for different sections (Fig. 1.3). The chief pathologist must have casual access to all sub-units of the laboratory. If possible, he should be able to directly see into the cabins either through glass windows or through closed circuit cameras. In the cabins again, the central region should be kept free and benches be placed against the walls and away from the doors.

- Hygiene is of utmost importance. The whole facility should be absolutely clean, uncrowded and devoid of any hindrances to movement of men and materials. Never, should a chance arise where two people would clash or contaminated material would be spilt all over
- Scratch proof matt finish vitrified floor (slip resistant) should be provided. The walls should preferably have white ceramic tiles. Such provisions are resistant to chemicals and disinfectants
- All benches should be preferably 2½ feet high and those to be used while standing should be at least 3 feet high. The bench surfaces should be solvent and acid proof.

Every laboratory and/or its section must have at least one sink and one hand wash basin. The hand wash basin should not be used for any other purpose, the sink can be utilized for laboratory purposes like washing off stains from slides or washing glassware or discharging non-contaminated laboratory refuse.

Sero-immunology ELISA's, PCRs, drugs, Cancer markers	Biochemistry	Microbiology
Pathologist's chamber	Collection of specimens and report delivery	Hematology + Clinical pathology
Histopathology Cytopathology		Toilet

FIG.1.3: A typical large/complete laboratory plan

Physical Aspects of a Laboratory

- The ambient temperature should be within the comfort zone of a human body. It should between 21 and 27°C. If the laboratory is in a cold zone, it must have heating provision, and conversely, if it is in a hot zone, it must have cooling or air conditioning. The environment control appliances like air conditioners or heaters must not directly discharge air at the working bench zone
- A good exhaust system is a must for all laboratories. This removes dirty air (aerosols), which may at times be foul smelling. The sample collection zone too, must have excellent exhaust provision
- Adequate ventilation is also essential but without strong currents of air
- Lighting should be more than adequate and places where very delicate or fine processes are being conducted should have additional lighting provision. As far as possible, do not use excessive heat producing bulbs and lamps. The new CFLs are ideal
- Windows that are exposed to bright sunlight can be internally fitted with reflective films or blinds
- There should be sufficient running water for the laboratory and all must have sufficient number of sinks and hand wash basins
- As most machines consume a lot of electricity, sufficient power load (a little in excess) must be available to the laboratory

Provisions and Precautions

Every working room or cabin should have adequately spaced provision of water, electricity, gas, sinks lighting and exhausts. All aspects, whether plumbing, electrical systems or gas connection must pass through regular inspections and a log book should be maintained of such preventive exercises. Preventive maintenance should be carried out by knowledgeable and qualified persons.

Fire Prevention

- Install appropriate fire extinguishing system and timely testing of such a system be conducted at regular intervals
- Color code and place firefighting equipment at an easily visible and reachable location. Check the working capability of all such systems at regular intervals
- Provide adequate ventilation in zones where flammable chemicals are used. Before these substances reach combustible or explosive concentration, they should be removed by mechanical exhausts

- Post "No-smoking" signs in zones where smoking can be hazardous
- Lastly, mark clearly the emergency exit points. Keep the emergency exit route free from obstructions.

Electrical Installations

- Hire a proper, qualified electrical engineer and explain to him the purpose of the premises being taken. As far as possible, all points where sparks can be generated should be kept out of room/cabins where explosive chemicals are likely to be used
- Use earthing everywhere and install fire-resistant cables in the laboratory
- Employ only certified products
- Use one electrical socket for a single device or machine. Overloading is usually the cause of accidents.

Liquefied and Compressed Gases

- Color code and identify each gas container. Check their valves regularly
- Keep all such cylinders away from sources of heat and electrical sparks
- When not in use, replace protection/safety caps back on the cylinder mouths.

Chemicals and Radioactive Substances

- Label all bottles with proper names of contents and affix warning signs and symbols as applicable to them
- Clearly display the warning charts (both chemical and radioactive) next to such containers. All staff members working in such areas should be well trained to handle accidents of any kind that can happen
- A stringent record of stocks should be maintained of all persons and radioactive substances being used in the laboratory. A bottle lost or stolen is invitation to problem.

Stores

- Every bottle/container should be labeled. Affix the hazard intensity on the bottle or the container
- Ensure in every possible way that the containers cannot under any circumstances fall or spill. This can be done by placing the most dangerous chemical at the bottom or at the floor level
- Proper ventilation should be ensured in storage zones that house flammable chemicals. Keep fire extinguishing equipment handy. Post "No smoking" signs that are clearly visible. Make sure that the place remains free from pests.

Staff Safety and Facilities

The most important asset of any institution is the man-power that works for it. It holds true for laboratories too. Absence of staff due to morbidity or mortality can stifle your working capacity, capability and reputation. Provide adequate facilities to your team. (Designate a room or space meant exclusively for retiring or resting and consuming foodstuffs).

- Hot and cold running water with soap and disinfectants should always be provided. Clean hand towels should be replaced daily
- A clean toilet for use by staff members is mandatory as are the changing rooms. If possible, separate units for male and female members should be provided
- Biomedical wastes and non-biomedical wastes should be discarded properly and safely. Chemical treatment of liquid wastes and incineration of solid wastes should not be overlooked. Wastes handled properly ensures good health of your working team
- Designate a room or space meant exclusively for retiring or resting and consuming foodstuffs. Under no circumstances, laboratorians should eat or drink on their workbenches. Provide safe drinking water to all
- Each room/cabin must have a first-aid box kept at an identified place that is easily accessible. Every person working in the laboratory must be aware of all hazards that exist and must also know about the remedial measures that should be taken if something happens. What can be managed in house should be managed, when required, assistance of other specialists must be taken. Contact numbers of such institutions/specialists must be displayed prominently
- All members of your team must be immunized as relevant to the laboratory work. Make sure no single person works alone in a room or cabin. Two compatible persons should work together always.

Basic Laboratory Safety

- Use only certified safe equipment in the laboratory
- Decontaminate all equipment regularly and before their servicing or maintenance, use appropriate disinfectants correctly
- As far as possible, use disposable plasticware to avoid contamination (chemical, biological, etc.) and breakages with ensuing dangers
- Regularly test and service biological safety cabinets and fume cupboards.

Appropriate safety measures taken by you will go a long way in enhancing productivity.

As a rule, the place for receiving or withdrawing the specimens should be separate from the working compartment. To avoid specimen mixing (hazardous), each sample should be carefully labeled. The label should clearly mention the alloted specimen number, the date and time of receipt of specimen, the investigations to be done and most important the name of the patient.

Both, the clinical and the paraclinical workers are equally at risk of acquiring transmissible diseases through the patient or through the test samples. The risk of these can be lessened by taking appropriate vaccinations. In addition, one should attend to one's general hygiene and prevent fomite transmission of any infectious disease. Disinfect the working benches and as far as possible autoclave (or chemically disinfect) various glassware used in the laboratory. Use a rubber teat for sucking/filling the pipettes. To avoid strain on the eyes, keep both eyes open while doing microscopic work. Before leaving the laboratory, one should thoroughly wash one's hands with soap and water, and then rinse them well in a disinfectant lotion.

CODE OF CONDUCT FOR MEDICAL LABORATORY PERSONNEL

1. Place the well-being and service of the sick above your own interests.
2. Be loyal to your medical laboratory profession by maintaining high standards of work and strive to improve your professional knowledge.
3. Work scientifically and with complete honesty.
4. Do not misuse your professional skills or knowledge for personal gain.
5. Never take anything from your place of work that does not belong to you.
6. Do not disclose to a patient or any unauthorized person the result of your investigations.
7. Treat with utmost confidentiality and personal information that you may learn about a patient.
8. Respect and work in harmony with the other members of your hospital staff or health center team.
9. Be at all times courteous, patient, and considerate to the sick and their relations.
10. Promote health care and the prevention and control of disease.
11. Follow safety procedures and know how to apply first aid.
12. Do not drink alcohol during laboratory working hours or when on emergency stand-by.
13. Use equipment and laboratory-ware correctly and with care.
14. Do not waste reagents or other laboratory supplies.
15. Fulfil reliably and completely the terms and conditions of your employment.

Always remember that you can be a patient tomorrow. Treat others as you would want them to treat you.

ACCIDENTS

Safety Measures in the Laboratory

You must remain alert and cautious while working in the laboratory. You must know that careless handling of reagents, glassware or specimens to be tested in the laboratory can cause serious injury and is dangerous to life.

Hazards in the Clinical Laboratory

Clinical laboratory workers may encounter three types of hazards:

1. Physical,
2. Chemical, and
3. Biological hazards.

Physical Hazards

Physical hazards are present in ordinary equipment or surroundings. Electrical equipment, open flames, laboratory instruments and glassware can all be hazardous if improperly used.

Electricity

- All electrical equipment must be properly grounded following the manufacturer's instructions
- Even minor repairs, such as replacement of the microscope bulbs, require that instrument be disconnected from the power supply before the work is begun
- All electrical cords and plugs be kept in good shape and order with no frayed cords or exposed wires
- Avoid overloaded circuits
- Extension cords present several safety hazards and should not be used except in emergency.

Fire

Fire is a potential danger in the workplace:♥

- Though rare, they can occur when open flames are used in the vicinity of flammable liquids
- Make sure that loose clothing and long hair do not catch fire
- Instead of open flames, use hot plates, microwave ovens, electric incinerators and slide warmers
- Store flammable chemicals in a flameproof cabinet, away from heat sources and well-ventilated area. A flameproof cabinet can protect flammable chemical

from flames until firefighters arrive and also allow workers more time to escape
- All laboratory workers must know about the escape route and procedure to follow if that exit is blocked
- All workers must know the location of fire extinguishers and how to use them
- Inspect all fire extinguishers periodically and log the date of inspection.

Usual Causes of Fire in the Laboratory
- Naked flames (do not work with loose clothing and long hair near naked flames). Naked flames can also ignite flammable liquids and gases
- Electrical overloading. Use one socket for one equipment only. Do not operate a 15 amp equipment from a 5 amp socket
- Poor electrical maintenance. No frayed or open/exposed wires be ever used
- Leaving equipment switched when not in use. Out of sight is out of mind
- Deteriorated gas tubing. Leakage of gas is an open invitation to fire hazard. If you suspect gas leakage, do not operate any electrical equipment (do not ever switch on a light or a fan)
- Smoking in the laboratory
- Misusing matches. Use carbonized matches as far as possible
- Storing flammable and explosive chemicals in an ordinary refrigerator.

When a Fire Occur

- For tiny blazes; water, sand and a fire blanket can be employed to put out the fire. For larger blaze, a fire extinguisher can be used
- Never use water on an electrical fire or one caused by organic solvents (ether, alcohol, petrol, etc.). For electrical fires, use carbon dioxide fire extinguisher. For organic solvents, use sand or halon
- Escape via the fire exit route. Stay close to the floor, cover your mouth and nose with a damp cloth to filter out some of the harmful fumes
- Inform firefighting department of your area if you feel the fire can go out of hand. Medium to large fires should be reported irrespective of your preparedness to handle them.

Laboratory Equipment (Table 1.1)
- Use all laboratory equipment as per manufacturer's recommendation
- Any instrument with moving parts, such as a centrifuge, must be operated with a special regard for safety. Latch, the lid before turning it on. On turning it off, do not open the lid before it has come to a complete stop
- Autoclaves present special hazards. Strictly adhere to manufacturer's instruction to prevent explosions and burns. Use insulated gloves while removing hot items from the autoclave.

Glassware
- Use glassware that is free of chips and cracks. Damaged glassware is weakened and may break, resulting in injury
- Broken glass should be cleaned with a brush and dustpan and not with bare hands
- Glass should not be discarded into regular trashcans, but into rigid cardboard or plastic containers
- Wherever possible, replace glassware with plasticware.

Equipment Related Hazards
- Hypodermic needles: Accidental inoculation, aerosol or spillage
- Centrifuges: Aerosols, splashing and tube breakages
- Culture stirrers, shakers, agitators: Aerosols, splashing and spillage
- Refrigeration: If flammable chemicals are stored within them, the light switches, thermostats, etc. can provide sparks to ignite them
- Water baths: Provide ground for microorganismal growth

(The risk of acquiring hepatitis B from a needle stick is 30%, hepatitis C is 2 to 10% and HIV is 0.3%).

Equipment/Materials Employed to Eliminate/Reduce Hazards
- Laboratory apron: Assists in diminishing skin contacts to a certain extent

TABLE 1.1: Fire fighting equipment

Fire fighting material	*Used for*	*Contraindicated for*
Fire blanket	Clothing fire, G small blaze	Electrical fires, flammable liquids, a small blaze burning metals, alkali metal
Water	Paper, wood, fabric	Electrical fires, flammable liquids, burning metal, alkali metal
CO_2 fire extinguisher	Flammable liquids and gases, electrical fire	—
Dry powder	As above	—
Foam	Flammable liquids	—
Halon spray	All kinds of fires	—

- Biological safety cabinets: Prevent dangers arising out of aerosols and splatters
- Splatter shields: Provide protection from splatter of specimen and chemicals
- Pipetting aids (teat or electromechanical devices). Prevent from hazards arising out of mouth pipetting
- Goggles: Protect eyes from impacts and splashes
- Face shields: Protect the face from impacts and splashes.

Safety with Chemicals/Reagents

Excepting just a couple of reagents, almost all chemicals/reagents used even in the most basic laboratory are lethal poisons if consumed by anyone. Even if they are splashed on the skin/eye, they can cause irreversible damage. There is an appropriate way of handling and storage of hazardous chemicals to avoid injury and damage to self and others. In our country (and other tropical nations), excessive heat can decompose many chemicals, cause explosions, or lead to the formation of toxic fumes.

Labeling of Hazardous Reagents/Chemicals

At appropriate places, display the prohibition signs; and on all dangerous reagents or chemicals, stick *Hazard* warning symbols. In the following pages, important signs and symbols as related to safety in the laboratory are given.

Incompatible Chemicals

Fair number of common laboratory chemicals react dangerously if they come in contact with specific chemicals. Ensure that you keep such chemicals away from each other. A few examples are listed below:

Acids

- Acetic acid with chromic acid, nitric acid, hydroxyl compounds, ethylene glycol, peroxides and permanganates
- Chromic acid—with acetic acid, alcohol, glycerol and other flammable liquids
- Sulfuric acid—with chlorates, perchlorates, permanganates and water.

Vaporizing Substances

- Acetone—with sulfuric acid and nitric acid
- Flammable liquids—with chromic acid, hydrogen peroxide, nitric acid, ammonium nitrate and halogens.

Others

- Alkali metals, e.g. calcium, potassium, sodium (these form hydroxides on coming in contact with water) and with other chlorinated hydrocarbons
- Chlorine—with ammonia, hydrogen, benzene and other finely divided metals
- Copper—with azides, hydrogen peroxide and acetylene
- Cyanides—with all acids and alkalies
- Hydrogen peroxide—with copper, iron, chromium and most other metals
- Iodine—with acetylene and ammonia
- Sodium azide—with lead, copper and other metals.

Flammable Chemicals

These include ether, xylene, toluene, methanol, ethanol, glacial acetic acid, acetic acid, acetone, acetic anhydride, alcoholic Romanowsky stains and acid alcohol, etc.

Storage

These should be stored in a fire-proof metal box at ground level, preferably in a cool store. A container well lined with tin foil can also be used. Store only small quantities of such solvents on the shelves.

Safe Use

Ensure that there is no open flame nearby while opening a bottle containing flammable solvent. Nearest flame should be at least 10 feet away. Never heat a flammable liquid over any flame. Use a water bath or electric hot plate.

Control of Fire Caused by Flammable Chemicals

Best controlled by smothering them. Use sand, thick blanket or the now available multipurpose fire extinguishers. Pouring water on such fires will spread them. Every laboratory should be equipped with the commercially available fire extinguishers. If these are not available, there should be sand buckets in accessible places.

Corrosive Chemicals

These include strong acids, e.g. concentrated sulfuric acid, hydrochloric acid, nitric acid, glacial acetic acid, trichloroacetic acid, orthophosphoric acid, and strong alkalies like sodium hydroxide and potassium hydroxide.

Storage

Store these at low levels.

Safe Use

Never attempt mouth pipetting. Accidental swallowing can be lethal as these chemicals cause destruction of living tissue. Always pour a corrosive chemical at below eye level, slowly, and with great care to avoid splashing. Wear protective eye glasses/eye shields while opening such containers. Always add the corrosive substance to water and that too slowly. The addition of small amount of water to sulfuric acid is enough to produce sufficient heat to break a glass container.

Toxic, Harmful, and Irritating Chemicals

These are chemicals that can cause death or serious ill-health if swallowed or inhaled or if they come in contact with skin. Examples are potassium cyanide, mercuric nitrate, sodium azide, sodium nitroprusside, formaldehyde solution, chloroform, barium chloride and methanol. Iodine and sulfuric acid also fall in this category. Skin and mucous membrane irritants are xylene, formaldehyde and ammonia vapors.

Storage

Store highly toxic chemicals, e.g. potassium cyanide in a locked cupboard. Stock solutions should also be stored safely in a cupboard, not on an open shelf.

Safe Use

Always wear protective gloves and after working with them immediately lock them up. Always wash your hands after using a toxic or harmful chemical. Keep fume forming chemicals in a fume cupboard. Never mouth pipette them.

Oxidizing Chemicals

These include chlorates, perchlorates, strong peroxides, potassium dichromate, and chromic acid.

Storage

Keep these away from organic materials and reducing agents. They can produce much heat when in contact with other chemicals, especially flammable chemicals.

SIGNS FOR MEDICAL LABORATORIES

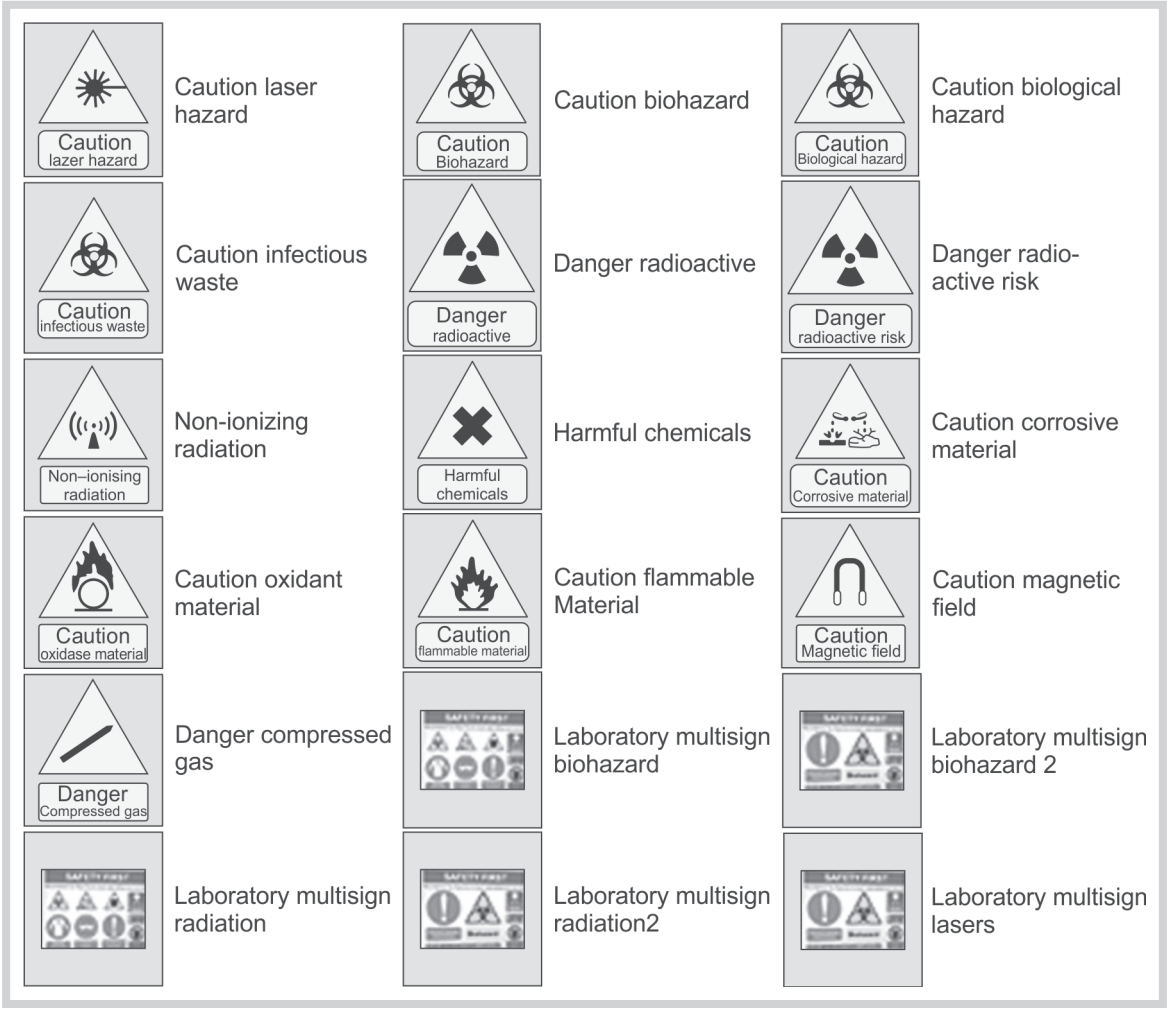


FIG. : General laboratory

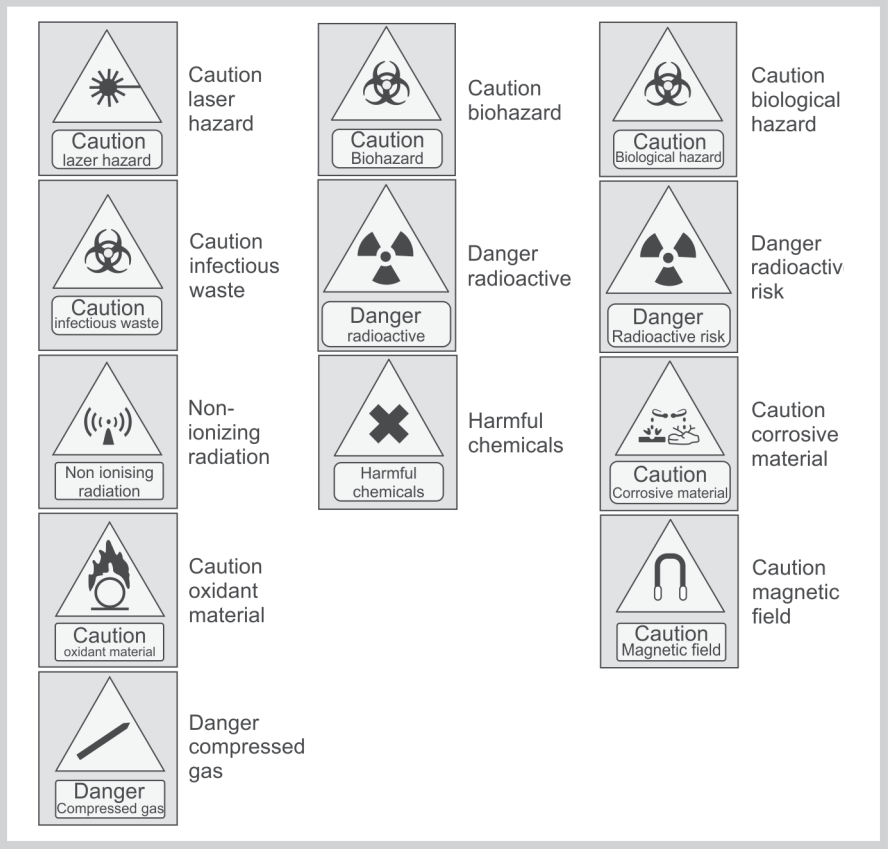


FIG. : Laboratory cautionary

Safe Use

Handle them with utmost care. Most of them are dangerous to skin and eyes and when in contact with reducing agents.

Explosive Chemicals

These chemicals can explode on being heated or on getting exposed to flame or friction. A good example is picric acid, which must be stored under water. If picric acid is allowed to dry, it can explode.

Carcinogens

These chemicals can cause cancer by ingestion, inhalation, or by skin contact. Such chemicals include benzidine, O-toluidine, O-dianisidine, α and β naphthylamine, nitrosamines, nitrosophenols, nitronaphthalenes, and selenite. The carcinogenic risk is directly proportional to the length and frequency of exposure and the concentration of the chemical.

Storage

Label their containers "CARCINOGENIC" and handle with special precautions.

Safe Use

Must wear protective plastic or rubber gloves, a facemask and eyeshields when handling carcinogenic chemicals. Do not let them come in contact with skin. After handling a carcinogen, wash well in cold water all the apparatus, bench, bottles and protective gloves (before removing them) and change your overall. Rinse your hands in cold running water before using soap. Should a carcinogen come in contact with skin, wash the affected part in cold running water for 5 minutes.

ACCIDENTS IN THE LABORATORY

They may be caused by:

1. Acids or Alkalis
   - Splashes on the skin
   - Splashes in the eye
   - Swallowing
2. Toxic substances
3. Heat
   - Open flames
   - Hot liquids
   - Inflammable liquids
   - Explosions

FIG. : General prohibition

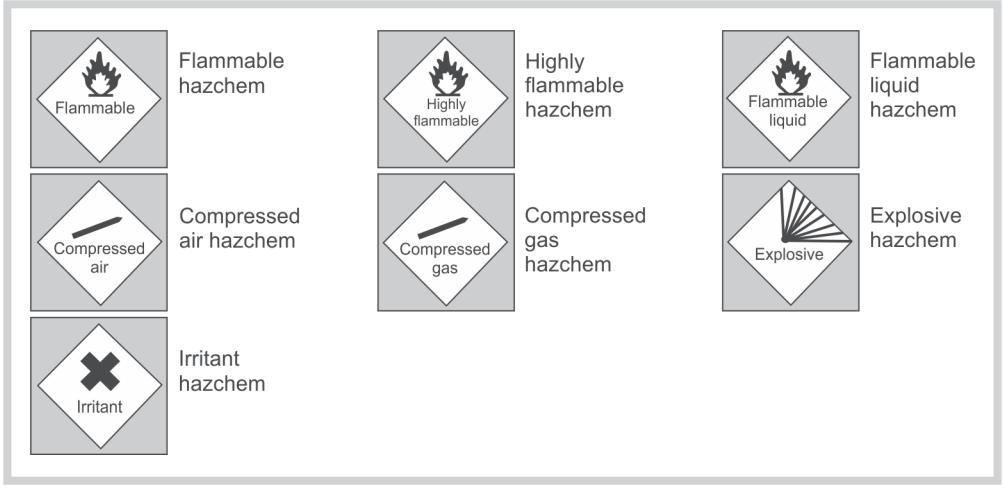


FIG. : General laboratory

5. Broken glass
6. Contamination by infected material
7. Electric shock.

A suggested list of first aid equipment is given later in the chapter. The items should be readily available in the laboratory. They must not be kept in a locked cupboard.

First Aid in Laboratory Accidents

Acid Burns

Nitric, sulfuric, hydrochloric and trichloroacetic acids.
In all cases: Wash immediately with large quantities of water.

Acid Splashes on the Skin

a. Wash thoroughly and repeatedly with water.
b. Bathe the affected skin with cotton wool soaked in 5% aqueous sodium carbonate.

Acid Splashes in the Eye

a. Wash the eye immediately with large quantities of water sprayed from a wash bottle or rubber bulb. Squirt the water into the corner of the eye near the nose (Figs 1.4 and 1.5).
b. After washing, put 4 drops of 2% aqueous sodium bicarbonate into the eye.
c. Refer the patient to a physician. Continue to apply bicarbonate solution to the eye while waiting for the doctor. Alternatively, hold the eye under the running tap.

Swallowing Acids

Accidental swallowing while using a pipette:

a. Call a physician.
b. Make the patient drink some 5% soap solution immediately. Alternatively, give him two whites of egg mixed with 500 mL of water or milk. If neither of these is available, he should drink ordinary water.
c. Make him gargle with the soap solution.
d. Give him 3 or 4 glasses of ordinary water.
e. If the lips and tongue are burned by the acid:
   - Rinse thoroughly with water
   - Bathe with 2% aqueous sodium bicarbonate.

Alkali Burns

Sodium, potassium and ammonium hydroxide.
In all cases: Wash immediately with large quantities of water.
Important: Alkali burns are as serious as, and often more serious than, acid burns.

Alkali Splashes on the Skin

a. Wash thoroughly and repeatedly with water.
b. Bathe the affected skin with cotton soaked in 5% acetic acid (or undiluted vinegar).

FIG. 1.4: Eye washing upright

FIG. 1.5: Eye wash lying

Alkali Splashes in the Eye

a. Wash immediately with large quantities of water sprayed from a wash bottle or rubber bulb. Squirt the water into the corner of the eye near the nose.
b. After washing with water, wash the eye with a saturated solution of boric acid (apply drops repeatedly).
c. Refer the patient to a physician at once.

Swallowing Alkalis

Accidental swallowing while using a pipette:

a. Send for a physician.
b. Make the patient drink at once:

- A 5% solution of acetic acid or lemon juice or dilute vinegar (1 part vinegar to 3 parts water).

c. Make him gargle with the same acid solution.
d. Give him 3 or 4 glasses of ordinary water.
e. If the lips and tongue are burned by the alkali:
   - Rinse thoroughly with water
   - Bathe with 5% acetic acid.

Poisoning

This can be caused by:

- Inhaling toxic vapors or gases (e.g. chloroform)
- Accidental swallowing while pipetting a poisonous solution.

In all cases

a. Send for a physician or qualified nurse, specifying the toxic substance involved
b. Place the victim in the open air while waiting for the physician.

Burns Caused by Heat

They fall into two categories:

- Severe burns—affecting large areas of skin, e.g. burns caused when burning ether or boiling water is spilled over the victim
- Minor burns—affecting a small area of skin, e.g. burns caused by hot glassware or a Bunsen flame.

Severe Burns

a. If the victim is on fire, e.g. if splashed with burning ether or other inflammable solvent, roll him in a blanket or overall to smother the flames.
b. Inform the physician on duty immediately.
c. Lay the victim on the ground. Do not remove his clothing. Cover him if he is cold.
d. Do not apply any treatment to the burns. This must be left to the physician.

Minor Burns

a. Plunge the affected part into cold water or ice-water to soothe the pain.
b. Apply mercurochrome or acriflavine ointment to the burn.
c. Apply a dry gauze dressing loosely.
d. If the burn becomes infected or does not heal, refer the patient to a physician.

Note: Never tear off the blisters that form over the burns.

Injuries Caused by Broken Glass

These are caused by broken test tubes, syringes or other glassware.

a. Wash the wound immediately to remove any glass pieces.
b. Apply mercurochrome or acriflavine ointment to the wound.
c. Cover with gauze and adhesive tape.
d. If the cut bleeds profusely, stop the bleeding by pressing down on it with a compress. Refer the patient to a physician.
e. If the cut bleeds heavily with the blood spurting out at intervals, try to stop the bleeding with a compress and call a physician or qualified nurse.
f. Continue to press on the wound while awaiting the physician's or nurse's arrival. He or she will decide whether a tourniquet should be applied.

Contamination by Infected Material

Wounds caused by broken glassware containing stools, pus, etc.

a. Wash the wound immediately.
b. Check whether the cut is bleeding. If not, squeeze hard to make it bleed for several minutes.
c. Bathe the whole area, i.e. the edges of the cut and inside the cut, with antiseptic lotion.
d. Wash thoroughly with soapy water.
e. Bathe again with antiseptic lotion.
f. Refer the patient to a physician, if the material involved is known to be very infective, e.g. pus.

If infected material is accidentally sucked into the mouth:

a. Spit it out immediately.
b. Wash out the mouth with diluted antiseptic lotion.
c. Wash out the mouth thoroughly with large amounts of clean water.

Bodily Damage by Electric Shock

A low-voltage alternating electric current (220 V) is usually used in the laboratory and electric shocks are rare. They may occur when faulty equipment is being handled, particularly with wet hands. The symptoms are fainting and asphyxia.

a. Before doing anything else, put off the main switch.
b. Send for a physician.
c. Begin giving mouth-to-mouth respiration immediately if required (Fig. 1.6).

Precautions for the Avoidance of Accidents

1. Handling acids and alkalis
   a. Diluting sulfuric acid with water: Always add the sulfuric acid to the water drop by drop, stirring

the mixture after each drop. Do this preferably in a sink. Never pour water into sulfuric acid (because of the danger of splashing).

b. Bottles of acids and alkalis: Keep them on the lower shelves of the cupboards. When you take one out, hold it firmly upright with a dry hand. Do not keep acids and alkalis in bottles with ground glass stoppers as they may get stuck.

c. Pipetting: Where possible, use small measuring cylinders for measuring acids and alkalis. If more accurate measurement is required, use a pipette plugged with non-absorbent cotton wool or with a rubber tube attached. Pipette slowly, watching the level of the liquid.

2. Heating glassware and liquids

a. Test tubes: Never heat the bottom of a test tube. The liquid inside might sputter. Heat the middle of the tube, shaking gently. The mouth of the tube should be facing away from the worker and any other person, towards an empty space or a sink.

b. Ordinary glass and Pyrex: Only Pyrex glassware and porcelain receptacles can be heated over a Bunsen flame. Ordinary glass will break.

c. Inflammable liquids: Only small quantities of inflammable liquids such as ether, ethanol, acetone, benzene, toluene and carbon disulfide should be kept in the laboratory.

Warning: Ether will ignite at a distance of several meters from a flame. Never place a bottle of ether on a workbench where there is an open flame (Bunsen burner, spirit lamp, etc.). Carbon disulfide is even more dangerous.

FIG. 1.6: Mouth-to-mouth respiration

d. Butane gas: When lighting a gas burner, always light the match and hold it to the burner before turning on the gas tap. Turn off the main valves of all butane gas cylinders every evening. Replace the rubber connecting pipes once a year.

3. Do not use broken, cracked or chipped laboratory glassware.
4. Put clear labels on poisons. Keep them in a locked cupboard.
5. Do not use nylon clothes while working as these are easily inflammable. Always use a laboratory apron.
6. Always ensure that electrical wiring and electrical appliances are in good condition.

Suggested List of First Aid Equipment for Laboratory

1. 5% aqueous sodium carbonate
2. 2% aqueous sodium bicarbonate in an eye drop bottle
3. 5% acetic acid
4. Saturated solution of boric acid in an eye drop bottle
5. Soap powder solution (5 g per liter of water)
6. Acriflavine ointment
7. Mercurochrome 2%
8. Antiseptic lotion
9. Cotton wool
10. Gauze
11. Roller bandage
12. Adhesive tape
13. Scissors.

Contamination from Infective Material

If contamination has occurred, then:

1. Disinfect the part with the disinfectant available in the laboratory. Thoroughly clean the affected area with a stream of running water.
2. Sucking the contaminated material: Spit out all that has been sucked. Use a disinfectant liquid (e.g. diluted dettol) for mouth washing. If the infected material has been swallowed accidentally, forced vomiting to be done, ascertain the kind of infection and take advise from a medical person.
3. If skin is infected by highly virulent organisms, touch the involved part with pure carbolic acid.

Precautionary Measures

1. A fire extinguisher should always be handy.
2. Keep sand bucket in the laboratory.
3. Take measures to prevent electrical short circuiting.
4. No smoking in the working zone of the laboratory.

5. Breakable items should be kept in proper racks and never at the edge of the working table.
6. Do not suck anything with the mouth, use rubber teats and bulbs for sucking.
7. Do not place eatables on the working bench.
8. Keep fingernails short.
9. At the end of the day, clean all working benches with a disinfectant. See that nothing except the required electrical appliance is on.
10. Dispose all infected material properly. Can put such material in hypochlorite solution or in an acidic solution, e.g. diluted sulfuric acid (25%). Burn off all dried contaminated articles, e.g. filter papers.
11. The glassware should be disinfected with a suitable disinfectant and be cleaned thoroughly with running water.
12. Use rubber gloves and a nose mask while working with infective samples, e.g. serum of viral hepatitis patient.

UNIVERSAL WORK PRECAUTIONS (UWP) FOR LABORATORY PERSONNEL (ESPECIALLY IN RELATION TO HIV TRANSMISSION)

Introduction

Healthcare personnel (HCP) can acquire certain illnesses beyond those acquired by all others who live and work in our society, by virtue of their profession. HCPs are at risk of acquiring any of the whole gamut of infections from patients/specimens, which may be viral, bacterial, parasitic or fungal. However, this risk due to occupational exposure can be minimized if not obliterated altogether, if we follow universal work precautions (UWP).

Today, with the WHO estimates of above 5 million HIV positive persons in India, there is an urgent need to review UWP. Besides HIV, there is the very real danger of acquiring Hepatitis B and Hepatitis C in exactly the same way as HIV and could also be fatal. Hepatitis B is 100 times more infectious than HIV. Besides, Hepatitis B is also far more prevalent in India in comparison to HIV with estimated carriers being between 30 and 40 million, a considerable number being infectious. However, fortunately, effective vaccination is available for hepatitis B; therefore, it is strongly recommended for all levels of healthcare workers.

Much of the contamination in the laboratory occurs as a result of penetrating injuries caused by sharp objects and the spilling and splashing of specimen materials.

Components of UWP

1. Handwashing.
2. Barrier precautions (mask, cap, plastic apron and protection of feet).
3. Careful handling of all kinds of sharps and needles.
4. Effective infection.
5. Sterilization.
6. Correct disposal of different kinds of wastes generated in a health care facility.

Guidelines of Basic Practices and Procedures

- Prevention of puncture wounds, cuts and abrasions and protection of existing wounds, skin lesions, conjunctiva and mucosal surfaces
- Application of simple protective measures designed to prevent contamination of the person and his/her clothing
- Good basic hygiene practices, including regular handwashing
- Control of surface contamination by containment and disinfection procedures
- Safe disposal of contaminated waste.

Biosafety Regulations for Laboratory Procedures

- Wear gloves when handling infectious materials or where there is a possibility of exposure to blood and other body fluids. All laboratories that work with material that is potentially infected with HIV require a generous supply of good quality gloves.
- Discard gloves whenever they are thought to have become contaminated or perforated, wash your hands and put on new gloves. Alternatively, where there are economic constraints, wash gloved hands whenever they get contaminated with blood/body fluids before collecting further samples
- Do not touch your eye, nose, or other exposed membranes or skin with your gloved hands.

Sterilization (for Nondisposable Items)

- For sharps, reusable blades, cystoscopy instruments, endoscopy instruments, use CIDEX (2% glutaraldehyde) or 5% Korsolex. Disinfection usually occurs in 30 minutes
- Use autoclaving for other reusable items (e.g. needle holders, gowns, etc.)
- Wherever, autoclaving is not possible, boiling must be for 30 minutes at the least.

Waste Disposal

Divide waste into three parts at source.

i. Household type noninfectious waste:
   - Not to be decontaminated
   - To be disposed off as such.

ii. Infected sharp waste disposables (needles/surgical instruments):
   - Place in puncture-proof container containing disinfectant (1% bleach prepared every morning). Needles should ideally be burnt (machines are available that operate on electricity)
   - Final disposal.

iii. Infected nonsharp waste:
   - Is to be decontaminated
   - Placed in disinfectant 5 to 10% bleach as the case may be (left over blood, tissues, etc.).

Final Disposal

- Purchase of needle destroyer if resources permit
- Incineration of all infected waste
- Deep burial in controlled land fill sites (protected from all sides)
- Shredding of disposable plasticware waste.

Postexposure Care

- Minor bleed with percutaneous inoculation, open skin wound, breached skin, exposed mucous membranes.

First Aid

- Allow to bleed by squeezing
- Wash with water
- Antiseptic.

Report

- Employee identification, date, time with place of accident
- Circumstances around accident
- Action taken.

Initial Consultation

- Easy access to medical advice with counseling. Consult, physician for AZT prophylaxis regime if medication available.

Laboratory Testing

- After consent with counseling within 2 weeks, 5 weeks, 12 weeks, or 24 weeks.

Clinical Follow-up

- For fever, pharyngitis, rash, malaise, lymphadenopathy, myalgia and arthralgia within 6 months
- Do not leave the workplace or walk around the laboratory while wearing gloves
- Wash hands with soap and water immediately after any contamination and after work is finished. If gloves are worn, wash your hands with soap and water after removing the gloves. This is a vital and simple precaution that is often overlooked
- Wear a laboratory gown or uniform when in the laboratory. Wrap-around gowns are preferable. Remove this protective clothing before leaving the laboratory
- When work with material that is potentially infected with HIV is in progress, close the laboratory door and restrict access to the laboratory. The door should have a sign BIOHAZARD: NO ADMITTANCE
- Keep the laboratory clean, neat and free from extraneous materials and equipment
- Disinfect work surfaces when procedures are completed at the end of each working day. An effective all-purpose disinfectant is a hypochlorite solution with a concentration of at least 0.1% available chlorine (1 g/L, 1000 ppm)
- Whenever possible, avoid using needles and other sharp instruments. Place used needles, syringes and other sharp instruments and objects in a puncture resistant container. Do not recap used needles and do not reuse needles from syringes for disposal
- Never pipette by mouth
- Perform all technical procedures in a way that minimizes the risk of creating aerosols, droplets, splashes or spills
- Use a biosafety cabinet while working on aerosolizing specimen
- Do not eat, drink, smoke, apply cosmetics or store food or personal items in the laboratory
- Make sure that there is an effective insect and rodent control program
- If a laboratory personnel has lesions on hand and feet, then:
   - If superficial, he or she should wear protective dressing and wear gloves over it
   - If wound is deep or raw then the concerned person should not handle samples till the wound heals.
- If there is a pregnant healthcare worker then in view of the occupational risk to the woman and the developing fetus, on compassionate grounds, where possible she should be involved in clerical tasks or stay away from work for the duration of her pregnancy.

Containing Spills

- Cover the spill immediately with absorbent material to avoid aerosolization
- Soak the material by pouring disinfectant on it
- Leave the area for 30 minutes
- Mop with more adsorbent material after wearing gown, mask and gloves

- Place material in appropriate bin for disposal (autoclaving or incineration).

Collection of Specimen

- Always keep labeled bottle ready on the bedside
- Wear disposable gloves
- Keep adequate cotton with spirit at collection site
- Keep a bucket full of disinfectant [CIDEX (glutaraldehyde)], one for at the most 5 beds.

Transport of Specimen

- Specimens should be collected in plastic; screw-capped containers prelabeled with patient identification data, should be packaged and transported in puncture resistant containers in upright position with the sign of biohazard on the container.

MEDICOLEGAL ASPECTS OF CLINICAL PRACTICE

Under the Consumer Protection Act (CPA), India, 1986; any patient, registered consumer organization, state or central government or patient's legal heirs can sue the undermentioned persons for shortcomings in "service" provided by them.

- A technician, microbiologist, biochemist or pathologist running a laboratory
- Any private polyclinic, nursing home or hospital, registered or otherwise
- As government hospitals provide service without consideration (free of cost), they cannot be held responsible under CPA 1986
- Doctors appointed by the government, however, can be held accountable under other civil and criminal laws for proven negligence
- Medical practitioners delivering new service without any consideration in a charitable hospital or medical camps are exempted from the provisions under CPA
- As per clause 2(d) (ii) of the CPA 1986, a consumer implies any person who hires or avails of any service for a consideration, which has been paid or promised, or partly paid and partly promised, or under any system of deferred payment, and includes any beneficiary of such services other than the person who hires or avails of the service for consideration paid or promised or partly paid and partly promised or under any system of deferred payment, when such services are availed of with the approval of the first mentioned person
- The time limit stipulated for filing a complaint is 2 years from the date of alleged negligence
- Patients can be dealt with severely if they file frivolous and false complaints just to harass the medical practitioner
- Free services provided are exempted under CPA
- A laboratorian is also a consumer as he buys various instruments, equipment, diagnostic kits/reagents/devices. He too can file a complaint under CPA for any defect or deficiency in service related to that purpose
- Ignorance is not held as an excuse as an established legal principle. Concurrently, law does not expect a very high degree of knowledge but expects only average knowledge from a medical practitioner
- Medical negligence is a civil wrongdoing classified as 'tort', where a medical practitioner fails to take proper care in respect of examination, diagnosis, investigation, treatment, etc. resulting in injury or mortality
- Laboratorians are expected to keep all reports confidential (legally and ethically). The reports can be divulged to the referring clinician or to the patient or the relatives of the patient (with patient's onsent). Reports pertaining to sexually transmitted diseases or HIV/AIDS should be handed over only to the patient
- Legally, only authorized or registered blood banks can supply units of blood. All mandatory information must be clearly mentioned on the bottle label legibly
- These days doctors have 'Malpractice Insurance Covers'. In case a legal notice is received by such a doctor, he should immediately notify the insurance company. The insurance company must take all necessary actions in such a case. The company should appoint a lawyer to give reply or to take legal steps and inform the doctor about it. The doctor, by permission of the company, can appoint a lawyer of his choice
- What constitutes a legal notice? Any letter received by a medical practitioner from a patient or a voluntary registered organization or an advocate, demanding explanation about treatment given or demanding some explanation about treatment for alleged injury or death constitutes a legal notice
- Section 27 of the Civil Procedure Code provides that when a suit has been duly instituted, a summon may be issued to the defendant to answer the claim, and such summon is to be served in the prescribed manner. When a complaint is lodged before the commission or the forum, the defendant practitioner is informed by a registered letter by the office, which is called a summon in legal parlance. In this summon, time for the reply and date of hearing is mentioned. Usually, the time given for filing the reply is 30 days.

LABORATORY INSTRUMENTS

Microscope

Micro = Small, Scope = to view.

It magnifies the image of the object to be visualized through it. Normally, the laboratory microscopes provide

a magnification of 40x (scanner), 100x (low power), 400x (high power) and 1000x (oil immersion). The total magnification is obtained by multiplying the magnification of the objective with that of the eyepiece.

Parts of the Microscope

It has three sets of parts. They are the:

1. Stand,
2. Mechanical adjustments, and
3. Optics or the lenses.

Stand

It consists of:

1. The tube—supports objectives and eyepiece.
2. The body—gives support to the tube.
3. The arm—gives correct height and angulation to the body and the tube.
4. The stage with a pair of spring clips or a mechanical stage.
5. The substage holds the condenser lens with its iris diaphragm and a holder for light filters and stops.
6. The foot on which other parts rest, can be in tripod or horseshoe shape.

Mechanical Adjustments

Focusing Adjustments
These are coarse and fine adjustments.

Coarse Adjustment
Controlled by a pair of large knobs, one on each side of the body. On rotating this, the tube moves with its lenses. Some microscopes have this attached to the stage; so that instead of the tube, the stage moves up and down. Coarse adjustment is enough for low power lenses.

Fine Adjustment
Necessary for high power and oil immersion lenses. This is usually controlled by two smaller knobs on each side of the body. They may be graduated to indicate the movement in microns.

Draw Tube
It is used to adjust the distance between the objective lens and the eyepiece lens.

Inclination
The arm can be tilted upon the foot by a hinge.

Condenser Adjustments
Focusing of condenser is done by rotating a knob present on one side below the stage.

Aperture Adjustment
It is done by the iris diaphragm (made up of leaves).

Centering of Condenser
It is done to bring the light beam accurately through the instrument. In some microscopes, it is permanently fixed.

Mechanical Stage
It has knobs for moving the slide across or along the stage.

Monocular, Binocular and Digital Microscopes
Monocular—has only one eyepiece (Fig. 1.7).

Binocular—has 2 eyepieces, the only advantage it offers is that it causes less strain on the eyes (Fig. 1.8A). Nowadays digital microscopes are available, here digital image is projected onto a digital display device (Fig. 1.8B).

FIG. 1.7: Monocular microscope with substage lamp

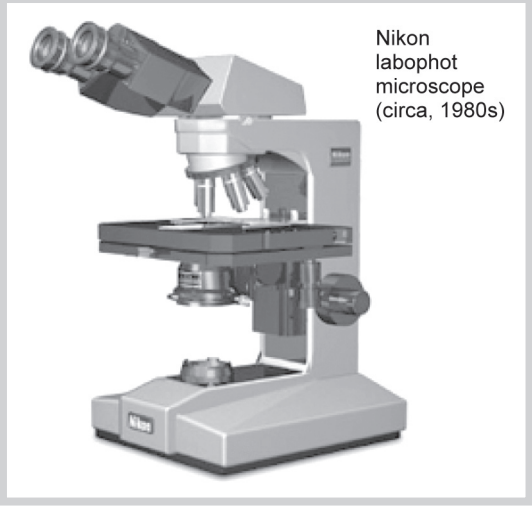


FIG. 1.8A: Binocular microscope with substage lamp

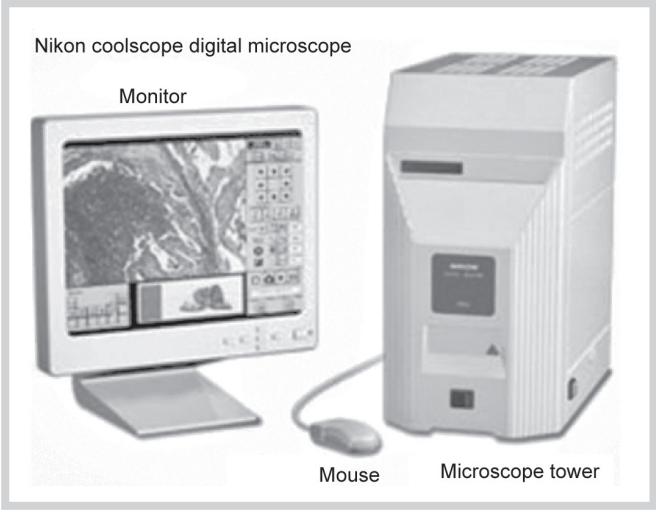


FIG. 1.8B: Digital microscope

Microscope Optics

Objective
On objective quality, depends, the quality of the image. These are usually made up of more than one lens. On each objective is engraved the magnification power.

Numerical Aperture
Numerical aperture (NA) of the objective is important, for on this, depends, among other things, the amount of light which the lens passes and the detail which it can make visible, on which it is said to resolve.

Oil Immersion Objectives
They are used to avoid bending of light beam (with higher magnification). The oil used should have the same optical properties as glass, e.g. cedar wood oil. Liquid paraffin can also be used.

Objective Aberrations
With increasing magnification certain optical aberrations creep in:

1. Spherical aberration—edge of the lens gives slightly higher magnification than its center.
2. Chromatic aberration—blue light is magnified slightly more than red.

These aberrations can be avoided by using a series of lenses made of special glass, carefully calculated and designed.

Objective Qualities

1. Achromatic—are the usual average quality lenses and are good enough for routine laboratory work.
2. Fluorite (Fi)—are highly corrected and expensive, have a wider field and are good for searching blood films.
3. Apochromatic (Apo)—are very highly corrected and costly and are only of value in special work.

Spring-loaded Objectives
The high power objectives (40X and 100X) of most modern microscopes are spring loaded, i.e. the front mount of the objective will be pushed in rather than pushed through a specimen, if such an objective is accidentally pressed against a specimen when focusing (Fig. 1.9).

Working of Oil Immersion Objectives
A beam of light passing from air into glass is bent; and while passing from glass to air, it is bent back again. The bending effect and its limitations can be avoided by replacing the air between the specimen and lens with an oil which has optical properties similar to that of glass, i.e. immersion oil. When an appropriate oil is used, the light passes in a straight line from glass through the oil and back to glass as though it were passing through glass all the way. Whenever possible, the immersion oil recommended by the manufacturer of a microscope should be used (Fig. 1.10).

Eyepiece
The most commonly used eyepiece is known as Huygens eyepiece which has 2 lenses mounted at a correct distance apart, with a circular diaphragm between, which give a sharp edge to the image. These are available in different magnifications. Lesser the magnification, brighter and sharper is the image. For routine work, a 10X Huygens is good enough. The 15X eyepieces are also available, as are wide field ones.

Condenser and Iris
Condenser is a large lens mounted below the stage, with an iris and diaphragm. There may be 2 or more lenses. Its function is to deliver the light beam to the objective at a sufficiently wide angle.

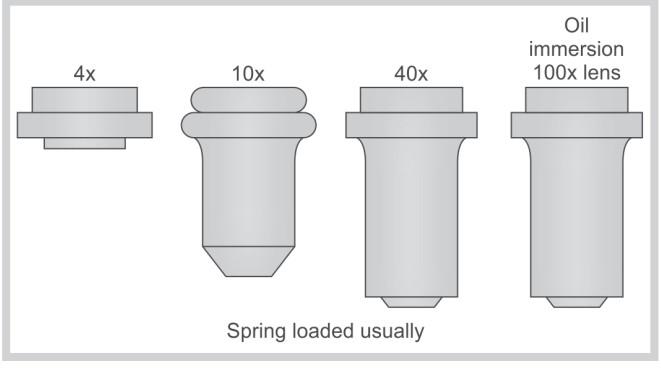


FIG. 1.9: Microscope objectives

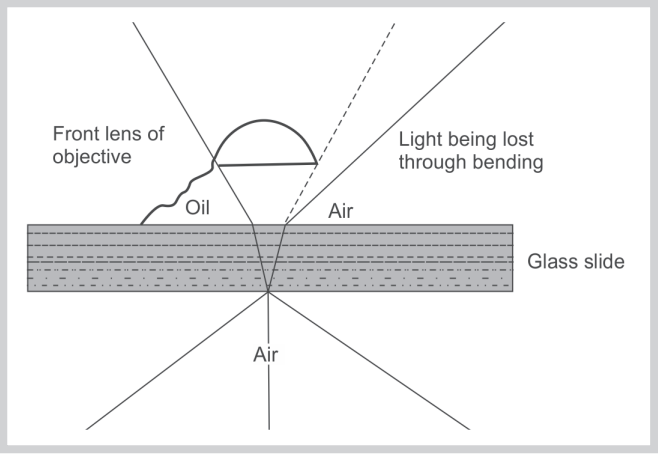


FIG. 1.10: Working of an oil immersion objective

The Mirror

It is placed below the condenser and iris, it can be turned in any direction. It reflects the light beam from the source to the iris and condenser. It usually has two mirrors mounted back to back, one flat and the other concave. Flat mirror is used in the presence of condenser and the concave without the condenser.

Light Source

Daylight

Use of direct sunlight is bad for the microscope and the eye. It is best to use reflected sunlight of a dull white background. It is not sufficient for oil immersion lens and it is not available during evening or night.

Electric Light

A 60 watt frosted electric lamp placed 18" away from the microscope is sufficient for most routine work. Many microscopes are now provided with built-in sources of illuminations. In the absence of electricity, a battery lamp or an oil lamp can be utilized. The light from these artificial sources is rather yellow but may be used. Best, however, are halogen lamps.

Special Applications of the Microscope

Phase Contrast Illumination

This is needed to visualize transparent microorganisms suspended in a fluid. Ray of light travels in a wave form in a straight line. Two such rays traveling together are said to be in phase, and they produce a brighter illumination. If, however, these rays are out of step with each other, they are said to be out of phase. They interfere and produce less bright illumination. Phase contrast microscopy makes use of this property of rays to help or hinder each other and thereby resulting increased contrast in the microscopic image.

The desired effect is brought about by placing an annulus in the condenser and a phase plate in the objective. A circle is engraved in the phase plate which matches the ring of beam coming through the condenser and annulus. This circle makes the wave take a longer or a shorter step, so becoming out of phase with those aves which pass through the rest of the plate.

Supposing that the specimen is suspension free fluid, the only light that reaches the eye is that which goes from the annulus through the phase plate. Whereas presence of organisms would diffract and scatter the light. The light passing through the fluid gets out of phase with the light that has the organisms stand out in contrast to their background.

Equipment Needed

An annulus, a phase plate and a telescope that is needed for adjusting the rings of both annulus and the phase plate.

Method

1. Focus the specimen with the right objective after illuminating the microscope.
2. Place the matching annulus at its position.
3. Remove the eyepiece and put the telescope in its place, adjust it till the two rings, one bright and one dark are in focus.
4. Adjust condenser screws till the bright annulus ring fits exactly into the darker ring of the phase plate.
5. Remove the telescope, replace the eyepiece, focus and examine the specimen.

Importance

This method is made use of for examining live organisms, for examaple,

a. Cholera vibrios
b. Amebae
c. Trypanosomes
d. Trichomonas, and
e. Other flagellates.

It can also be used for platelet counting and for examining routine urine specimens.

Demerits

a. A halo is seen around each particle, it gives a false appearance of its structure.
b. In addition, some resolution power is lost but this is more than compensated for by the increased contrast that is produced.

Dark Ground Illumination

This method too, is used for visualizing organisms suspended in fluid, both the structure and the motility

of the organisms can be seen. In this method, the light enters the special condenser which has a central blacked-out area so that light cannot pass directly through it to enter the objective. Instead the light is reflected to pass through the outer rim of the condenser at a wide angle which illuminates the microorganisms by a ring of light surrounding them (Fig. 1.11).

In this method, the light that is seen comes only from the microorganisms themselves and not from the light source. Hence, the organisms are brightly illuminated against a dark background. Though useful, this method is rather cumbersome.

Equipment Needed

1. An oil immersion dark ground condenser with the centering screws.
2. A funnel stop for insertion in 100X objective to reduce its NA and exclude light coming directly from the source.
3. A brightly illuminated microscope lamp.
4. Scratchless slides not more than 1 mm thick.

Method

1. Fit the dark ground condenser and raise it to stage level.
2. Place the coverslipped specimen on the thin polished glass slide. Both, the coverslip and the slide should be absolutely clean.
3. Place a drop of immersion oil between the condenser and the slide.
4. Adjust light source and the mirror properly.
5. Focus 10X objective and observe.
6. Focus condenser up or low, so that the ring ultimately becomes just a spot of light. Focus this spot right in the center.
7. Use 40X objective; if needed, use the 100X oil immersion by inserting the funnel stop into it.

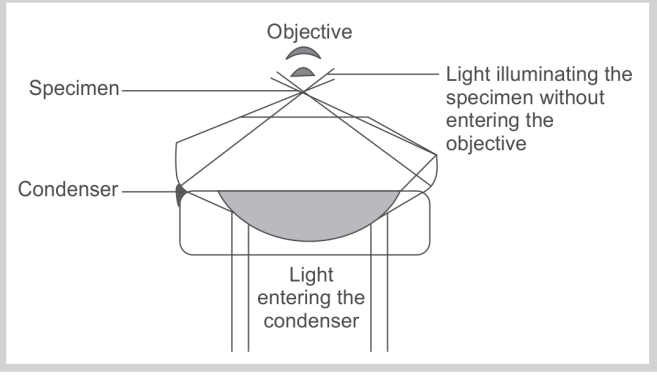


FIG. 1.11: The principle of dark ground illumination

Demerits

1. Focusing and/or centering of condenser is difficult as is the alignment of the lamp.
2. Difficulties may arise under the following circumstances:
   - Smear traces on the slide or coverslip
   - If the specimen is dense
   - A bubble is present in the immersion oil
   - Insufficient oil contact below or above the slide.

Importance

1. This method is of particular importance for the examination of *Treponema* group of organisms.
2. It can also be of use for microfilariae, for the sheath of the pathogenic forms can be clearly seen which otherwise needs to be stained.
3. For examining the rapid movement of *Vibrio cholerae*.
4. In addition, this method can be used for:
   - *Leptospira*
   - *Borrelia*, and
   - *Spirillum* species.
   - The ideal objective for dark ground illumination is the 50X fluorite as this lens gives a clear, sharp and a well-illuminated image.

Fluorescence Microscopy

This method entails the illumination of particles/microorganisms (previously stained with a fluorescent dye) with ultraviolet (UV) light into visible light (yellow or orange), by lengthening their wavelength. This procedure is made use of for visualizing, besides other things, mycobacteria glowing against a black background.

All other wavelengths emitted by the lamp except the ultraviolet (UV) are to be filtered off (by using appropriate optical filters) and no harmful rays of UV light should reach the observer's eye (by using an immersion dark ground condenser as described for previous method). Again, another filter is used to remove all unwanted fluorescent light by placing a secondary or a barrier filter above the eyepiece (Fig. 1.12).

Equipment Needed

1. A fluorescent lamp (mercury vapor or quartz iodine, the latter is better, being cheaper, lighter and easier to use).
2. A blue (primary or exciting) filter, generally a BG 12.
3. A yellow (secondary or barrier) filter.
4. An immersion dark ground condenser.
5. A nonfluorescent immersion oil, e.g. liquid paraffin.

Importance

1. For identifying mycobacteria.
2. It is used extensively in fluorescent antibody techniques used in parasitology and bacteriology.

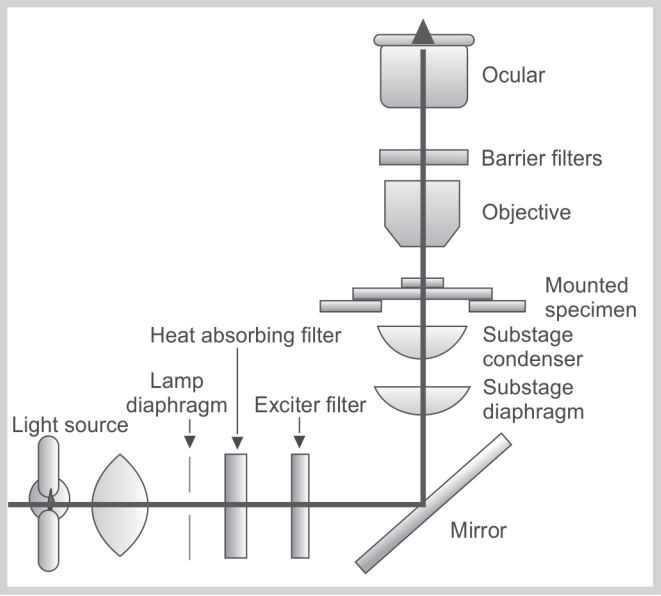


FIG. 1.12: Components of fluorescence system

3. It is also used widely in histopathology of kidney, skin, etc. where immune/autoimmune basis of disease is expected. In fact, anything can be confirmed with high degree of sensitivity and specificity, if antibodies against it (later tagged with a fluorescent dye) can be produced.
4. Used widely in cytogenetics.

Electron Microscope

Basic Principle

The resolution of the light microscope has been shown to be limited by the NA and the wavelength of light employed. As the degree of correction in glass lenses is very high, the main limitation is imposed by the light (e.g. half wavelength of light), giving a normal resolution of approximately 250 nm; and when UV light is used, a resolution of about 100 nm. By the substitution of an electron beam for light rays, a much greater degree of resolution can be obtained; since at an acceleration of 50,000 volts, electrons have a wavelength of only 0.001 nm; therefore, a theoretical resolving power of 0.0005 nm could be attained, which would enable molecules to be seen. Unfortunately, the degree of correction that is currently feasible with transmission electron microscope (TEM) lenses will permit a resolution of only 0.25 nm, but this is still a thousand times greater than that possible with the light microscope. A further difficulty with the TEM is that, since electrons have poor penetrating power, the sections to be examined must be very thin, less than 50 nm thick. This necessitates the use of special hard embedding media (plastics) and special ultra-microtomes to cut such thin sections. Steel knives cannot be used to cut these sections; either glass or diamond knives are used.

Weighing Scales or Analytical Balance

Weighing scales: For weighing large quantities.

Analytical balance: For accurate weighing of smaller quantities.

Use and Care

1. The weighing equipment must be placed on a firm bench, away from vibration, draughts, direct sunlight and dust.
2. It should be kept perfectly horizontal by altering the screws on which the equipment stands.
3. Chemicals, etc. should never be placed directly on the pans. Weigh them in a container.
4. Never touch the weights with hands, handle them with forceps.
5. The balance should be at rest before adding or removing the weights or chemicals.
6. Before taking the reading, the glass window of the instrument should be closed.

Electronic analytical balances are also available. Made by various companies, these are very accurate.

Centrifuge

Centrifuge is used to sediment or deposit rapidly particles such as cells which may be suspended in a fluid. The speed is expressed as rpm, i.e. revolutions per minute.

Relative Centrifugal Force (RCF)

More important than rpm is relative centrifugal force (RCF). RCF is expressed as the acceleration due to gravity or G (dynes per cm). The formula is:

$$G = 0.00001118 \times (r) \times (n)2$$

where r = radius in centimeters

and n = revolutions per minute.

The time of centrifugation is equally important. The tubes should be spun for a definite period to obtain the desired effect.

Types of Centrifuge

Hand Centrifuge

Fixed to the bench, the handle is rotated manually. It gives low speeds only.

Motor-driven Centrifuge

Operated through mains electricity supply. The tubes may be kept in a fixed angle head or in a swing out head (Figs 1.13 and 1.14).

Microhematocrit Centrifuge

Also motor driven for finding out packed cell volume (PCV) of red blood cells (RBCs). In this, blood-filled capillary tubes are spun and later the percentage of RBC-filled column is estimated (Figs 1.15 and 1.16).

Use and Care

1. Use centrifuge tubes made of strong glass and they should not be too long.
2. The opposite tubes should be balanced properly.
3. The centrifuge speed should be increased gradually.
4. The instrument should be kept clean. If something spills over inside, it should be cleaned and the instrument disinfected, if necessary.

FIG. 1.13: Swing out head centrifuge
(*Courtesy:* Yorco Sales Pvt. Ltd)

FIG. 1.15: Dual centrifuge routine centrifuge with microhematocrit attachment
(*Courtesy:* Yorco Sales Pvt. Ltd)

FIG. 1.14: Motor driven centrifuge with rpm. indicator and auto (timed) shut off
(*Courtesy:* Yorco Sales Pvt. Ltd)

FIG. 1.16: Microhematocrit centrifuge and its parts
(*Courtesy:* Yorco Sales Pvt. Ltd)

Glassware (Many Items are now Made of Plastic)

1. *Flasks*—are of different sizes and shapes.
   a. Erlenmeyer or conical flasks—for heating and boiling liquids (Fig. 1.17).
   b. Volumetric flasks—are graduated for getting exact volume of liquids (Fig. 1.18).
   c. Round and flat-bottomed flasks for preparing solutions (Figs 1.19A and B).
2. *Beakers*—available in different sizes (Fig. 1.20).
3. *Bottles*
   a. Specimen bottles—with top screws, e.g. the universal type containers.

FIG. 1.17: Conical flasks

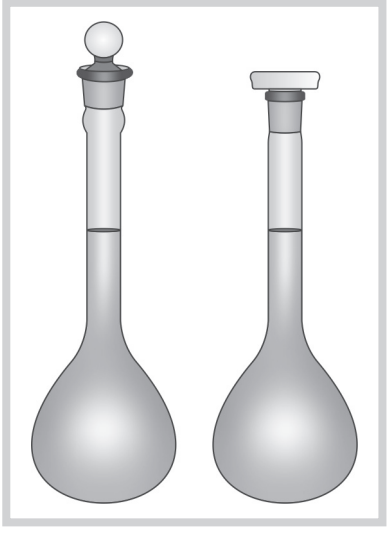
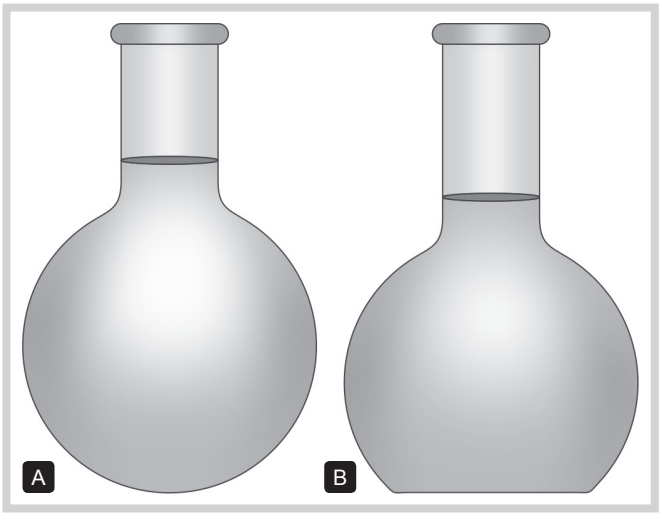


FIG. 1.18: Volumetric flasks



FIGS 1.19A AND B: (A) Round bottomed flask and (B) Flat bottomed flask

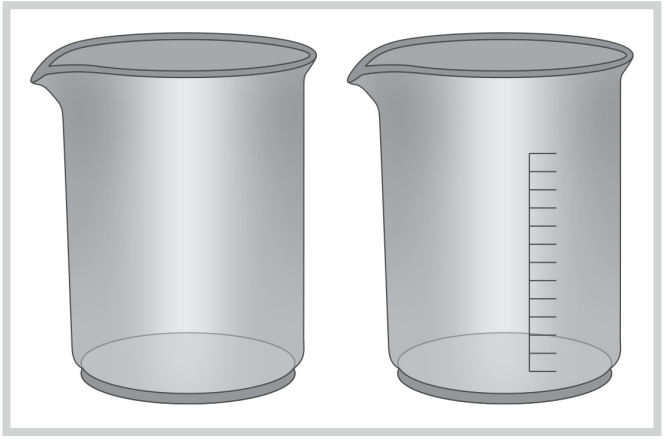


FIG. 1.20: Beakers

   b. Reagent bottles—have ground glass or plastic stoppers, available in different sizes and may be made of amber colored glass (Figs 1.21A and B).
   c. Drop bottles—fitted with special tops through which drops can be delivered (Fig. 1.22).
4. *Funnels*—used to hold filter papers when filtering fluids or for pouring liquids into narrow neck containers (Figs 1.23A and B).
5. *Cylinders*—used for measuring liquids, they have a pouring spot (Fig. 1.24).
6. *Tubes*—are of various sizes; of the test tube or centrifuge (conical) type, with or without a top rim (Figs 1.25 and 1.26).
7. *Pipettes*—are used to measure and deliver a given volume of fluid.

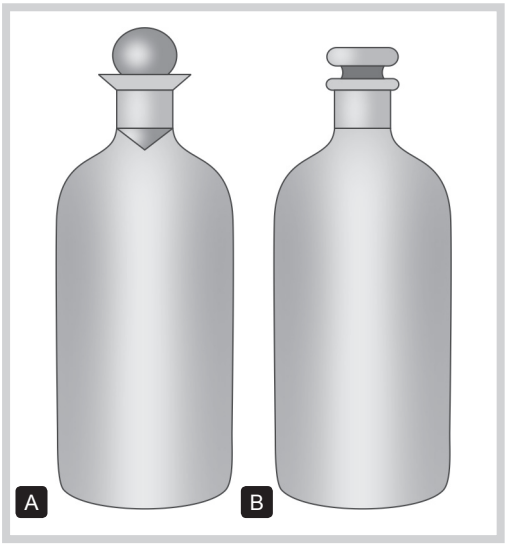
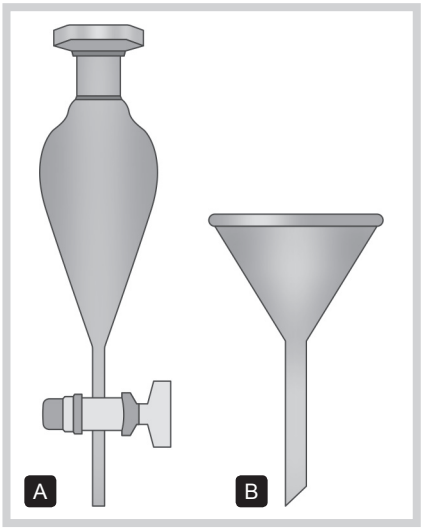


FIG. 1.21A AND B: (A) Specimen bottles and (B) Reagent bottles



FIGS 1.23A AND B: (A) Separating funnel and (B) Funnel

FIG. 1.22: Drop bottles

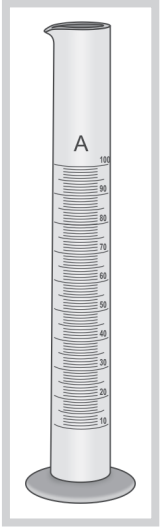


FIG. 1.24: Measuring cylinder

a. Volumetric pipettes—have a bulb shape in the stem. Each pipette is marked to show the given volume of fluid, it contains or delivers (Figs 1.27A and B).
b. Graduated pipettes—are of various sizes. They may be of the non-blow out or the blow out type.
c. Blood pipettes—have a white back and include the 0.02 mL pipette used for hemoglobin, red cell and platelet counts, and also the 0.05 mL pipette for white cell counts (Fig. 1.28A).
d. Pasteur pipettes—have multiple uses. They are not graduated or marked. These can be bought or made in the laboratory (Fig. 1.28B).

Other Necessary Equipments

Serological Water Bath

It is electrically heated and has a thermostatic temperature regulator. It can provide temperature ranging from room temperature to 100°C. Various sizes to suit various workloads are available (Fig. 1.29).

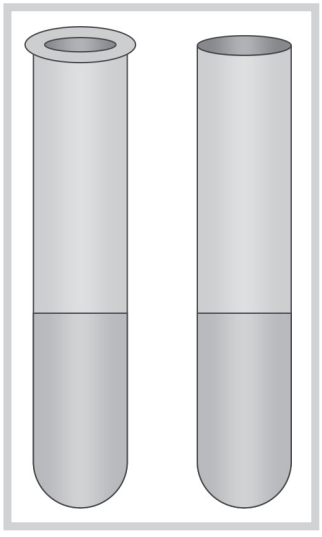
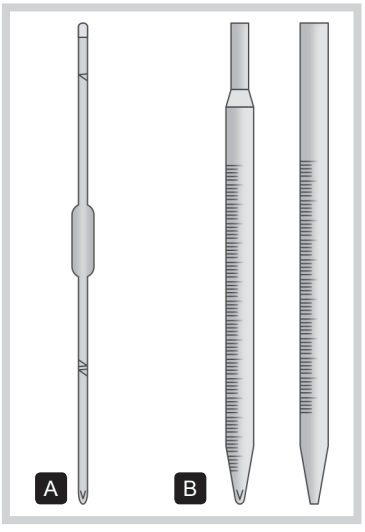


FIG. 1.25: Test tubes



FIGS 1.27A AND B: (A) Volumetric pipette and (B) Measuring pipette

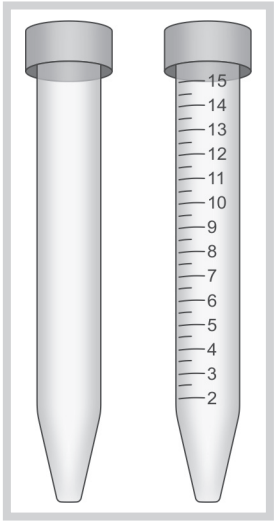
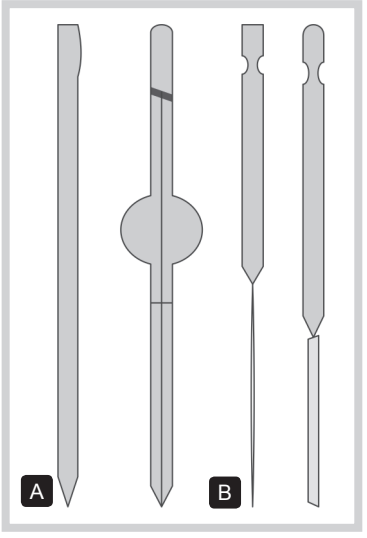


FIG. 1.26: Centrifuge tubes



FIGS 1.28 A AND B: (A) Blood pipettes and (B) Pasteur pipttes

Incubator

Works on electricity and regulates temperature thermostatically. Necessary for various investigations where body temperature 37°C (or otherwise) incubation is required (Fig. 1.30A).

Hot Air Oven

This is used for drying and sterilizing glassware. This too is thermostatically controlled and electrically heated. It looks like an incubator (Fig. 1.30B).

Reporting Laboratory Tests and Keeping Records

Standardization

Standardization in the reporting of laboratory tests contributes to the efficiency of the laboratory service and is of great value when patients are referred from one place to another. Whenever possible, request forms and other laboratory printed stationery should be prepared and issued by a central stationery office.

FIG. 1.29: Serological water bath
(*Courtesy:* Yorco Sales Pvt. Ltd)

FIG. 1.30A: Incubator
(*Courtesy:* Yorco Sales Pvt. Ltd)

FIG. 1.30B: Hot air oven
(*Courtesy:* Yorco Sales Pvt. Ltd)

Use of Rubber Stamps

When stationery is not supplied from a central source, standardization in presenting and reporting results can be achieved by the use of rubber stamps. Adequate ink must be used and the stamp must be positioned carefully.

Format

The top part of the report card must prominently give the name, address and telephone numbers of the laboratory. It should then have place for printing the patient's name, age, sex, name of the referring doctor, the laboratory reference number and date. Next, the title of the report should be mentioned, e.g. urinalysis, stool examination, hematology, biochemistry, etc. After this, print the investigation name, leave space for patient's values, print normal values followed by the units. The report must end with the signatures of the person in-charge of the laboratory.

Keeping Records in the Laboratory

A record of all test results must be kept by the laboratory as carbon copies, work sheets, or in simple exercise books. A day book is ideal as it has the necessary ruled lines. In your record put the date, reference number, patient's name, name of the referring doctor, investigations asked for, reports given and payment status (if privately owned laboratory).

Laboratory Reporter

An ideal laboratory computer program helps in reporting and recording diagnostic center, pathology lab and other diagnostic imaging fields. The program should also keep history records of the patients. It must have facilities of making and reporting profiles, e.g. lipid, renal, cardiac, hepatic and diabetic profile. A program can be called ideal if:

It Reduces Overload

- Avoids manual operations by printing booking slips, receipts, bills, envelopes, etc.
- Prints daily register of patients
- Prints rate lists
- Reports only the tests required and not the whole group of tests
- A comprehensive reporting option for day end operations including daily collection report and doctor wise daily collection.

- It helps referencing doctor
- Provides clear reports with normal values
- Abnormal values are underlined or highlighted automatically
- Prints history reports of the patients.

It Makes Working Easy

- Reports as per your own method of grouping of tests, profiles, etc.
- Automatic calculation of charges
- Keeps list of referencing doctors
- Maintains daily collection on referencing doctor/ institution
- Provides workload report.

The Computerized System is Easy to Operate

- Simple menu-based operations and does not require any detailed knowledge of computers
- Help facility at every stage of working for beginners
- Can find a patient detail based on of reference number, name, date and referencing doctor.

Features and Provisions

- Keeps the results for as long as you want
- Keeps normal values for male/female and adult/child for all tests
- Can change any normal value as per your equipment, techniques and methods
- Provision for reporting by different doctors
- Reports can be printed on simple paper or on preprinted letter heads (computer stationery).

Graphs

- Prints graphs, e.g. GTT for to the point reporting
- Can make/design your own graphs
- Can see the graph on screen as well as print.

Accounts

- Maintains your bank, cash accounts
- Provides all ledgers
- Makes trial balance for final accounts.

Address Manager

- A mail list program, keeps address details.
- Provides easy working on the basis of name
- Keeps addresses for Labmate program of referencing doctors, patients and reporting doctors
- Prints address directory with telephone numbers, etc.
- Prints labels for sticking on your mail
- Can group your addresses as per nature of address such as friend, relative, doctor, patient, etc.

The features given above are complete. All records can be retrieved date wise or name wise. Any program that provides the above-mentioned capabilities can be considered as an ideal laboratory reporter.

Caution: All medical electronic diagnostic devices need a stable constant voltage, therefore, proper protective cover must be provided. CVT (constant voltage transformer), servo stabilizers, and UPS (uninterrupted power supply) should be installed in the mainline or with specific instruments.

CHAPTER 2

Sterilization

The terms *sterilization* and *disinfection* are used to indicate the treatment of material so as to destroy or otherwise eliminate any living organisms present. However, the term sterilization is used where physical methods are used and disinfection is used where chemical agents are made use of.

METHODS COMMONLY USED FOR STERILIZATION

The methods used commonly in practice are:

1. *Killing organisms by heat:* Heat may be dry or moist
2. *Destroying organisms by employing chemical antiseptics,* e.g. lysol, phenol, perchloride of mercury, etc.
3. *Removing organisms mechanically by filtration,* e.g. Seitz, unglazed porcelain.

Sterilization by Heat

Adequate heat is the most certain and rapid method for sterilization. The time needed for sterilization is inversely related to the temperature of exposure—the higher the temperature, the shorter the time needed. High temperature kills bacteria by coagulating their proteins. Different types of bacteria show considerable differences in heat susceptibility. In general, vegetative forms are destroyed at lower temperatures, whereas high temperatures are needed for sporing organisms.

Dry Heat

This is the preferred method for sterilizing glassware, e.g. of glass syringes and of materials such as oils, jellies and powders which are impervious to steam. Dry heat requires a much higher temperature or a much longer time at the same temperature than does moist heat. Dry heat can be used in the following ways:

Flaming

The articles are passed through the Bunsen flame, without letting them become red hot. It is used for scalpels, needles, mouths of culture tubes, glass slides, coverslips and points of forceps. Only the surfaces actually touched by the flame are sterilized.

Red Heat

Platinum loops, inoculating wires and needles are heated in the Bunsen flame until red hot.

Hot Air Oven

These are electrically heated and thermostatically controlled. The oven itself is a double-walled steel chamber with a stout door. The top or side contains a ventilator which is left open during sterilization to disperse any moisture or volatile matter. Air circulates within the oven by convection currents. Suitable sterilizing times in the hot air oven are 3 hours at 140°C, 1 hour at 160°C and 30 minutes at 180°C. All dry glassware, such as test tubes, petri-dishes, flasks, pipettes and throat swabs, etc. are made sterile by using hot air oven.

This method is not suitable for sterilizing culture media, liquids, rubber connections, glass to metal fitting and fabrics, e.g. masks, towels or gowns.

Moist Heat

Temperature

A temperature of 60 to 65°C kills most vegetative bacteria (made use of in pasteurization of milk and preparation of vaccines).

Boiling

Boiling is frequently used for sterilizing syringes, etc. but is not adequate as many spores withstand this temperature.

Steam

Steam is the most effective technique of moist heat sterilization. Steam may be employed in three ways.

Steam at 100°C

The apparatus used commonly is called Koch's steamer. It has a vertical metal cylinder with a conical lid. It is fitted with a thermometer and has a small opening for escape of steam.

Sterilization by free steam can be done in two ways.

Prolonged exposure: For 1½ hours, used for broth or nutrient agar.

Intermittent heat or tyndallization: It involves exposure for 20 minutes on three successive days and is used to sterilize sugars and gelatin which decompose on higher temperatures.

Principle: Spores would germinate after first steaming and destroyed on the next, three steamings would eliminate all spores and their vegetative forms.

Low Temperature Steam

This method is employed for sterilizing materials (blankets, polyethene tubing, etc.), which would be damaged at higher temperatures.

Steam at Temperatures above 100°C (Autoclaving)

Autoclaves are made of strong metal jackets; strong enough to withstand high pressures required (Figs 2.1A to C). The autoclave door is hermetically sealed. It has a safety valve set to blow off at a predetermined pressure. The principle is that water boils when its vapor pressure is equal to the pressure of the surrounding atmosphere. If the pressure is raised inside a closed vessel, the temperature at which water boils will rise above 100°C. At 15 lbs pressure water boils at 120°C.

Following are the measures that must be taken care of during autoclaving:

a. The steam must be saturated.
b. There must be complete discharge of air from the sterilizing chamber.
c. The autoclave must be loaded in such a way that all the materials to be sterilized can be adequately penetrated by steam.
d. The duration of autoclaving would depend on the pressure inside and hence on the steam temperature.

FIG. 2.1B: Horizontal autoclave
(*Courtesy:* Yorco Sales Pvt. Ltd)

FIG. 2.1A: Vertical autoclave
(*Courtesy:* Yorco Sales Pvt. Ltd)

FIG. 2.1C: Precision autoclave
(*Courtesy:* Yorco Sales Pvt. Ltd.)

Method

1. Fill boiler with water to a point just below the basket bottom.
2. Place articles within the basket, bottles should not be more than 3/4 full and should have loosely screwed on caps.
3. Close the lid and tighten the screws.
4. Open outlet valve and adjust safety valve to the required pressure.
5. Turn on the heat source and when steam flows smoothly, close the vent-cock and let the internal pressure rise. See that all air has been expelled from the cylinder.
6. Let pressure rise to the required level and maintain at that level for the required period of time.
7. Switch off the heat source and let the pressure meter register zero. Open the vent-cock and the lid slowly.

(If the autoclave pressure is taken down very quickly—the fluid-filled bottles may burst).

Timings

10 lbs Pressure for 10 minutes—culture media.
15 lbs Pressure for 20 minutes—infected material.
20 lbs Pressure for 30 minutes—rubber gloves.

Inspissation

Used to sterilize serum containing media, e.g. Loeffler's for diphtheria and Dorset's, or Lowenstein's media for TB. The inspissator consists of a double-walled copper box, with water flowing between the 2 walls; the temperature is controlled between 75 to 80°C thermostatically. Sterilization is done for 2 to 3 hours on each of 3 successive days. A higher temperature may cause bubbling of the surface of the media.

Cold

Not used clinically.

Cold Shock

A sudden drop of temperature (e.g. 45 to 15°C) without actual freezing causes irregular contraction of cytoplasmic organelles leading to disorganization of cellular structures (95% drop in *E. coli* viable number is reported by using this method).

Freezing

This helps by (1) formation of ice crystals outside the cell by the withdrawal of water from the cell interior, increases the intracellular salt concentration—protein denaturation, and (2) formation of ice within the cell.

Ultraviolet Radiation

Ultraviolet (UV) rays of wavelength 2400 to 2800 Angstrom units are most effective. Low pressure mercury vapor type lamps can be used to produce UV rays. Take care that the UV rays do not directly enter the eyes. Gram-negative bacteria are destroyed more rapidly than gram-positive bacteria, spores are highly resistant and susceptibility of viruses is variable.

Ionizing Radiations

Cathode rays and gamma rays are the most effective and are being increasingly used to sterilize disposable items. These radiations have considerable disinfectant action.

Filtration

It may be used for the preparation from cultures of cell-free bacterial products, e.g. toxins and enzymes, to free virus containing fluids from bacteria and for the sterilization of media or media ingredients, which would be damaged by heating. For this purpose, filters with pores sufficiently small to hold back bacteria must be employed. Filtration is usually carried out under negative pressure, the fluid being sucked through the filter into a receiving flask, which is connected to an exhaust pump. During filtration, the filter surface may adsorb material carrying an opposite charge—the material adsorbed may be the one desired in the filtrate.

Seitz Filter

This employs filter that consists of a flat disk or asbestos material of special composition and is inserted into metal holders, which ensure a tight joint (Fig. 2.2). The disk is

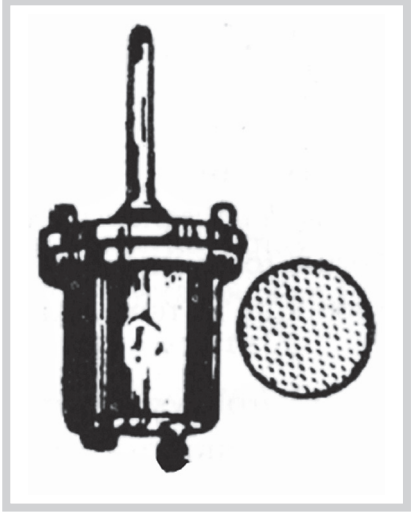


FIG. 2.2: Seitz filter
(*Courtesy:* Yorco Sales Pvt. Ltd)♥

used only once. The disks are available in three grades, viz. (1) clarifying (2) normal, and (3) special. This method is good for obtaining bacteria free filtrates. The large pore filters are good for filtration of serum for making media.

Collodion or Gradocol Membranes

These are virtually free of any adsorptive effects and have replaced Seitz filters considerably. These, too, are replaced after a single use.

Berkefeld and Mandler Filters

These are made from diatomaceous earth. Grades available are V—coarse, N—medium and W—fine.

Chamberland and Doulton Filters

These are made of unglazed porcelain. Available in various grades:

L/a—coarse clarifying.
L/a L2 and L3—medium.
L5 to L15—very fine.

Sintered Glass

It is made of a pad of finely ground glass fused into a glass cup.

Sand and Paper Pulp Filter

They are used for removing large particles and clearing emulsions, etc.

Chemical Sterilization

Chemical agents can exert bactericidal or bacteriostatic effect. The bactericidal agents in lower concentrations exert bacteriostatic effect. The bactericidal effect is probably because of enzyme inactivation either by protein denaturation, oxidation or by a combination of the antibacterial agent with specific groups of enzyme proteins.

Chemicals Used

Various chemicals used are:

1. *Chloroform (volatile antiseptic):* Used in preservation of serum for culture media at 0.25% concentration. Can be removed by heating to 56ºC.
2. *Phenol group:* Cresol, lysol (strong antiseptics) are mainly employed for surgical instruments, discarded routine cultures, and pipettes, slides, etc. and disinfecting hands. Phenol 0.5% is used for preserving sera and vaccines.
3. *Metallic salts:* Perchloride of mercury in 1:1000 strength solution.
4. *Glycerol:* A 50% solution is used for preservation of certain viruses. Glycerol also kills contaminating organisms.
5. *Formalin:* It is the only method for sterilizing Perspex or polythene and for killing cultures on plates. Can also be used for fumigation purposes.
6. *Sulfur:* Burning in air forms sulfur dioxide (SO_2) for fumigation.
7. *Halogens:* Chlorine and iodine. Chlorine for water disinfection and iodine for skin.
8. *Acids and alkalies:* Most bacteria grow in pH range of 5 to 9 and many grow at pH 7. Strong acids and alkalies can be used to disinfect contaminated materials.
9. *Alcohols:* These are used for disinfecting skin before injecting and before operations. Alcohols act by protein denaturation.
10. Two groups of dyes (a) the aniline dyes, and (b) the acridines have been widely used as skin and wound disinfectants.
11. Quaternary ammonium compounds are active against both gram-positive and gram-negative species but are not effective against spores and mycobacteria. They are used for sterilizing food utensils in restaurants and hotels and for disinfecting blankets in hospitals.
12. Glutaraldehyde (Cidex) affects even spores and mycobacteria. It is employed as 2% solution and is recommended for sterilizing cystoscopes, etc.
13. Ethylene oxide gas is being widely used to sterilize disposable plastic syringes, petri dishes, etc.

Glassware Preparation for Use

Selection

Grade A glassware needs no testing. Grade B is satisfactory for most routine work. Others should be heat resistant, have a low coefficient of expansion and be free from soluble metals and free alkali; in addition, they should be mechanically strong.

Cleaning Glassware

New: Look for cracks if any. Soak in 2% HCl for overnight to neutralize any alkali present. Wash in running water. Boil in synthetic detergent for 30 minutes, rinse in tap water and finally in distilled water.

Used glassware should be rinsed immediately after use. Boil in a detergent for 30 minutes and clean thoroughly with a brush, rinse in tap water and finally in distilled water. Dry them in the oven with temperature not exceeding 80°C.

Dichromate Cleaning Solution

Dissolve 25 g potassium dichromate in 25 mL of water. Add 50 mL concentrated sulfuric acid (slowly, always add acid to water and not (vice-versa), cool, store it in a stoppered bottle; discard when it starts turning green.

Petri Dishes

- Autoclave to remove infected material
- Wash in soapy water
- Rinse in running water, let dry
- Rinse in methylated spirit, let dry
- Sterilize in hot air oven.

Pipettes

- Soak in chromic acid solution overnight
- Wash in running water
- Rinse in distilled water
- Dry on suction pump using methylated spirit, or ether, or methylated acetone
- To sterilize—plug mouth piece with nonabsorbent cotton wool, wrap in kraft paper and hot air sterilize (160°C for 1 hour).

Test Tubes

- Autoclave to remove infected material
- Boil in detergent solution for 30 minutes
- Clean with a brush
- Rinse in running water, rinse in distilled water and place them in a wire basket upside down and dry in an oven
- Cotton plug them and sterilize in the hot air oven.

Pasteur Pipettes

- Soak in 3% lysol for 1 hour
- Wash as before (as for test tubes).

Screw-capped Bottles

- Wash the liners separately
- Dry quickly to avoid corrosion
- Sterilize metal caps and their bottles in hot air oven
- Rubber liners—autoclaved.

Glass Slides

New

- Boil in a detergent for 30 minutes
- Place in dichromate for overnight
- Wash in running water
- Keep in methylated spirit
- For using, take them out with a forceps and hold them only by the edges.

Used

- As for used glassware.

Infected Slides

- Should be autoclaved and then cleaned as mentioned earlier (never use slides used for examining acid fast bacilli for the same purpose).

Infected Glassware

Contaminated material—may be disposed in paper or cardboard wrappers and incinerated.

Autoclave the glassware that has been contaminated. Having autoclaved, wash and prepare in the usual way.

Syringes

Complete bacterial sterility can be achieved either by sterilizing them in the hot air oven or in an autoclave. Keep injection syringes separate from blood withdrawing syringes. Fresh syringes (sterilized) should be used for withdrawing blood for each patient. Before reusing them, clean them properly and then sterilize them. Needles used should be sharp and not with blunted ends.

Choice of Syringes and Needles

All glass syringes are preferred over glass and metal ones. Preferably keep size 5 mL or more syringes for withdrawing blood. Needles should be of size equal to or less than 21 (SWG). A needle with a smaller diameter would cause lysis of blood when used for blood withdrawing. Withdrawing needles should be at least an inch long.

New Syringes

These are washed in the usual way. Dried with acetone. Wrap the plunger and the barrel in a paper and sterilize in hot air oven.

Used Syringes

Immediately after use, wash them thoroughly with cold water (hot water will coagulate proteins and will make the syringes difficult to clean). Clean them thoroughly in a detergent, brush the barrel properly, rinse in tap water and then in distilled water. Rinse in acetone and let dry. Sterilize in hot air oven as mentioned above.

Infected Syringes

These should be washed at first with cold 2% lysol solution and then clean as above. Syringes infected with highly virulent material should at first be autoclaved. The syringes should be placed in the cold oven and be heated at 160°C for 90 minutes. Syringes not used for 3 months should be resterilized before use.

Glass Barrel and Metal Plunger Syringes

The only precaution to be taken here is that metal corrosion should be avoided and the barrel and the plunger should be sterilized separately (kept in a wrapper) by autoclaving them.

Needles

- Should first be rinsed in cold water
- Clean the mounts with a cotton-wool swab
- Wash again, rinse in acetone
- Pass a stylet through the hole to remove any plugs if present
  (It is important to discard all needles with blunted tips, a hand lens can be used to examine needle tips)
- Serum hepatitis and HIV can be transmitted through using imperfectly cleaned and sterilized needles
- The needle should be sterilized in hot air oven.

Disinfection of Syringes by Boiling

In an emergency, syringes can be effectively sterilized by boiling them in distilled water for at least 5 minutes after having cleaned them in the usual way.

Disposable Sterile Syringes

This is the world and time of disposables. In the interest of the patient every laboratory should ideally use disposable syringes only.

As far as possible use disposable, sterilized plasticware instead of glassware.

MODERN DAY DISINFECTION

(Commercially available from Bioshields)

Prevention before Cure

Air, land and water are the essential elements around which diverse life forms our planet "Earth" thrive and survive.

Precious human life needs to be protected against the challenge mounted by microbes in day to day life as well as professional settings of infectious agents. Build up of resistance and development of resistant strains continues to challenge preventive health care and infection control professional globally.

To overcome these challanges and to empower infection control professional, scientists have researched, designed and developed potent, effective and safe disinfectant and antiseptic solution for medical, industrial and general use.

Icons Used

Bioshields has created appropriate icons for easy visualization and understanding of the product application, intended use of any relevant product and product highlights. These icons are especially useful to understand the usage potential of products, as many products have multiple applications. These icons are displayed prominently on the product labels.

Hand Care

For generations, handwashing with soap and water has been considered a measure of personal hygiene. The concept of cleansing hands with an antiseptic agent probably emerged in the early 19th century. In 1846, Ignaz Semmelweis observed that physicians who went directly from the autopsy suite to the obstetrics ward has a disagreeable odor on their hand despite washing their hands with soap and water upon entering the obstetrics clinic. He postulated that the puerperal fever that affected so many parturient women was caused by cadaverous particles transmitted from the autopsy suite to the obstetrics ward via the hands of the students and physicians. Perhaps because of the known deodorizing effect of chlorine compounds, as of May 1847, he insisted that students and physicians clean their hands with a chlorine solution between each patient in the clinic. The maternal mortality subsequently dropped dramatically and remained low for years. This intervention by Semmelweis represents the first evidence indicating that cleansing heavily contaminated hands with an antiseptic agent between patient contacts may reduce health care associated transmission of contagious diseases more effectively than handwashing with plain soap and water.

To understand the objectives of different approaches to hand cleansing, knowledge of skin and normal bacterial skin flora is essential. The skin is often known as the largest organ in the human body. The basic structure of skin includes the superficial region (i.e. the stratum corneum), the viable epidermis, the dermis and the hypodermis. The primary function of the skin is to reduce water loss, provide protection against abrasive action and microorganism, and also act as permeability barrier to the environment. Normal human skin is colonized with bacteria; different areas of the body have varied bacterial counts. Total bacterial counts on the hands of medical personnel have ranged from 3.9×10^4 to 4.6×10^6. Price (1938) divided the bacteria found on skin onto two types, namely, those normally permanent (resident flora) and those normally temporary (transient flora). Resident flora, which are attached to deeper layers of the skin, are more resistant to removal. The resident flora consists of species that can resist both the antimicrobial substances excreted on skin and in sweat and also moderate desiccation. They also have an innate ability to adhere to epithelial cells. The predominant flora is composed of coagulase negative staphylococci, mainly *Staphylococcus epidermidis.* Other species implicated are *Acinetobacter*, *Klebsiella,*

Corynebacteria and *Propionibacteria species*. The resident flora (Noble, 1981) forms microcolonies on skin and is attached to skin scales, which tend to be shed into the environment at a great rate, the whole superficial layer of the skin being shed every few hours. The bacterial flora is normally harmless but when transferred to an immunocompromised person could result in clinical conditions. The resident flora is also more resistant to easy removal by mechanical means. Hence, a minimum of 5 minutes of diligent hand wash is often required to result in significant reduction. Transient flora, which colonizes the superficial layers of the skin, are more amenable to removal by routine handwashing. They are often acquired by HCWs during direct contact with contaminated environmental surfaces within close proximity of the patient. They are most frequently associated with health care associated infections. The flora can be varied; they include *Staphylococcus aureus, Pseudomonas*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus* (VRE).

The hygienic hand wash aims on mechanically remove dirt and loosely adherent transient flora and, simultaneously, to inactivate strongly adherent transient flora and parts of the resident skin flora. While the aim of the hygienic handrub is to reduce the release of members of the transient microbial skin flora, without regard of the resident flora, with maximum efficacy and speed to render hands safe after known or suspected contamination. It involves the elimination of a substantial part of the transient flora by 'killing' it on the hands rather than by mechanical removal. A surgical hand scrub procedure involves the aims at a marked reduction of the resident flora.

Strategies for the prevention of hand associated microbial transfer must consider the microbial flora to be of importance in a given situation. In the wards, the transient flora is often accidentally picked up from an infections source and must be prevented from being transmitted via hands to a susceptible target. The normal skin resident skin flora is often of little consequence in this situation. However, in the operating area and in some special situations such as reverse isolation or a hemodialysis unit or during the outbreaks of hospital infection, the resident flora may play an additional important role as a cause of nosocomial infection.

Various agents used are:
- 0.5% w/v triclosan
- Chlorhexidine
- Isopropyl alcohol.

Antiseptics

General Antisepsis

The word hygiene comes from Hygeia, the Greek goddess of health, who was the daughter of Asclepius, the god of medicine. The discovery of the germ theory of disease in the second half of the 19th century, hygiene and sanitation have been in the forefront of the struggle against illness and death. Advances in scientific medicine hygiene and sanitation have resulted in unprecedented longevity and improved quality of life in the last century and a half of medical history. Of particular importance in medical history, puerperal fever was one of those dreaded diseases that intrigued and baffled doctors in the 19th century. Just as Dr Semmelweis had predicted, the disease was conquered when obstetricians began washing their hands between deliveries. Puerperal fever was simply eradicated with cleanliness.

The skin is often known as "the largest organ in the human body". The skin weights more than any single internal organ, accounting for about 15% of body weight and a surface area of 1.5 to 2.0 square meters, most of it between 2 to 3 mm thick. It is an organ of the integumentary system made up of a layer of tissues that protect underlying muscles and organs. As the only interface with the surroundings, it plays the most important role of protection against pathogens. However, the skin also supports its own ecosystem of microorganisms. In general, these organisms keep one another in check and are harmless but certain factors like pH imbalance, skin stripping or breach in the epithelial lining of the skin could result in infections of these microorganisms.

The word 'antiseptic' has acquired the special meaning of an antimicrobial agent (microbicidal/microbistatic), suitable for application to living tissues and intended to reduce the viable count or inhibit the growth of the microbial flora. Skin antisepsis has moved on from the confines of hospital care and treatment to now play an essential role in secondary and tertiary health care setups, thereby also going a large footage in home care and maintenance of general personal hygiene.

A preoperative antiseptic shower/bath decreases skin microbial colony counts. In a study of > 700 patients who received preoperative antiseptic showers, chlorhexidine reduced bacterial colony counts nine fold (2.8×10^2 to 0.3). Systematic studies proving the role of antiseptics in controlling various hospital infections like the above-mentioned one have generated an awareness and appreciation of the role of antiseptics in infection control.

Specialized Antisepsis

Hairdressing: Microorganisms are everywhere and are continually introduced into the environment. They live on skin, in food and dirt. Infection can also occur during hairdressing procedures. Items such as razors, scissors, combs, clippers and hairpins can accidentally pierce the skin. Blood and body fluids do not have to be visible on instruments or working surfaces for infection to be transmitted. In such cases, microorganisms are easily spread between clients and operators and are easily transferred by contact with unwashed hands, soiled equipment or contact with blood and body substances. Both clients and operators are at a risk.

Successful hairdressing businesses supply their clients with professionally competent, safe and hygienic services in clean and congenital premises. To do otherwise by following unhygienic or unsafe procedures or to allow premises, furnishing/fittings to become dirty or poorly maintained will not only threaten the commercial success of the business, but can lead to conditions that jeopardize the health of both clients and operators and thereby contribute to the spread of highly infectious diseases.

It is essential to know and understand the health implications of the procedures carried out and the precautions that must be taken to minimize health risks. In developing effective infection control strategies in the hairdressing industry, operators must identify situations where there is a significant risk of spreading harmful microorganisms and intervene at an appropriate time to prevent the spread.

Instruments: Skin that is intact without cuts or abrasions is a natural protective barrier against infection but cutting, piercing, nicking the skin can introduce infectious microorganisms into the body. Some bacterial infections can occur without breaking the skin and for this all equipment must be cleaned between each client. The patient at risks may be the next client on whom the contaminated instrument is used. Operators may also be at risk if they have any open cuts, sores/broken skin that comes in contact with the contaminated instrument. Some of the infections that can be spread in hairdressing premises include.

Skin infections (including scalp face and neck)

- Staphylococcal infections such as impetigo
- Fungal infections on the scalp such as *Tinea capitis*.

Blood infections

- HIV
- Hepatitis B and C.

It therefore, becomes necessary to also use the right disinfectants and sterilization methods in order to obtain effective infection control. Very often it has been observed that the disinfectant solution used are inappropriate which are not capable of providing complete protection.

Hospitality industry: Hospitality industry related infections date back to the early 70s when two cases gained considerable mileage. A hotel in Philadelphia was the site of the outbreak that made 221 people ill and killed 24, leading to the discovery of "legionnaires disease" a disease caused by contaminated water. Prior to that, Mary Mallon was identified as the first healthy carrier of typhoid who carried over the infection owing to her cooking profession. Both these separate incidents served as an eye opener to the increasing need for efficient disinfection policies in this industry.

The hotel and food industry is an important industry closely linked tourism, business travel and conventions which from a significant part of the economy. In providing a high standard of service to customers, it becomes an essential prerequisite to ensure a safe and healthy environment.

As the Hazard analysis and critical control points (HACCP) defines it, a "hazard" is anything that could cause harm to the consumer. There are three main hazards that arise with food served in catering premises. These are contamination of food by:

- Bacteria/other microorganisms that cause food poisoning *(Salmonella, Shigella, Campylobacter, Aspergillus)*, virus infections (Hepatitis, Creutzfeldt Jacobs disease), parasites (nematodes, herrings and other relevant worms).
- Chemicals for example by cleaning materials or pest baits.
- Foreign materials such as glass, metal, plastic and so on.

Of these, the most likely to be harmful are bacteria/other germs.

A number of critical care points if addressed efficiently could drastically reduce the number of food hazards that occur annually which along with huge economic losses lead to impending ill-health.

Various agents used are:

- Chloroxylenol
- Terpineol
- Triclosan
- Isopropyl alcohol
- Cetrimide.

Skin Preparatives

Long before the discovery of bacteria and the introduction of antiseptic surgery, a variety of substances had been

used to prevent infections. Pasteur's initiation of the science of bacteriology was probably the foundation of the development and use of skin antisepsis. Joseph Lister an academic surgeon who was greatly influenced by Pasteur's works and bacteria causing infection causing infection ventured into 'antiseptic surgery'. His solution was to apply some chemical substance 'in such a manner that not only would the microbes already present be destroyed, but also the germ killing substance would act as a barrier between the wound outside source of infection'. Lister hit on the idea of using carbolic acid, which was first used on compound fractures. Later, the method was refined by use of different concentrations of carbolic acid and extended to instruments, ligatures and even room air.

Among surgical patients, surgical side infections (SSIs) were the most common nosocomial infection accounting for 38% of all such infections. When surgical patients with nosocomial SSI died, 77% of the deaths were reported to be related to the infection, and the majority (93%) were serious infections involving the organs or spaces accessed during the surgery. Microbial contamination of the surgical site is a necessary precursor of SSI. Quantitatively it has been shown that if a surgical site is contaminated with > 10^5 microorganism per gram of tissue, the risk of SSI is markedly increased. For most SSIs, the source of pathogens is the endogenous flora of the patient's skin, mucous membranes or hollow viscera. When mucous membranes or skin is incised, the exposed tissues are at risk of contamination with endogenous flora. These microorganisms are usually aerobic gram-positive cocci (e.g. staphylococci) but may also include fecal flora (e.g. anaerobic bacteria and gram-negative aerobes). The flora may also change as per the site of the incision of the type of organ exposed during surgery. An important SSI prevention measure would include techniques directed at reducing microbial flora by localized skin prepping (at the surgical site). Before the skin preparation of a patient is initiated, the skin should be free from gross contamination (i.e. dirt, soil or any other debris). The patient's skin is prepared by applying an antiseptic in concentric circles, beginning in the area of the proposed incision. This procedure is a vital step in removing all transient microorganisms and ensuring an extremely sub minimal population of resident flora (Table 2.1).

Since the days of Lister, antiseptic development has been in a state of flux. Despite this, SSIs remain a substantial cause of morbidity and mortality among hospitalized patients. Thus, to reduce the risk of SSI, a systematic but realistic approach must be applied with the awareness that this risk is influenced by characteristics of the patient, operation, personnel and hospital.

TABLE 2.1: Summary of CDC recommendations

1.	Preoperative: Preparation of the patient Hand/forearm antisepsis Management of infected/colonized surgical personnel (Anti -microbial prophylaxis)
2.	Intraoperative: Ventilation Cleaning and disinfection of environmental surfaces Microbiologic sampling Sterilization of surgical instruments Surgical attire and drape Asepsis and surgical technique
3.	Postoperative incision care
4.	Surveillance

Source: CDC guidelines for prevention of SSI, 1999.

Environment and Surfaces

The health care environment contains a diverse population of microorganisms, but only a few are significant pathogens for susceptible humans. Microorganisms are present in great numbers in moist organic environments, but some can also persist in dry conditions. Although, pathogenic microorganisms can be detected in air and water and on fomites, assessing their role in causing infection and disease is difficult. The surface and environment therefore would be considered one of a number of potential reservoirs for the pathogen but not the 'de facto' source of exposure. An understanding of how infection occurs after exposure based on the principles of the "chain of infection", is important in evaluating the contribution of the environment to health care associated diseases.

Chain of infection components comprises of (a) adequate number of pathogenic microorganisms, (b) pathogenic microorganisms of sufficient virulence, (c) a susceptible host, (d) an appropriate mode of transmission or transferal of the microorganism in sufficient numbers from the source to host, (e) the correct portal or entry into the host. The presence of the susceptible host is one of these components that underscore the importance of healthcare environment and opportunistic pathogens on fomites and in air and water. All of the components of the 'chain' must be operational for the infection to occur.

A variety of airborne infections in susceptible hosts can result from exposure to clinically significant microorganisms that are released into the air when environmental reservoirs (i.e. soil, water, dust and decaying organic

matter) are disturbed. Once these materials are brought indoors into a health care facility by any of a number of vehicles (e.g. people, air currents, water, construction materials and equipment), the attendant microorganisms can proliferate in various indoor ecological niches and if subsequently disbursed into the air, serve as a source for airborne health care associated infections. It can be observed that the infection cycle then is completed very quickly resulting in extensive infections.

As a result of advances in medical technology and therapies (e.g. cytotoxic chemotherapy and transplantation medicine), more patients are becoming immunocompromised in the course of treatment and are therefore at an increased risk of acquiring health care associated opportunistic infections. Trends in health care delivery (e.g. early discharge of patients from acute care facilities) also change the distribution of patient populations and increase the number of immunocompromised patients in non-acute care hospitals.

The environment is often overlooked as a passive player in hospital acquired infections owing to the emphasis laid on other more risky modes of transmission. However, with the ever-changing face of modern medicine disrupting the existing environmental stability could open a Pandora's box of dangerous environmentally linked infections.

A couple of such cases observed in the recent years (a) transmission of infections caused by *Mycobacterium tuberculosis, Varicella Zoster Virus (VZV),* and Measles facilitated by inappropriate air-handling systems in health care facilities; (b) disease outbreaks caused by *Aspergillus* species, *Mucoraceae*, and *Penicillium* species associated with the absence of environmental controls during periods of health care facility associated construction; (c) infections and/or colonization of patients and staff with vancomycin resistant. *Enterococcus faecium* [VRE] and *Clostridium difficile* acquired indirectly from contact with microorganisms present on environmental surfaces in health care facilities; and (d) outbreaks of pseudoepidemics of *Legionellae*, *Pseudomonas aeruginosa*, and the non-tuberculous mycobacteria (NTM) linked to water and aqueous solution in health care facilities.

In many instances, it is still difficult to decide on the appropriate method of decontamination even after taking into consideration the nature and risk involved. However, it is useful to remember that the risk of transmitting infection from a surface that has been thoroughly cleaned and disinfected is very small. Thorough cleaning removes potential bacterial nutrients (organic matter) as well as a significant load of bacteria. Studies have shown prior cleaning achieves approximately a 4-log reduction of microorganisms. Disinfection achieves a 99.9% reduction if performed using the right disinfectant as per the standardized procedure. Disinfection of hard surfaces is performed by combination of a cleaning and disinfecting agent. Thus, it can be seen that cleaning is an essential prelude effective disinfection and one should not underplay the importance of either of the processes.

Various agents used are:

- Silver nitrate
- H_2O_2 (hydrogen peroxide)
- Benzalkonium chloride
- Isopropyl alcohol.

Instruments

Formal procedures for sterilization of instruments and medical devices, liquids, and other materials used in hospitals have developed over a century. The initiation of these procedures began during a time when microorganisms were strongly implicated in the transmission of infectious diseases and hence the need to use sterile materials in surgery and other hospital related activities. Although the concept of hospital infection control was in its infancy, hospitals and medical-device industry began to sterilize instruments and materials used to treat patients.

Earlier methods of sterilization used different forms of heat such as dry heat, moist heat to sterilize medical devices and instruments. The 50s marked the increased use of heat sensitive devices and hence the need for low temperature sterilization methods or liquid chemical germicides.

Sterilization and disinfection are two terms, which are often used interchangeably. However, sterilization is a more absolute term, which implies complete elimination or destruction of all forms of microbial life. Disinfection on the other hand describes a process that eliminates many or all pathogenic microorganisms, with the exception of bacterial spores. The success of these processes is largely dependent on the right method of choice, which often became a difficult decision for health care professionals. In 1968, Dr EH Spaulding devised a rational classification scheme that could be used to decide whether a medical instrument needs to be sterilized or disinfected. His classification was extremely simple yet logical and has been endorsed and adapted by the CDC, FDA and numerous other reviewers and professional societies today. The Spaulding classification revolved around the central idea that medical devices or items need to be categorized based on the risk of infection involved in their use (Table 2.2 and 2.3).

TABLE 2.2: Spaulding classification system

Device classification	*Devices*	*Spaulding process classification*	*EPA product classification*
Critical (enters sterile tissue or vascular system)	Implants, scalpels, needles, other surgical instruments, etc.	Sterilization-sporicidal Chemical: prolonged contact	Sterilant/disinfectant
Semi-critical (touches mucous membranes; except dental)	Flexible endoscopes, laryngoscopes, endotracheal tubes, and other similar instruments. Lab instruments Thermometers, hydrotherapy tanks	High level disinfection-sporicidal. Chemical: Short contact Intermediate-level disinfection	Sterilant/disinfectant Hospital disinfectant with label claim for tuberculocidal activity
Non-critical (touches intact skin)	Stethoscopes, tabletops, bedpans, etc.	Low level disinfection	Hospital disinfectant without label claim for tuberculocidal activity

TABLE 2.3: Modified spaulding scheme (dental)

Classification	*Area of use*	*Dental instrument/item*
Critical	Penetrates soft tissue, contacts bone, enters into or contacts the bloodstream or other normally sterile tissues	Surgical instruments, periodontal sealers, scalpel blades, surgical dental burs
Semi critical	Contacts mucous membrane or non-intact skin; does not penetrate soft tissue, contact bone, enters into or contacts the blood stream or other normally sterile tissues	Dental mouth mirror, amalgam condenser, reusable dental impression trays, dental handpieces
Noncritical	Contacts intact skin	Radiograph head/cone, blood pressure cuff, facebow, pulse oximeter

Undoubtedly, the Spaulding classification is an oversimplification especially when applied to the new mantra of modern medicine endoscopes.

Endoscopes are notorious to nosocomial infections. Reports of nosocomial infections related only to endoscopes state a wide variety of infections transmitted by various scopy procedure like gastrointestinal endoscopies and bronchoscopy. The clinical spectrum of these infections ranged from asymptomatic colonization to death. Major reasons for transmission are inadequate cleaning and improper selection of a disinfectant. Very often the fear of the dreaded blood borne infectious disease like HIV and HBV results in overlooking the more challenging microorganisms. It thus, becomes helpful to know the resistance pattern of some frequently encountered microorganisms.

Prions — **Hard to kill**
Spores
Mycobacteria
Non-enveloped viruses
Fungi
Bacteria
Enveloped viruses — **Easy to kill**

As more and more heat labile instruments enter the medical arena, foolproof disinfection of these instruments owing to their heat sensitivity and complex structures is a challenge to health care professionals and manufactures alike. Numerous disinfectants are available in the market; glutaraldehyde continues to be the most commonly used disinfectant owing to its effectiveness and broad range of material compatibility. Other chemicals like per acetic acid, hydrogen peroxide and more recently orthophthaldehyde are also used for high-level disinfection or cold sterilization.

Laboratories have become an integral part of health care, industrial and pharmaceutical organizations. The function of laboratories in each of these areas varies. A laboratory could cater to diagnostic, research and quality control procedures. As the complexities of the procedures increase disinfection and infection control acquires a more important role.

Laboratorians working with infectious agents are subject to laboratory acquired infections as a result of accidents, unrecognized incidents and improper disinfection. The degree of hazard depends upon the virulence of the biological agent concerned and host resistance. Other than infections contamination of work material often lead

to valuable waste of time, money and may also result in false interpretation of results.

Modern medicine has undoubtedly reduced mortality and morbidity rates with prompt diagnostic and therapeutic measures. The blood related infectious diseases gained a new face of terror among health care workers when reports of the first needle stick related infections hit headlines. On one hand as health care aims at combating existent health perils, on the other it faces new challenges such as HIV and HBV.

Awareness camps, systematic waste segregation, safe handling of laboratory wastes including laboratory samples and glassware; culture of stocks of infectious agents became important aspects of infection control. In all these wastes, the major concern is to prevent accidental transmission of infection.

There is growing trend in health care settings to provide or reuse disposable materials to reduce cost factor. Such practices could further lead to transmission of diseases unless appropriate changes are made in the routine handling.

Thus arises the million-dollar question; "Should one decontaminate before disposal?" Yes, in order to ensure complete protection to personnel handling laboratory waste prior and post disposal it becomes necessary to decontaminate. The United states environment protection agency (USEPA) recommends the cleaning and decontamination of laboratory glassware prior to sterilization as a key step to effective and foolproof sterilization and adequate protection.

The practice of density involves high risk of infection by both cross contamination amongst patients and direct transmission to the dental health care professionals. They are often exposed to potent organisms including Cytomegalovirus, HBV, HCV, Herpes simplex virus, HIV, *Mycobacterium* spps, *Staphylococcus, Streptococcus* and other viruses and bacteria that colonize or infect the oral cavity and respiratory tract. The most frequent routes of transmission in dental settings are (1) direct contact with blood/oral fluids or other patient materials, ♥(2) indirect contact with contaminated objects (e.g. instruments, equipments, environmental surfaces) (3) contact of conjunctival, nasal/oral mucosa with droplets (e.g. spatter), containing microorganisms generated from an infected person and propelled at a short distance (by coughing, sneezing or talking) (4) inhalation of airborne microorganisms that can remain suspended in the air for long periods.

Infection control is an important element of safe dental practice. Whilst many of the disinfection and sterilization issues relevant to dentistry are generic and no different from those in other areas of healthcare, dental practice raises some particular problems. These include high patient turnover, use of large numbers of small intricate devices, varying degrees of invasiveness and point of use sterilization.

The emergence of Human Immunodeficiency Virus (HIV) in the early 1980s prompted a major review of infection control procedures in dentistry.

The revised CDC emphasizes on the use of appropriate sterilization and disinfection methods to curb infections via contaminated instruments, although the basic Spaulding scheme still forms the basis of the right choice and use of disinfectants. It has been customized to meet the specific requirements of dental health care personnel and patients to ensure complete control of infections (Table 2.2).

The choice of specific disinfection agents is largely a matter of judgment, guided by product label claims and instrumentation and government regulations. A single liquid chemical germicide might not satisfy all disinfection requirements in a given dental practice or facility. Realistic use of liquid chemical germicides depends on consideration of multiple factors including the degree of microbial killing required; the nature and composition of the surface, item or device to be treated; and the safety, cost and ease of use of the available agents.

Various agents used are:

- Glutaraldehyde
- Benzalkonium chloride
- 5% phenol.

CHAPTER 3

SI Units

The SI units (*Système International d'Unités*) have replaced the old system of reporting and measurements. This is in accordance with a World Health Organization resolution which recommends the adoption of the International System of Units by the medical community throughout the world. Consequently, reports and measurements from any corner of the world can be safely understood anywhere else.

The SI system is based on meter-kilogram-second system and replaces both the foot-pound-second system and the centimeter-gram-second system. There are seven SI base units, i.e. meter, kilogram, second, mole, ampere, Kelvin and Candela. The symbols for these units and what they measure are listed in Table 3.1.

LITER

The SI unit of volume is cubic meter (m^3). This is a very large unit, hence, the liter (L) although not an SI unit, has been recommended for use in the laboratory.

The liter is equal to a cubic decimeter (1 dm^3). Volume measurements are made in liters or multiples and submultiples of the liter, e.g. dL (10^{-1}L), mL (10^{-3}L), µL (10^{-6}L).

SI unit	*Old unit*
dL	100 mL
mL or cm^3	cc
µL	lambda
nL	—
pL	µ µ L

One liter is, therefore, equivalent to 10 dL, 1000 mL or 1000 000 µL. One dL is equivalent to 100 mL, and 1 mL to 1000 µL.

GRAM

The kilogram (kg) is the SI unit for mass and the gram (g) is the working unit.

Formerly, the gram (g) was written as *gramme*, or gm.

Mass measurements are made in grams or in multiples and submultiples of the gram, e.g. mg (10^{-3}g), µg (10^{-6} g), ng (10^{-9}g), pg (10^{-12}g).

One g is, therefore, equivalent to 1000 mg, 1000 000 µg, or 1000 000 000 ng. One mg is equivalent to 1000 µg.

SI unit	*Old unit*
nm	m µ
µ m	µ (micron)

MOLE (MOL)

The mole (mol) is the SI unit for amount of substance and measurements of the amounts of substances are made in moles, or in mmol (10^{-3} mol), µmol (10^{-6} mol), or nmol (10^{-9} mol).

One mol is, therefore, equivalent to 1000 mmol, 1000 000 µmol, or 1000 000 000 nmol. One mmol/L is equivalent to 1000 µmol/L.

Earlier, the results of tests expressed in mmol/L or µmol/L were expressed in mg/100 mL or µg/100 mL.

TABLE 3.1: The symbols of units and what they measure

SI base units			*SI unit prefixes*			
	Symbol	*Quantity measured*	*Prefix*	*Symbol*	*Function*	*Divide by*
Meter	m	Length	deci	d	10^{-1}	10
Kilogram	kg	Mass	centi	c	10^{-2}	100
Second	s	Time	milli	m	10^{-3}	1000
Mole	mol	Amount of substance	micro	µ	10^{-6}	1000 000
Ampere	A	Electric current	nano	n	10^{-9}	1000 000 000
Kelvin	K	Temperature	pico	p	10^{-12}	1000 000 000 000
Candela	cd	Luminous intensity	femto	f	10^{-15}	1000 000 000 000 000
SI derived units						*Multiply by*
Square meter	m^2	Area	deca	da	10^{1}	10
Cubic meter	m^3	Volume	hecto	h	10^{2}	100
Meter per second	m/s	Speed	kilo	k	10^{3}	1000
Named SI derived units			mega	M	10^{6}	1000 000
Hertz	Hz	Frequency	giga	G	10^{9}	1000 000 000
Joule	J	Energy, quantity of heat	tera	T	10^{12}	1000 000 000 000
Newton	N	Force	peta	P	10^{15}	1000 000 000 000 000
Pascal	Pa	Pressure				
Watt	W	Power				
Volt	V	Electric potential difference				
degree Celsius	°C	Celsius temperature				

SI unit	*Old unit*
mol	M
mmol	mEq
µmol	µM
nmol	nM

The formula used to convert mg/100 mL to mmol/L is as follows:

$$\text{mmol/L} = \frac{\text{mg/100 mL} \times 10}{\text{molecular weight of substance}}$$

where the molecular weight of a substance cannot be accurately determined (e.g. albumin), results are expressed in g/L.

INTERNATIONAL UNIT (U)

This unit is used to express enzyme activity. An International Unit of enzyme activity is that amount of enzyme which under defined assay conditions will catalyze the conversion of 1 µmol of substrate per minute. Results are expressed in International Units per liter (U/L).

CONVERSION FACTORS BETWEEN CONVENTIONAL AND SYSTEM INTERNATIONAL UNITS (SIU)

This list is included to assist the reader to convert values between conventional units and the newer SI units that have been mandated by many journals. Only common analytes are included (Tables 3.2 to 3.5).

TABLE 3.2: Hematology

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
WBC count (leukocytes) (B)	µL or/cu mm or/mm^3	cells 10^9/L	0.001	1000
(CSF)	/cu mm or	10^6/L	1	1
	→ cu µL	10^6/L	10^6	10^{-6}
(SF)	#/µL	#/L	10^6	10^{-6}
Platelet count	10^3/cu mm	10^9/L	1	1
Reticulocytes	/cu mm	10^9/L	0.001	1000
RBC count (erythrocytes) (B)	10^6/µL or /cu mm	10^{12}/L	1	1
(CSF)	or/mm^3/cu mm	10^6/L	1	1
Hematocrit [packed cell volume (PCV)]	%	Volume fraction	0.01	100
Mean corpuscular volume (MCV)	μ^3 (cubic microns)	fL	1	1
(volume index)				
Mean corpuscular hemoglobin (MCH)	pg (or µg)	pg	1	1
(color index)	pg	fmol	0.06206	16.11
Mean corpuscular hemoglobin	g/dL	g/L	10	0.1
concentration (MCHC)	g/dL	mmol/L	0.6206	1.611
(saturation index)				
Hemoglobin	g/dL	g/L	10	0.1
(whole blood)	g/dL	mmol/L	0.155	6.45
(plasma)	mg/dL	µmol/L	0.155	6.45
Fetal hemoglobin	%	mol/mol (may omit symbol)	0.01	100
Haptoglobin	mg/dL	mg/L	10	0.1
Fibrinogen	mg/dL	g/L	0.01	100

TABLE 3.3: Chemistry

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
Adrenocorticotropic hormone (ACTH)	pg/mL pg/mL	ng/L pmol/L	1 0.2202	1 4.541
Aldosterone				
(S)	ng/dL	nmol/L	0.0277	36.1
(U)	mEq/24 h	mmol/d	1	1
(U)	µg/24 h	nmol/d	2.77	0.36
Angiotensin	ng/dL pg/mL	ng/L ng/L	10 1	0.1 1

Contd...

Contd...

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
Angiotensin-converting enzyme (ACE)	nmol/min/mL	U/L	1	1
Antidiuretic hormone (ADH)	pg/mL	ng/L	1	1 (vasopressin)
Albumin				
(S)	g/dL	g/L	10	0.1
(CSF, AF)	mg/dL	mg/L	10	0.1
Alpha antitrypsin	mg/dL	g/L	0.01	100
Alpha-fetoprotein (AFP)	ng/mL	µg/L	1	1
(S)	ng/dL	ng/L	10	0.1
	mg/dL	g/L	0.01	100
	mg/dL	mg/L	10	0.1
	µg/dL	µg/L	10	0.1
Ammonia	µg/dL	µmol/L	0.714	1.4
(P)	µg/dL	µmol/L	0.5872	1.703
Anion gap	mEq/L	mmol/L	1	1
Base excess	mEq/L	mmol/L	1	1
Bicarbonate	mEq/L	mmol/L	1	1
Bilirubin	mg/dL	µmol/L	17.1	0.0584
Calcitonin	pg/mL	ng/L	1	1
Catecholamines (U)	µg/24 h	nmol/d	5.91	0.169
Norepinephrine	µg/mg creatinine	µmol/mol creatinine	669	0.00149
	pg/mL	pmol/L	5.91	0.169
	ng/mL	nmol/L	5.91	0.169
Epinephrine	µg/24 h	nmol/d	5.46	0.183
	µg/mg creatinine	µmol/mol creatinine	617	0.00162
	pg/mL	pmol/L	5.46	0.183
	ng/mL	nmol/L	5.46	0.183
Normetanephrine	ng/mL	nmo]/L	5.46	0.183
Dopamine	µg 24 h	nmol/d	6.53	0.153
	µg/mg creatinine	µmol/mol creatinine	783	0.00136
	pg/mL	pmol/L	6.53	0.153
	ng/mL	nmol/L	6.53	0.153
Human chorionic gonadotropin (hCG),	mU/mL	IU/L	1	1
beta, subunit	U/24 h	IU/d	1	1
Calcium	mg/dL	mmol/L	0.25	4.0
(S)				
	mEq/L	mmol/L	0.5	2.0

Contd...

Contd...

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
(U)	mg/24 h	mmol/d	0.025	40
Carbon dioxide total (content; CO_2 + bicarbonate)	mEq/L	mmol/L	1	1
CO_2 partial pressure, tension (PCO_2)	mm Hg	kPa	0.133	7.52
Standard bicarbonate (hydrogen carbonate	mEq/L	mmol/L	1	1 (carbonate)
Chloride	mEq/L or mg/dL	mmol/L	1	1
CEA	ng/mL	µg/L	1	1
	µg/mL	mg/L	1	1
Ceruloplasmin	mg/dL	mg/L	10	0.1
Cholesterol	mg/dL	mmol/L	0.0259	38.61
HDL-cholesterol	mg/dL	mmol/L	0.0259	38.61
LDL-cholesterol	mg/dL	mmol/L	0.0259	38.61
Copper				
(S)	µg/dL	µmol/L	0.157	6.37
(U)	µg/24 h	µmol/d	0.0157	63.69
Coproporphyrins (I and III)	µg/dL	nmol/L	15	0.067
(U)	µg/24 h	nmol/d	1.5	0.67
(F)	µg/g	nmol/g	1.5	0.67
Porphobilinogen (PBG)				
(U)	mg/24 h	µmol/d	4.42	0.226
Cortisol				
(S)	µg/dL	µmol/L	0.028	35.7
	ng/mL	nmol/L	2.76	0.362
17-OHKS (cortisol)	mg/24 h	µmol/d	2.759	0.3625
(U)	µg/24 hr	nmol/d	2.759	0.3625
Creatine	mg/dL	µmol/L	76.3	0.0131
(S)				
Creatinine				
(S, AF)	mg/dL	µmol/L	88.4	0.0113
(U)	g/24 h	mmol/d	8.84	0.1131
(U)	mg/24 h	mmol/d	0.00884	113.1
(U)	mg/kg/24 h	µmol/kg/d	8.84	0.113
(C)	mL/min/1.73 m^2	mL/sec/m^2	0.00963	104
cAMP (cyclic adenosine monophosphate)				
(S)	µg/L	nmol/L	3.04	0.329
(B)	ng/mL	nmol/L	3.04	0.329
(U)	mg/24 h	µmol/d	3.04	0.329

Contd...

Contd...

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
(U)	mg/g creatinine	µmol/mol creatinine	344	0.00291
Dehydroepiandrosterone sulfate (DHEA-S)				
(S)	µg/mL	µmol/L	2.6	0.38
(AF)	ng/mL	nmol/L	2.6	0.38
17-Ketosteroids (as DHEA)				
(U)	mg/24 h	µmol/d	3.467	0.2904
17-Ketogenic steroids (as DHEA)				
(U)	mg/24 h	µmol/d	3.467	0.2904
17-Hydroxycorticosteroids (17-OHCS)				
(U)	mg/d of creatinine	mg/mol of creatinine	113.1	0.00884
11-Deoxy corticosterone (DOC)				
(S)	pg/mL	pmol/L	3.03	0.33
Glucose	mg/dL	mmol/L	0.0555	18.02
Ferritin	ng/mL	µg/L	1	1
Gastrin	pg/mL	ng/L	1	1
Growth hormone	ng/mL	µg/L	1	1
Homovanillic acid (HVA)				
(U)	mg/24 h	µmol/d	5.49	0.182
	µg/24 h	µmol/d	0.00549	182
	µg/mg of creatinine	mmol/mol of creatinine	0.621	1.61
5-Hydroxyindoleacetic acid (5-HIAA)				
(U)	mg/24 h	µmol/d	5.2	0.19
Hormone receptors (T)				
Progesterone receptor assay (PRA)	fmol/mg of protein	nmol/kg of protein	1	1
Estrogen receptor assay (ERA)	fmol/mg of protein	nmol/kg of protein	1	1
Iron	µg/dL	µmol/L	0.179	5.587
Iron-binding capacity	µg/dL	µmol/L	0.179	5.587
Iron saturation	%	fraction saturation	0.01	100
Lactate	mg/dL	mmol/L	0.111	9.01
Lead				
(S)	µg/dL	µmol/L	0.0483	20.72

Contd...

Contd...

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to Conventional units*
(S)	mg/dL	µmol/L	48.26	
(U)	µg/24 h	µmol/d	0.00483	
Lipids (total)	mg/dL	g/L	0.01	100
Magnesium	mEq/L	mmol/L	0.5	2
	mg/dL	mmol/L	0.411	2.433
Osmolality	mOsml/kg	same		
O_2 partial pressure (PaO_2)	mm Hg	kPa	0.133	7.5
Parathyroid hormone	pg/mL	ng/L	1	1
Phosphate (inorganic phosphorus)				
(S)	mg/dL	mmol/L	0.323	3.10
(U)	g/24 h	mmol/d	32.3	0.031
pH	nEq/L	nmol/L	1	1
Porphobilinogen	µg/d	µmol/d	4.42	0.226
Potassium				
(S)	mEq/L	mmol/L	1	1
(U)	mEq/24 h	mmol/L	1	1
(U)	mg/24 h	nmol/d	0.02558	39.1
Protein, total				
(S)	g/dL	gm/L	10	0.1
(U)	mg/24 h	gm/d	0.001	1000
CSF	mg/dL	mg/L	10	0.1
Renin [plasma-renin activity (PRA)]	ng/mL/h	µg/L/hr	1	1
Sodium				
(S)	mEq/L	mmol/L	1	1
(U)	mEq/24 h	mmol/L	1	1
(U)		mmol/d	0.0435	22.99
Serotonin				
(S)	ng/mL	µmol/L	0.00568	176
Testosterone (total)				
(S)	ng/dL	nmol/L	0.0347	28.8
Thyroid-binding globulin (TBG)	mg/dL	mg/L	10	0.1
	µg/dL	µg/L	10	0.1
Thyroglobulin	ng/mL	µg/L	1	1
TSH (thyroid-stimulating hormone)	µU/mL	mIU/L	1	1
Thyrotropin-releasing hormone (TRH)	pg/mLL	ng/L	1	1
Triiodothyronine, total (T_3)	ng/dL	nmol/L	0.0154	65.1
Reverse T_3 (rT_3)	ng/dL	nmol/L	0.0154	65.1

Contd...

Contd...

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to Conventional units*
Thyroxine, total (T_4)	µg/dL	nmol/L	12.9	0.0775
Transferrin (TIBC)	mg/dL	g/L	0.01	100
Triglycerides	mg/dL	mmol/L	0.0113	88.5
Urea nitrogen				
(S)	mg/dL	mmol/L	0.357	2.8
(U)	g/24 h	mol/d	0.0357	28
Uric acid				
(S)	mg/dL	mmol/L	0.05948	16.9
(U)	mg/24 h	mmol/d	0.0059	169
VanillylmandelicacidVMA)				
(U)	mg/24 h	µmol/d	5.05	0.198
	µg/mg of creatinine	mmol/mol of	0.571	1.75
		creatinine		
Viscosity (S)	centipoise	same		
Vitamin B_{12} (cyanocobalamin)	pg/mL	pmol/L	0.738	1.355
Unsaturated B_{12} binding capacity				
(S)	pg/mL	pmol/L	0.738	1.355
Vitamin C (ascorbic acid)	mg/dL	µmol/L	56.78	0.176
Vitamin A	µg/dL	µmol/L	0.0349	28.65
Vitamin D (calcitriol; 1,25-dihydroxy)	pg/mL	pmol/L	2.4	0.417
Xylose (U)	mg/dL	mmol/L	0.0666	15.01
	g/5 h	mmol/5 h	6.66	0.15

(µ = microns; µmol = micromoles; mmol = millimoles; nmol = nanomoles; fmol = fentamoles; mg = milligrams; g = grams; pg = picograms; ng = nanograms; L = liter; mL = milliliter; mEq = milliequivalent; mL/sec = milliliter/second; mL/min = milliliter/minute; U = units; mU = milliunits; IU = international units; d = day; 24 h = 24 hours; S = serum; U = urine; B = blood; C = clearance; F = feces; AF = amniotic fluid; SF = synovial fluid; T = tissue. All references are to serum unless otherwise indicated).

TABLE 3.4: Enzyme

Conventional unit	*IU/L Equivalent*
Acid phosphatase (prostatic)	
Bodansky	5.37
Shinowara-Jones-Reinhart	5.37
King-Armstrong	1.77
Bessey-Lowry-Brock	16.67
Alkaline phosphatase	
Bodansky	5.37
Shinowara-Jones-Reinhart	5.37
King-Armstrong	7.1
Bessey-Lowry-Brock	16.67 0.14
Babson	1.0

Contd...

Contd...

Conventional unit	*IU/L Equivalent*
Aldolase	
Sibley-Lehninger	0.74
Amylase	
Somogyi (saccharogenic)	1.85
Somogyi	20.6 0.541
Creatine kinase (CK)	1.0
Hydroxybutyric dehydrogenase (d-HBD)	
Rosalki-Wilkinson	0.482
Isocitrate dehydrogenase (ICD)	
Wolfson-Williams-Ashman	0.0167
Taylor-Friedman	0.0167
Lactate dehydrogenase (LDH)	
Wroblewski	0.482
Lipase	
Cherry-Crandal	278
Malic dehydrogenase (MD)	
Wacker-Ulmer-Valee	0.482
Transaminases	
Reitman-Frankel	0.482
Karmen	0.482

TABLE 3.5: Therapeutic and toxic drugs

			Conversion factors	
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
Acetaminophen	µg/mL	µmol/L	6.62	0.151
Amikacin	µg/mL	µmol/L	1.71	0.585
Amitriptyline	ng/mL	nmol/L	3.61	0.277
Amobarbital	µg/mL	µmol/L	4.42	0.226
Amphetamine	ng/mL µg/mL	nmol/L µmol/L	7.4 7.4	0.135 0.135
Bromide	µg/mL	mmol/L	0.0125	79.9
Caffeine	µg/mL	µmol/L	5.15	0.194
Carbamazepine (Tegretol)	µg/mL	µmol/L	4.23	0.236
Carbenicillin	µg/mL	µmol/L	2.64	0.378
Chloral hydrate	µg/mL	µmol/L	6.69	0.149
Chloramphenicol	µg/mL	µmol/L	3.09	0.323
Chlordiazepoxide (Librium)	ng/mL	µmol/L	0.00334	300
Chlorpromazine (Thorazine)	ng/mL	nmol/L	3.14	0.319
Chlorpropamide (Diabinese)	µg/mL	µmol/L	3.61	0.227

Contd...

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			Conversion factors	
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
Cimetidine (Tagamet)	µg/mL	µmol/L	3.96	0.252
Clonazepam (Klonopin)	ng/mL	nmol/L	3.17	0.316
Clonidine (Catapres)	ng/mL	nmol/L	4.35	0.230
Cocaine	ng/mL	nmol/L	3.3	0.303
Codeine	ng/mL	nmol/L	3.34	0.299
Demerol (Meperidine)	ng/mL	nmol/L	4.04	0.247
Desipramine (Norpramin)	ng/mL	nmol/L	3.75	0.267
Diazepam (Valium)	ng/mL	µmol/L	0.0035	285
Digitoxin	ng/mL	nmol/L	1.31	0.765
Digoxin	ng/mL	nmol/L	1.28	0.781
Dilaudid	ng/mL	nmol/L	4.85	0.206
Disulfiram	µg/mL	µmol/L	12.12	0.0761
Doxepin (Sinequan)	ng/mL	nmol/L	3.58	0.279
Ethanol	mg/dL	mmol/L	0.217	4.61
Ethchlorvynol (Placidyl)	µg/mL	µmol/L	6.92	0.145
Ethosuximide (Zarontin)	µg/mL	µmol/L	7.08	0.141
Gentamicin	µg/mL	µmol/L	2.09	0.478
Glutethimide (Doriden)	µg/mL	µmol/L	4.60	0.217
Haloperidol (Haldol)	ng/mL	nmol/L	2.66	0.376
Ibuprofen	µg/mL	µmol/L	4.85	0.206
Imipramine (Tofranil)	ng/mL	nmol/L	3.57	0 28
Isoniazid	µg/mL	µmol/L	7.29	0.137
Kanamycin (Kantrex)	µg/mL	µmol/L	2.06	0.485
Lidocaine (Xylocaine)	µg/mL	µmol/L	4.27	0.234
Lithium	mEq/L	mmol/L	1	I
Lorazepam	ng/mL	nmol/L	3.11	0.321
LSD (lysergic acid diethylamide)	µg/mL	µmol/L	3.09	0.323
Meprobamate	mg/L	µmol/L	4.58	0.218
Methadone	ng/mL	µmol/L	0.00323	309
Methaqualone (Quaalude)	µg/mL	µmol/L	4.0	0.250
Methotrexate	ng/mL	nmol/L	2.2	0.454
Methsuximide	µg/mL	µmol/L	5.29	0.189
Methyldopa (Aldomet)	µg/mL	µmol/L	4.73	0.211
Morphine	ng/mL ng/mL	nmol/L µmol/L	3.5 0.0035	0.285 285
Nortriptyline	ng/mL	nmol/L	3.8	0.263
Oxazepam	µg/mL	µmol/L	3.49	0.287
Paraldehyde	µg/mL	µmol/L	7.57	0.132

Contd...

Contd...

				Conversion factors
Analyte	*Conventional units*	*SI units*	*Conventional to SI units*	*SI to conventional units*
Pentobarbital (Nembutal)	µg/mL	µmol/L	4.42	0.179
Percodan	ng/mL	nmol/L	3.17	0.315
Phenacetin	µg/mL	µmol/L	5.58	0.179
Phenobarbital (Luminal)	µg/mL	µmol/L	4.31	0.232
Phenylbutazone (Butazolidin)	µg/mL	µmol/L	3.08	0.324
Phenytoin (Dilantin)	µg/mL	µmol/L	3.96	0.253
Primidone	µg/mL	µmol/L	4.58	0.218
Procainamide (Pronestyl), procaine (Novacain)	µg/mL	µmol/L	4.23	0.236
Propoxyphene (Darvon)	µg/mL	µmol/L	3.07	0.326
Propranolol	ng/mL	nmol/L	3.86	0.259
Quinidine	µg/mL	µmol/L	3.08	0.324
Quinine	µg/mL	µmol/L	3.08	0.324
Salicylic acid	µg/mL	µmol/L	7.24	0.138
Secobarbital (Seconal)	µg/mL	µmol/L	4.2	0.238
Theophylline (Aminophylline)	µg/mL	µmol/L	5.55	0.180
Tobramycin	µg/mL	µmol/L	2.14	0.467
Valproic acid	µg/mL	µmol/L	6.93	0.144
Vancomycin	µg/mL	mg/L	1	1
Warfarin (Coumadin)	µg/mL	µmol/L	3.24	0.308

CHAPTER 4

Fundamental Chemistry

INDICATORS

Indicators are usually acids of weak strength whose molecules in solution are of a different color than their anions.
(pH: Neutral pH = 7.0, less than 7.0 is acidic, more than 7.0 is alkaline).

The color of an indicator solution depends on the degree of dissociation of the indicator, and on the pH of the solution. Supposing the weak acid indicator is H Indic', it would dissociate as:

$$\underset{\text{(color-X)}}{H\ Indic'} \rightleftharpoons \underset{\text{(hydrogen ion)}}{H^+} + + \underset{\text{(Color Y)}}{Indic^-}$$

All acids contain hydrogen ions, so addition of an acid would make the reaction shift from right to left (change of color from Y to X) and addition of an alkali (alkalies contain OH^- or hydroxyl ions) would lead to production of water ($H_2O \rightarrow H^+ + OH^-$) as the OH^- radicals will associate with and remove H^+ ions causing a shift to the right of the reaction (change of color from X to Y). Indicators that just show whether a solution is an acid or alkali are called broad indicators while some indicators change color at a precise pH.

The list of commonly employed indicators are given in Table 4.1.

TABLE 4.1: Commonly employed indicators

Indicator	*Color in acid solution*	*Color in alkaline solution*	*pH ranges*
Litmus	Red	Yellow	5.5–8.0
Phenophthalein	Colorless	Red	8.0–9.8
Methyl red	Red	Yellow	4.2–6.3
Methyl orange	Red	Orange	3.1–4.4
Phenol red	Yellow	Blue	6.8–8.4
Bromothymol blue	Yellow	Blue	6.0–7.6

SOLUTES, SOLVENTS AND SOLUTIONS

Solute

Solute is any substance that dissolves in a liquid.

Solvent

Solvent is any liquid in which a solute dissolves.

Solution

A solvent becomes a solution after dissolving a solute.

Buffer Solution

At any given temperature, these solutions retain their definite pH and maintain it even after adding considerable amounts of acids or alkalies. These solutions generally consist of a weak acid mixed with its sodium or potassium salt.

Strength of a Solution

Solution strength can be expressed in four ways: (1) Percent solutions, (2) Part dilutions, (3) Molar solutions, and (4) Normal solutions.

Percent Solutions

This is the most usual way of expressing solution strength. Percent implies per hundred and a 30% solution of anything should contain 30 parts of solute per hundred parts of the final solution.

Percent solution can further be expressed in three ways. Weight per unit weight (w/w). This implies that the weight of both, solute and solvent add up to 100, regardless of the final volume produced. A 25% solution would be 25 grams of solute dissolved in 75 grams of solvent. These solutions

are made by weighing both the solute and the solvent and the resultant solution may not have a volume of 100 mL.

Weight per Unit Volume (w/v)
This is a commoner method of preparing laboratory solutions. In this method a weight of the solute is dissolved in a final volume of 100 mL (it is wrong to dissolve the weight of solute in 100 mL of solvent as is often done).

A volumetric flask is the most accurate and convenient container for preparing such solutions. Put the weighed solute in the flask, add the solvent (keeping volume less than the final volume required), dissolve the solvent completely and then make up the total volume by adding more of the solvent.

Wherever necessary, deduct the molecular weight of water if the amount indicated is of anhydrous solute.

Volume per Unit Volume (v/v)
This is used when the ultimate solution is to be prepared from liquids. A volume of the liquid solute is made up to 100 mL final volume with solvent. Here too, a volumetric flask/beaker should be used and the final volume be made up by adding the solvent. Care should be taken to work under temperature conditions as indicated on the glass equipment.

The type of percent solution should be ascertained first, as a different type used instead of the required way would cause substantial error ultimately, for instance, a 50% w/w solution of sodium hydroxide is 75% w/v; similarly, a 22½% w/v solution of sodium sulfate is only 10% w/w.

Part Dilutions

In this method, the dilution is expressed as that part of the whole volume into which one part (or more parts) of the solution is dissolved, e.g. WBC dilution is 1:20, implying that there is one volume or part of blood in 20 volumes or parts of final solution, though actually mixed with 19 volumes or parts of the diluting fluid.

Molar Solutions

A molar solution contains the molecular weight of the solute (dissolved substance) in grams per liter of solution. The molecular weight is found by adding the atomic weights of the different atoms present in the compound (Refer Periodic table).

Example: The molecular weight of sodium chloride is 58.454. Hence, 1 molar solution of the salt contains 58.454 grams of NaCl in 1 liter.

Formula to convert a percentage solution into a mol/L solution:

$$\text{mol/L solution} = \frac{\text{g\% (w/v) solution} \times 10}{\text{molecular weight of substance}}$$

To change a normal solution into a mol/L solution:

$$\text{mol/L solution} = \frac{\text{Normality of solution}}{\text{valence of substance}}$$

Normal Solutions

A normal solution is one which contains the gram equivalent weight (equivalent weight in grams) of a substance per liter of solution. The equivalent weight is the number of units of the substance which will combine with or replace a single unit of hydrogen, 35.5 units of chlorine, 8 units of oxygen, etc. or the number of units of that substance which will contain a single unit of hydrogen, 35.5 units of chlorine, 8 units of oxygen, etc.

1. The equivalent weight of an element is calculated by dividing the atomic weight by the valency (valency is the number of atoms of hydrogen one atom of the element will combine with or displace).
   Example: The atomic weight of sulfur is 32.006 and its valency is 2, the equivalent weight would be 32.006/2 = 16.003.
2. The equivalent weight of an acid is the weight of it in grams which contains 1.008 g (one atomic weight) of replaceable hydrogen. It is calculated by dividing the molecular weight by the number of replaceable hydrogen atoms in the molecule.
   Example: The molecular weight of sulfuric acid (H_2SO_4) is 98.082 and the number of replaceable hydrogen atoms is 2, hence its equivalent weight would be 98.082/2 = 49.041.
3. Equivalent weight of an alkali is that weight of it which will neutralize the equivalent weight of an acid. It is calculated by dividing the molecular weight by the number of OH^-(hydroxyl) radicals in the molecule.
   Example: Calcium hydroxide; $Ca(OH)_2$, has a molecular weight of 91.014 and 2 hydroxyl groups, its equivalent weight would be 91.014/2 = 45.507.
4. Equivalent weight of a♥ salt is calculated by dividing its molecular weight by the number of metal ions (cations) per molecule, multiplied by the valence of the ion (cation).
   Example: Sodium sulfate has a molecular weight of 142.060, has 2 cations and the valency of the cation is one, hence its equivalent weight would be:

$$\frac{142.060 \times 1}{2} = 71.030$$

Saturated Solution

In this, the weight of the solute is not specified nor is the volume of the solvent. It states that it contains as much as will dissolve.

Standard Solution

The exact strength of standard solution is known and is used for comparing strengths of other similar solutions.

PERIODIC TABLE OF ELEMENTS

Abbreviations and Definition

No. – Atomic Number
MP – Melting point
BP – Boiling point

- Density of elements with boiling points below 0°C is given in g/L
- Earth crust composition average values are from a report by FW Clarke and HS Washington, 1924. Elemental composition of crustal rocks differ between different localities
- *Group:* There are only 18 groups in the periodic table that constitute the columns of the table. Lanthanoids and Actinoids are numbered as 101 and 102 to separate them in sorting by group.

Atomic Number

The number of protons in an atom each element is uniquely defined by its atomic number.

Atomic Mass

The mass of an atom is primarily determined by the number of protons and neutrons in its nucleus. Atomic mass is measured in Atomic Mass Units (amu) which are scaled relative to carbon, 12C, that is taken as a standard element with an atomic mass of 12. This isotope of carbon has 6 protons and 6 neutrons. Thus, each proton and neutron has a mass of about 1 amu.

Isotope

Atoms of the same element with the same atomic number, but different number of neutrons. Isotope of an element is defined by the sum of the number of protons and neutrons in its nucleus. Elements have more than one isotope with varying numbers of neutrons. For example, there are two common isotopes of carbon, 12C and 13C which have 6 and 7 neutrons respectively. The abundances of different isotopes of elements vary in nature depending on the source of materials. For relative abundances of isotopes in nature see reference on Isotopic Composition of the Elements.

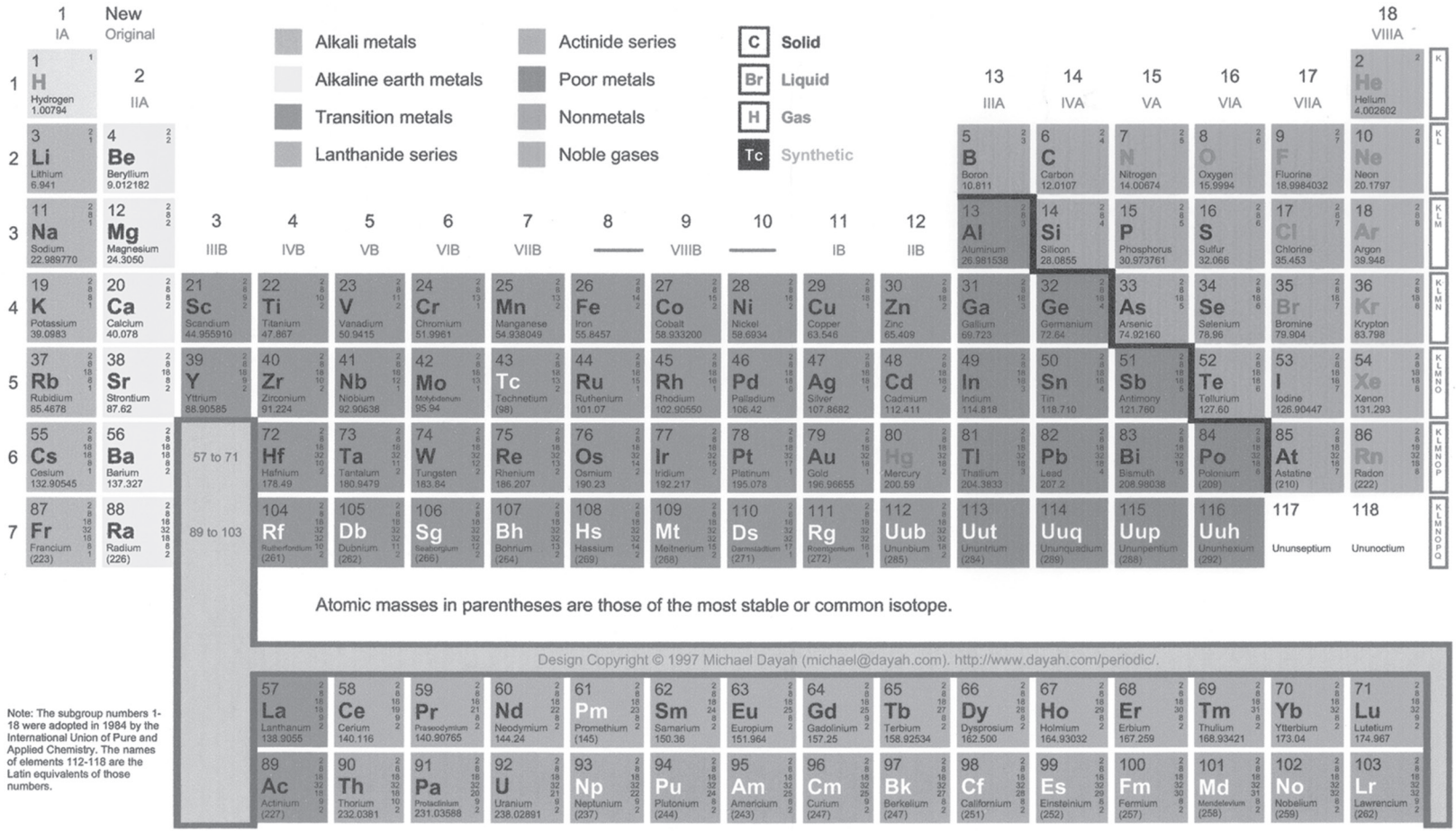
Atomic Weight

Atomic weight values represent weighted average of the masses of all naturally occurring isotopes of an element. The values shown here are based on the IUPAC Commission determinations (Pure Appl. Chem. 73:667-83, 2001). The elements marked with an asterisk have no stable nuclides. For these elements, the weight value shown represents the mass number of the longest-lived isotope of the element.

Electron Configuration

The distribution of electrons according to the energy sublevels (subshells) in uncharged atoms. The noble gas shown in square brackets (e.g. [He]), marks that all the subshells associated with that element are fully occupied by electrons.

PERIODIC TABLE OF THE ELEMENTS



CHAPTER 5

Urine Analysis

COMPOSITION OF URINE

Urine composition is affected mainly by three factors:

1. Nutritional status
2. State of metabolic processes
3. Ability of the kidney to selectively handle the material presented to it.

Physiochemical Characteristics of Urine

Dry weight	55–70 g/24 h
Osmolality	38–1400 mOsm/kg water (Average = 500–800 mOsm/kg water)
pH	4.6–8.0 (mean = 6.1)
Specific gravity	
Neonates	1.012
Infants	1.002–1.006
Adults	1.003–1.030
Volume	Per day
Neonates:	30–60 mL
10–60 days	250–450 mL
60–365 days	400–500 mL
Children:	
1–3 years	500–600 mL
3–5 years	600–700 mL
5–8 years	650–1000 mL
8–14 years	800–1400 mL
Adults:	600–2500 mL (Avg: 1200 mL)

Inorganic Constituents per 24 Hours

Iron	0.06–0.1 mg
Chlorides	6 (4–10) g on usual diet
Sodium	4 g on usual diet
Phosphate	0.8–1.3 g on usual diet
Sulfur	2 g
Calcium	< 150 mg.

Organic Constituents per 24 Hours

Nitrogenous—total	25–35 g
Urea 15–30 g	
Creatinine	1.4 (1–1.8) g
Ammonia	0.7 (0.3–1) g
Uric acid	0.45 (0.3–0.6) g
Protein (albumin)	0–0.1 g
Creatine, in children	10–50 mg (excreted in urine in adults in hepatic or muscle disorders or thyrotoxicosis)
Glucose (fasting range)	2–20 mg% (Diabetic may lose up to 100 g/day)
Amylase (diastase)	40–260 units/hour.

Cells and Casts (Table 5.1)

TABLE 5.1: As per Addis count

	Range	*Mean*
RBC	Up to 1 million /day (more in females)	130,000/day
Casts		
hyaline and occasionally granular	Up to 5,000/day	2,000/day
Leukocytes	Up to 5 million/hour (more in females)	108,000/h, females; 28,000/ h, males
Epithelial cells	Up to 2.5 lakh/hour (more in males)	68,000/h, females; 78,000/h. males.
Squamous cells epithelial	Variable	

Collection of Urine

The urine sample should be collected in a clean, dry container and should be examined fresh. For cultures, sterile containers should be used. With time, RBC, and leucocytes tend to be destroyed due to hypotonicity of the urine. Casts too tend to get decomposed. Bacterial contamination of stale urine is frequent and causes alkalinization of the urine due to conversion of urea to ammonia and loss of glucose. This rise in pH accelerates loss of leucocytes and epithelial cells. For ordinary qualitative, tests a random sample is enough. For diabetes mellitus, a 2-hour postprandial sample is desirable; for nephritis, a morning specimen is best as it has higher specific gravity and lower pH desirable for preservation of formed elements.

Repeated samples are necessary sometimes, as for orthostatic proteinuria.

Whenever needed, a 24-hour urine should be collected in a large container. Have patient void and discard urine at any particular time, save all urine for the next 24 hours, and then void at the same hour to finish the collection.

Preservation of Specimen

Urinary decomposition occurs quickly in warm temperatures. Hence, fresh specimens should be examined, if not, then it should be refrigerated. As far as possible, the need for preservation should not arise. However, the following preservatives can be used:

1. *Toluol* Best for preservation of chemical constituents. Add 2 mL toluol/100 mL urine.
2. *Thymol* A small floating lump of thymol can preserve the urine for several days in a bottle. Thymol may, however, cause a false-positive reaction for protein.
3. *Formalin* 1 drop/30 mL urine. Is good for preserving formed elements. It can precipitate proteins and can reduce Benedict's solution.
4. *Boric acid* 0.3 g/120 mL of urine. However, yeasts can still grow and uric acid crystals get precipitated.

GROSS EXAMINATION OF URINE

Color and Appearance

Normal urine is clear and pale yellow (straw) in color.

1. *Colorless:* Dilution; diabetes mellitus/insipidus, nervousness, diuretic or alcohol intake.
2. *Milky:* Purulent genitourinary tract disease; chyluria.
3. *Orange:* Urobilinogenuria, fever, excessive sweating, concentrated urine.
4. *Red:* Beetroot ingestion, hematuria, hemoglobinuria, phenolphthalein, pyridinium sulfo♥nate.
5. *Greenish:* Jaundice, phenol poisoning.
6. *Dirty blue or green:* Putrefying urine, in typhus or cholera, methylene blue.
7. *Dark brown, brown red, or yellow:* Very concentrated urine, acute febrile diseases, bilirubinuria.
8. *Brown-yellow or brown red (if acidic) or bright red (if alkaline):* Due to rhubarb, cascara, aloes.
9. *Brown, brown black or black*: Hemorrhage in urinary tract if urine is acidic (Acid-hematin); hemoglobinuria; porphyria, methemoglobinuria; myoglobinuria, melanin, phenol poisoning, homogentisic acid (alkaptonuria). In porphyria, urine turns dark brown on exposure to sunlight or boiling.

Interfering Factors

- Normally, urine darkens on standing. This occurs because of oxidation of urobilinogen to urobilin. Decomposition of urine commences in half an hour.
- Some foods cause change in urine color
  - Beets turn the urine red
  - Rhubarb changes color of urine to brown.
- Many drugs are also responsible for urinary color change
  - Cascara and senna laxatives in acidic urine will turn the urine reddish-brown, in alkaline urine they will turn the urine red
  - Phenazopyridine (pyridium), amido pyrine turn urine orange in color
  - Pyridium, ethoxazene turn urine to orange/orange red
  - Orange to purple red may occur due to chlorzoxazone
  - Salicylazosulfapyridine, anisindone, or phenindione turn urine color to orange-yellow in alkaline urine
  - Sulfonamides and nitrofurantoins produce rust-yellow to brownish color
  - Dilantin (diphenylhydantoin) dioctyl calcium sulfosuccinate, phenolphthalein and phenothiazine turn urine color to pink to red or red-brown
  - Phenolphthalein may also produce magenta color
  - Amidopyrine, pyridium, aniline dyes, BSP, PSP in alkaline urine or phenolphthalein and pyridium in acid urine or deferoxamine can produce red urine
  - Phenolphthalein in alkaline urine produces purple red color
  - Phenylhydrazine and phenolic drugs produce dark brown urine
  - Cascara may produce brown-black urine
  - Riboflavin or pyridium in alkaline urine produce bright yellow color

- Methylene blue and amitriptyline produce blue or green colored urine
- Levodopa causes urine to darken on standing
- Iron salt consumption produces dark colored urine
- Phenothiazine tranquilizers cause pink to brown color
- Triamterene causes pale blue colored urine.

Reaction

Average range: 4.6 to 8, Average pH = 6.0

Litmus paper or other pH indicator papers broad range (pH 1 to 12) or narrow range pH papers can be used. Another simple method is to add 2 drops of 0.4% alcoholic solution of methyl red to 5 mL of urine. Note the color change—if red = acidic; orange = neutral; yellow = alkaline. Digital electronic pH meters for better accuracy can be used—here, the electrode is dipped in urine and pH read off directly from the digital display.

Amongst urinary tract infections, *Escherichia coli* produce acidic urine, while *Proteus* (urea splitting) produces alkaline urine. Meat protein diet causes urinary acidification, while consumption of citrus fruits makes the urine alkaline.

TABLE 5.2: Urine pH

Finding and condition	*Causes and comments*
Acidic urine	
Ketosis	Diabetes, starvation, febrile illness in children.
Systemic acidosis	Except with impaired renal tubular function, respiratory or metabolic acidosis provokes intense urine acidity and decreased NH_4^+ excretion
Acidification	Used in treating urinary tract infections, and to prevent precipitation of calcium carbonate or phosphates or magnesium ammonium phosphate
Alkaline urine	
Postprandial alkaline tide	Normal finding in specimens voided shortly after meals
Vegetarianism	Meats produce fixed acid residue, vegetarian diet does not.
Systemic	As may occur in severe vomiting, hyperventilation, excess alkali ingestion
Urinary tract	*Proteus* or *Pseudomonas* infection, they split urea to HCO_3^- and ammonia
Alkalinization	Used to prevent crystallization of uric acid, oxalate, cystine, sulfonamides, streptomycin
Stale specimen	Bacterial overgrowth. If true infection exists, the sediment should show pus cells
Renal tubular	Impaired tubular acidification causes inappropriately high urine pH with systemic acidosis and low serum HCO_3^-

Interfering Factors

- On standing, urinary pH becomes alkaline because CO_2 will diffuse into the air
- Alkaline urine specimens tend to cause hemolysis of red cells and disappearance of casts
- High protein diets will cause excessively acidic urine
- Ammonium chloride and mandelic acid may produce acidic urine
- Alkaline urine after meals is a normal response to the secretions of HCl in gastric juices
- Sodium bicarbonate, potassium citrate, and acetazolamide may produce alkaline urine.

Be Careful

- Only a freshly voided sample is suitable for measuring pH. Refrigerate the sample if any delay is expected (Table 5.2)
- Alkaline urine occurs from vegetarian diets, citrus fruits, milk and other dairy products (Table 5.2)
- Highly concentrated urine such as that formed in hot, dry environments is strongly acidic and may be irritating
- While sleeping, decreased pulmonary ventilation causes respiratory acidosis and urine becomes highly acidic
- Chlorothiazide diuretic will cause acidic urine to be excreted
- Bacterial contamination and overgrowth will result in alkaline urine. Bacteria in urine will convert to ammonia.

Odor

Important in fresh specimens only and is aromatic because of volatile fatty acids. Bacterial action causes ammoniacal odor, while ketosis leads to a fruity odor in urine.

Specific Gravity

It depends upon the concentration of various solutes in the urine:

1. *Urinometer:* Urine should be foamless. Transfer urine (about 70 to 80 mL) into the urinometer container and let the urinometer float freely without touching the sides or the bottom of the container (Fig. 5.1). Read graduations at the lowest level of urinary meniscus. If the urine amount is less, dilute the urine to raise the volume till 70 to 80 mL, take the reading and multiply the last two digits by the dilution factor.
2. *Refractometer:* Only small amount of urine is needed. It measures the concentration of solutes (related to refractive index). In Goldberg refractometer, the

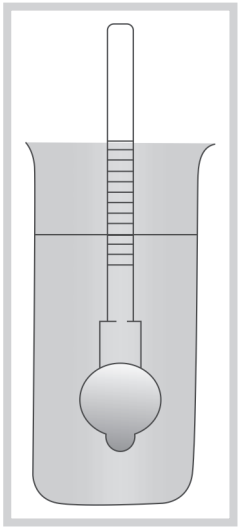


FIG. 5.1: Urinometer

specific gravity of urine can be read directly from the calibration.

3. Can be tested with *Dipsticks* also.
4. *Osmometry:* Gives the most accurate assessment.

Correction factor for temperature: While using urinometer, add or subtract 0.001 for each 3° C above or below the standardization temperature of the instrument.

Urines of low specific gravity are called hyposthenuric (< 1.007) while urines of fixed specific gravity of about 1.010 are known as isosthenuric.

High specific gravity

- Excessive sweating
- Glycosuria
- Acute nephritis
- Albuminuria
- All causes of oliguria.

Low specific gravity: (less than 1.010)

- Excessive water intake
- Chronic nephritis
- Diabetes insipidus
- All causes of polyuria except diabetes mellitus.

Low and fixed specific gravity: (1.010 to 1.012)

- Chronic nephritis (end-stage kidney) when concentration power of renal tubules is low
- ADH deficiency
- Arteriosclerotic kidney.

Interfering Factors

- Specific gravity is maximum in the first morning sample
- Specific gravity is increased whenever there is an excessive loss of water. It occurs in:
  - Sweating
  - Fever
  - Vomiting
  - Diarrhea.
- Drugs leading to false positive:
  - Dextran
  - Radiopaque contrast media used in X-rays of the urinary tract.
- Temperature of urine specimens affects specific gravity when specific gravity is measured in urine removed from the refrigerator. Specific gravity will be falsely higher
- Reagent strip testing of urine containing glucosed urea greater than 1% may cause a low specific gravity. Highly buffered alkaline urine may also cause a low reading
- Elevated reading may occur in presence of moderate (100 to 750 mg/dL) amounts of proteinuria.

Urinary Volume

The average 24 hours urinary output in an adult is around 1200 to 1500 mL and the night urine should not be more than 400 mL.

A volume more than 2000 mL is termed *polyuria*. *Oliguria* implies excretion of urine less than 500 mL and *anuria* is complete cessation. *Nocturia* is excretion by an adult of urine more than 500 mL with a specific gravity of less than 1.018 at night (characteristic of chronic glomerulonephritis).

Polyuria

- Neurotic polydipsia
- Diabetes mellitus/insipidus
- Diuretics
- Intravenous saline/glucose
- Chronic renal failure
- Addison's disease, decrease of adrenocortical hormones.

Oliguria

- Dehydration:
  - Vomiting
  - Diarrhea
  - Excessive sweating
- Renal ischemia
- Acute renal tubular necrosis
- Acute glomerulonephritis
- Obstruction to urinary outflow.

Turbidity

Normal—fresh urine is clear.

The appearance of cloudy urine provides a warning of possible abnormality such as the presence of pus, RBCs or bacteria. Sometimes, however, excretion of cloudy urine may not be abnormal since the change in urine pH may cause precipitation within the bladder of normal urinary constituents. Alkaline urine may appear cloudy because of presence of phosphates, and urine may appear cloudy because of urates.

- Pathologic urines are often turbid or cloudy, but so are many normal urines. Cloudy urine may appear from precipitation of crystals due to rapid cooling of the urine
- Occasionally, urine turbidity may result from urinary tract infections
- Abnormal urines may be cloudy on account of presence of RBCs, pus cells or bacteria.

Interfering Factors

- After ingestion of food, urates or phosphates may produce cloudiness in normal urine
- Vaginal contamination in female patients is often a cause of turbidity
- Greasy cloudiness may be caused by lipiduria
- Many normal urines will develop haziness or turbidity after being refrigerated or on standing at room temperature.

CHEMICAL EXAMINATION OF URINE

Tests for Protein

Normal values—negative (2 to 8 mg/dL)
If urine is not clear—filter or centrifuge the specimen. Both bile and protein cause urine to froth.

Heat and Acetic Acid Test

Take a test tube 2/3rd full with urine, boil upper portion of urine for 2 minutes (lower portion is not heated so that it can be used as a control for comparing). Now turbidity can arise because of phosphates, carbonates or protein. Add a few drops of 10% acetic acid, persistence or development of turbidity implies proteinuria.

False-positive tests may occur with X-ray contrast media and tolbutamide derivatives.

Sensitivity = 5 to 10 mg%

Interpretation

- – No cloudiness.
- ± Cloudiness barely visible.
- + Definite cloudiness, but no granularity and no flocculation.
- ++ Granular cloudiness, but no flocculation. Seen from above, the cloud is dense but not opaque. Protein content = about 0.1%.
- +++ Dense opaque cloud, clearly flocculated. About 0.2 to 0.3% protein.
- ++++ Very thick precipitation, almost a solid. Protein concentration > 0.5%.

Sulfosalicylic Acid Test

Urine should be clear and acid.

To 1 mL of urine, add 3 drops of 20% sulfosalicylic acid.

Absence of cloudiness means absence of protein. If the turbidity persists after boiling, it is due to protein. If the cloudiness vanishes on heating and reappears on cooling, it is due to Bence-Jones (BJ) protein.

False positive test may appear if the urine contains tolbutamide derivatives, high concentration of penicillin or X-ray contrast media.

Paper Strip Method

Paper strips impregnated with Bromophenol blue and salicylate buffer are dipped in urine. Presence of protein is indicated by change of color from light yellow to blue. Tolbutamide, X-ray contrast media and preservatives do not react, hence no false positive tests. However, highly alkaline urine may cause a false positive test; (sensitivity—30 mg% or more). Tablets of similar reagents producing the same color are also available.

Quantitative Estimation of Protein in Urine

1. *Turbidimetric and chemical procedures:* Provide an accurate estimation. Colorimetric readings taken against blanks and calculations done accordingly give the result (example; sulfosalicylic acid turbidity method).
2. *Esbach's quantitative method*: Acidify the urine if necessary. Cover the bottom of the Esbach tube with pumice, fill urine till the 'U' mark and add Esbach's or Tsuchiya's regent till the 'R' mark. Stopper the tube and invert it about a dozen times slowly.

Set the tube vertically and read after 30 minutes (if pumice has not been used, read after 24 hours). The tube is graduated to read in percent or in grams of protein per liter at the top of the sediment. Urine may be diluted for obtaining greater accuracy. After diluting, the Esbach tube reading may be multiplied by the dilution factor (Fig. 5.2). Dilutions can be made according to the specific gravity as follows:

1. 1.010 to 1.014—1:1 dilution.
2. 1.015 to 1.021—1:2: : urine : water.

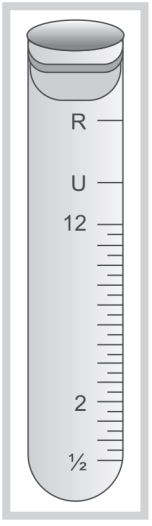


FIG. 5.2: Esbach's albuminometer

3. 1.022 or more—1:3: : urine : water.
4. If the qualitative test reading is +++, dilute as 1:4: urine: water.

Bence-Jones (BJ) Protein Tests

Seen in multiple myeloma classically.

1. Heat and Sulfosalicylic Acid

As for albumin, the precipitate formed will contain both BJ proteins and albumin. Mix the specimen of urine with the precipitate and divide equally in two test tubes. Place both in a water bath and heat to boiling. Remove one from the bath, cool to below 40°C and compare the turbidity in the two tubes in good light against a dark background. Cool the other hot tube and heat the cold one and compare again. If the cold tube both the times shows persistently a more densely turbid flocculum of protein, BJ protein is most likely present. If albumin is present also, add 10% acetic acid to a fresh urine sample (pH to be less than 6.0) and bring to boil, keep shaking and break the floc the BJ protein goes into solution. Filter off albumin while it is still hot, BJ proteins will come in the filtrate. Repeat the sulfosalicylic acid test as described above.

2. Toluenesulfonic Acid

Add 1 mL of TSA reagent to 2 mL of urine, let the reagent flow slowly by the side of the test tube. Mix. A precipitate appearing within 5 minutes indicates presence of BJ protein. A negative test excludes.

Sensitivity > 500 mg%.

3. Electrophoresis

Electrophoresis of concentrated urine for proteins would show the dense gammaglobulin band.

Bence-Jones protein is often seen in multiple myeloma and rarely in chronic leukemia, osteomalacia, osteosarcoma, cancer metastases to bone, and hypertension.

Interpretation of Proteinuria

Minimal Proteinuria (< 0.5 g/day)

- Following exercise or in highly concentrated urine, in healthy persons
- Fever, severe emotional/thermal stress, in otherwise healthy persons
- Postural proteinuria; young adults may pass protein while ambulatory but not while lying
- Hypertension
- Renal tubular dysfunction, including genetic and drug-induced
- Polycystic kidneys
- Lower urinary tract infections
- Hemoglobinuria with severe hemolysis.

Moderate Proteinuria (0.5–3 g/day)

- Chronic glomerulonephritis—moderate
- Congestive heart failure
- Diabetic nephropathy—mild
- Pyelonephritis
- Multiple myeloma
- Pre/eclampsia.

Marked Proteinuria (> 3 g/day)

- Acute glomerulonephritis
- Chronic glomerulonephritis—severe
- Lipoid nephrosis
- Severe diabetic nephropathy

} and other causes of nephrotic syndrome

- Renal amyloidosis
- Lupus nephritis.

Nonrenal Causes of Proteinuria

- Fever
- Trauma
- Severe anemias and leukemia
- Toxemia
- Abdominal tumors
- Convulsive disorders
- Hyperthyroidism
- Intestinal obstruction
- Cardiac disease
- Poisoning from turpentine, phosphorus, mercury, sulfosalicylic acid, lead, phenol, opiates and drug therapy.

Other Important Related Aspects

- Large numbers of leukocytes accompanying proteinuria usually imply infection at some level in the urinary tract. Large numbers of leukocytes and erythrocytes usually indicate a non-infectious inflammatory disease of glomerulus. Proteins with pyelonephritis may have as many RBCs as white blood cells.
- Proteinuria does not necessarily always accompany renal diseases. Pyelonephritis, obstructions, nephrolithiasis, tumors and congenital malformation can cause severe illness without causing protein leakage.
- Proteinuria is associated with the finding of casts on the sediment examination as protein is necessary for cast formation.
- Postural proteinuria is the excretion of protein by patients who are standing or moving during daytime. This proteinuria is intermittent and disappears when the patient lies down. Postural proteinuria has an incidence of 3 to 5% of all normal healthy subjects.

Collecting Specimen for Orthostatic Proteinuria

1. Instruct the patient to void at bedtime and discard sample.
2. Next morning sample is collected immediately as the patient awakes and assumes a standing posture.
3. A second specimen is collected after the patient has been standing or walking for a considerable period of time.

Differentiation from other types of proteinuria is done by testing for protein in two urine specimens; one collected before and one after the person is erect. In postural proteinuria the first sample would be devoid of protein while second one would be positive.

Interfering Factors

a. Functional, mild and transitory protein in the urine, because of renal vasoconstriction, is associated with:
   - Violent exercise
   - Severe emotional stress
   - Cold baths.

b. Increased protein in urine occurs:
   - After eating large amounts of protein
   - In pregnancy or immediately following delivery
   - In neonates
   - In premenstrual state
   - In orthostatic proteinuria.

c. False or accidental proteinuria may occur because of a mixture of pus and RBCs in urinary tract infections and the menstrual flow.

d. False positive results can occur from incorrect use and assessment of color strip test.
   - Prolonged dipping or allowing the strip to be held too long in the urine stream.
   - Failing to accurately match the reactive area with the color chart.

e. Alkaline urine can give a false positive test on the color strip test due to alkaline, highly buffered urine.

f. A very dilute urine may give a falsely low protein value.

g. Drugs that may cause false positive tests for protein (acid turbidity methods only) include:
   - Gold
   - Arsenic
   - Sodium bicarbonate
   - Acetazolamide
   - Radiopaque contrast media for up to 3 days (no false positives with dipsticks, only with sulfosalicylic acid test)
   - Sulfisoxazole
   - Thymol
   - Chlorpromazine
   - This list includes many other drugs also.

Mechanisms of Proteinuria

a. *Glomerular proteinuria:* There may be increased filtration of plasma proteins when there is disruption of normal glomerular capillary permeability—as occurs with antigen-antibody, with infiltrative processes such as amyloid or with ischemic glomerular injury.

b. *Tubular proteinuria:* There is increased renal excretion of plasma protein in the presence of normal glomerular permeability—increased filtered load of small proteins, light chain proteinurias, multiple myeloma.

   Normal filtered load of small proteins with a decreased capacity for tubular absorption of these proteins—chronic cadmium poisoning, Fanconi's syndrome, cystinosis, and some patients with Wilson's disease.

c. *Postglomerular proteinuria:* There is secretion of protein by the structures of upper and lower urinary tract—in response to infection or the presence of renal calculi.

Microalbuminuria

Definition

Microalbuminuria is the earliest sign of nephropathy before it manifests overtly as proteinuria, a condition where significant kidney damage has already occurred.

Microalbuminuria is classified as:

- Albumin excretion rate: 20–200 µg/min or 30–300 mg/day
- Albumin/Creatinine ratio: 2.5–25 mg/mmol
- Albumin/ Creatinine ratio: 30–300 mg/g
- Albumin concentration (early morning urine): 30–300 mg/L.

Microalbuminuria can only be detected by specific immunochemical assays for urinary albumin using antibodies to human albumin. The existing biochemical tests for detection of microprotein are nonspecific as they also detect other proteins apart from albumin.

Although dye binding and protein precipitation assays have been described, these are insensitive and nonspecific and should not be used.

Microalbuminuria: Diagnostic Relevance

Microalbuminuria indicates high probability of damage of the glomerular filtration capacity of the kidney and is of great diagnostic relevance;

- In diabetic patients for early diagnosis of nephropathy
- In hypertensive patients as indicator of end organ damage associated with lower life expectancy, and
- Is probably associated with cardiovascular diseases in general population.

Microalbuminuria Detection

It is recommended in the following subjects:

Diabetic Subjects:

- Insulin-dependent diabetes mellitus (IDDM): Annually in all patients suffering from diabetes for 5 years or more and over 12 years of age
- Non-insulin dependent diabetes mellitus (NIDDM): As soon as diabetes is diagnosed, and at regular intervals (1–2 times) annually thereafter in case of negative test results.

Nondiabetic Subjects:

- All patients with potential disease involvement of the kidneys for early diagnosis
- Patients at risk of cardiovascular complications.

Microalbuminuria Indirect Latex Slide Test, Microtex®

(*Courtesy:* Tulip Group of Companies)

Summary

Urinary albumin excretion between 30–300 mg/day (microalbuminuria), far below the levels found in clinical proteinuria (> 300 mg/day) is a strong predictor of development of diabetic nephropathy and vascular complications. These low levels of albumin excretion are detectable only by sensitive immunoassays for microalbuminuria.

Diabetic nephropathy leads to progressive loss of renal function or end-stage renal disease (ESRD) and may necessitate need for dialysis or transplantation in most cases. The progression of microalbuminuria is closely associated with progressive hypertension and loss of blood glucose control. The early presence of microalbuminuria can be reversed by strict metabolic control and timely intervention of drugs early in the course of disease can arrest the progression of diabetic renal disease. Annual screening of microalbuminuria is recommended by the **'WHO'** and **'International Diabetes Foundation'** in all patients with IDDM over the age of 12 years and who have had diabetes for 5 years or more.

Microalbuminuria is also a significant risk marker of cardiovascular diseases. Its presence can be regarded as an index of increased cardiovascular vulnerability and a signal for correction of known risk factors.

Microtex is a sensitive immunoassay useful for the detection of microalbuminuria.

Reagent

1. *Antihuman albumin reagent:* The concentration of antibodies to human albumin is adjusted to provide sensitivity of about 25 mg/L and above of microalbuminuria.
2. *Albumin latex reagent:* A uniform suspension of polystyrene latex particles to which human albumin has been chemically coupled. Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

Store the reagents at 2–8°C. Do not freeze.

The shelf life of the reagents is as per the expiry date mentioned on the reagent vial labels.

Principle

Microtex slide test for the detection of microalbuminuria is based on the principle of agglutination inhibition. The urine specimen to be tested is first mixed with antibody reagent containing antibodies directed against human albumin. The latex coupled with human albumin is added to the mixture and is allowed to react. When the urine specimen does not contain albumin, antibodies to human albumin would be free to react with the latex coupled with human albumin causing agglutination. When the urine specimen contains at least 25 mg/L of albumin, the antibodies to human albumin will be neutralized and will not be available to react with latex couple with human albumin. Hence, no agglutination will be observed.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the materials derived from human source have been tested for HBsAg and anti-HIV antibodies and found to be nonreactive.
3. The reagents contain 0.1% sodium azide as preservative. Avoid contact with skin or mucosa. On disposal, flush with large quantities of water.
4. The reagents can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of reagents should be verified with positive and negative controls provided with the kit.
5. Use reagents of same lot numbers. Do not interchange reagents of different lot numbers.
6. Do not interchange vial droppers.
7. Shake the albumin latex reagent vial well before use to disperse the latex particles uniformly and improve test readability.
8. Only a clean and dry glass slide must be used. Slide should be free from even traces of protein compounds.

Sample Collection and Preparation

For Qualitative Method

Though random urine specimens can be used, first morning urine specimen is preferable. Specimens should be collected in clean glass or plastic containers free of detergents. Specimens should be tested immediately preferably within 12 hours of collection. Should a delay in testing occur, add thimerosal (0.01%) or sodium azide (0.1%) to the specimen and store at 2–8°C up to 72 hours. Do not use grossly contaminated specimens. If specimen is cloudy or contains blood, centrifuge the specimen at 1000 rpm (revolutions per minute) for one minute and use clear supernatant for testing.

For Semiquantitative Method

Urine specimens collected over a 24-hour period should be pooled in a clean detergent free container and refrigerated at 2–8°C. Thimerosal (0.01%) or sodium azide (0.1%) are recommended as urine preservatives.

Materials Provided with the Kit

Reagents

Antihuman albumin reagent, albumin latex reagent, positive control reactive with antihuman albumin reagent, negative control non-reactive with antihuman albumin reagent.

Accessories

Glass slide with six reaction circles, pipettes for dispensing urine specimen, mixing sticks and rubber teats.

Additional Materials Required

A high intensity direct light source and stopwatch.

Test Procedure

Bring all reagents and samples to room temperature before use.

Qualitative Method

1. Place one drop of clear urine under test on the glass slide using disposable pipettes provided with the kit. Deliver the drop vertically.
2. Add one drop of antihuman albumin reagent to the drop of urine under test on the slide. Deliver the drop vertically.
3. Using a mixing stick, mix the antihuman albumin reagent and urine over the circle for 30 seconds.
4. Add one drop of well mixed albumin latex reagent to the mixture. Mix uniformly over the entire circle.
5. Immediately start the stopwatch, rock the slide gently back and forth observing for agglutination macroscopically at three minutes.

Semiquantitative Method

Measure and record the total volume of patient urine collected over a 24-hour period. Centrifuge an aliquot of the 24 hours urine specimen. Using isotonic saline, prepare progressive dilutions from the centrifuged urine specimen. Perform the qualitative test procedure using each dilution as specimen.

Interpretation of Results

Qualitative Method

Agglutination is a negative test result indicating the absence of detectable levels of albumin in urine signifying absence of microalbuminuria. No agglutination is a positive test result indicating the presence of albumin in concentrations above 25 mg/L in urine signifying microalbuminuria.

Semiquantitative Method

No agglutination in the highest urine dilution corresponds to the titer of microalbumin per liter of the specimen. To calculate the concentration of microalbumin in the specimen uses the following formula:

Microalbumin (mg/L) = S × D

where

S = Sensitivity of the test, i.e. 25 mg/L

D = Highest dilution of urine showing no agglutination.

Remarks

1. Microalbuminuria also occurs in response to acute inflammatory conditions such as ischemia, trauma and thermal injury, surgery, pancreatitis and inflammatory bowel disease. In many of these conditions, the albumin excretion increases within minutes or hours of the initiating stimulus and only lasts for 24–72 hours.
2. Use only urine as test specimen. Do not use serum.
3. Albumin excretion is increased after physical activity. It is, therefore, recommended to use urine sample that has been produced at rest whenever random urine specimen is used.
4. As albumin excretion is subject to physiological fluctuations, it is necessary to take two measurements in consecutive days; in case of contradictory results, three measurements on different days must be done preferably within a week.
5. Liquid intake of the patient must be in the normal range, i.e. 1.5–2 liters/day.
6. To diagnose incipient nephropathy microalbuminuria must be present in at least 2 out of 3 specimens over a 3–6 months period.
7. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
8. It is recommended that reagents should be tested with positive and negative controls periodically to validate their performance.
9. The agglutination pattern in negative urines will vary from sample to sample since it is affected by the salt concentration and pH of the urine under test.

Tests for Glucose

Normal values
Random : Negative
24-hour specimen: 100 mg/24 hours
Renal threshold level: 180 mg/dL.

Benedict's Qualitative (Semiquantitative) Glucose Test

In this method, the cupric ion is reduced to Cu_2O (cuprous oxide). If only 0.1% or less of glucose is present, the precipitate may not appear until cooling. To 5 mL of Benedict's qualitative reagent, add 8 drops of urine (0.5 mL). Heat to boiling and set in a boiling water bath for 5 minutes or else boil it over a flame for 2 minutes.
Read as follows:

Blue to cloudy		
Green color	=	Negative, 0
Yellow-green	=	+ (< 0.5% glucose)
Greenish yellow	=	++ (0.5-1% glucose)
Yellow	=	+++ (1-2% glucose)
Orange to brick red	=	++++ (over 2% glucose)

Sensitivity of this test is 50 mg% or more.

Glucose oxidase methods: Glucose oxidase reacts with glucose to yield gluconic acid and hydrogen peroxide. Hydrogen peroxide and orthotolidine yield a blue color. This is a specific test. The reagents may be impregnated on paper strips (as mentioned above) and dipping them in urine provide the result in lesser time as compared to Benedict's method (sensitivity = 0.1%).

Benedict's Quantitative Glucose Test

Place a small quantity of powdered pumice, 10 g of anhydrous sodium carbonate and 25 mL of quantitative Benedict's reagent in a 250 mL container and heat. While the mixture is boiling, add urine rapidly from a buret until the blue color begins to fade, then add urine drop by drop until all blue color is gone and only a gray color remains. At this point, all cupric ions originally in solution is reduced. The amount of urine used contains 0.05 gm of glucose. To calculate grams of glucose per 100 mL of urine, divide 5 by the number of mL of urine used.

Quantitative Method

Urinary sugar can also be detected by routine biochemical kits too.

Sugar Tests in Urine (Table 5.3)

Significance of Sugars in Urine

Glycosuria with Hyperglycemia

Diabetes mellitus
Other endocrine disorders: Acromegaly, Cushing's syndrome, hyperthyroidism, pheochromocytoma.

Pancreatic disease: Cystic fibrosis—advanced stage, hemochromatosis, severe chronic pancreatitis, carcinoma.

CNS dysfunction: Asphyxia, tumors or hemorrhage, especially of hypothalamus.

Massive metabolic derangement: Severe burns, uremia, advanced liver disease, sepsis, cardiogenic shock.

Drug induced: Corticosteroids and ACTH, thiazides, oral contraceptives.

Glycosuria without Hyperglycemia

- Renal tubular dysfunction
- Pregnancy (differentiate from gestational diabetes).

TABLE 5.3: Reactivity of usual methods

Sugars detected	*Benedict's qualitative test*	*Glucose oxidase strips*
	Glucose Galactose Lactose Fructose Maltose Pentose	*Glucose*
False positive	Ascorbic acid Homogentisic acid Many antibiotics (antitubercular drugs) Phenothiazines Salicylates Levodopa X-ray contrast media	Hydrogen peroxide or hypochlorite in container
False negative		Ascorbic acid, homogentisic acid, large amounts of salicylates

Nonglucose Sugars in Urine

Galactose: Detecting galactosemia in newborn period may prevent irreversible liver and CNS damage. Galactose spills into urine only if milk is being taken.

Fructose: Essential fructosuria (rare).

Pentose: Very high fruit intake may cause pentosuria in normal persons.

Interfering Factors

a. Pregnancy and lactation may cause a false positive in enzymatic tests. About 70% of women show a temporary glucosuria that appears to be of no clinical value.
b. Ascorbic acid, creatinine in concentrated urine, streptomycin and homogentisic acid may cause a false positive reduction test (Benedict's), usually it will only be a trace reaction.
c. Stress excitement, testing after a heavy meal, and following the administration of IV glucose may cause false positives of all tests. Usually, it is a trace reaction.
d. Ascorbic acid in large amounts may cause a false negative in the enzyme tests.
e. False negatives may be obtained if deteriorated reagents strips have been used, or directions not followed exactly.

Ketone Bodies in Urine

Normal values: Negative

The three ketone bodies that can be detected in urine are:

1. Acetone (2%)
2. Acetoacetic acid (20%)
3. β-Hydroxybutyric acid (78%).

$$\text{Acetoacetic acid} \xrightarrow{-CO_2} \text{acetone}$$

$$\text{Acetoacetic acid} \rightleftharpoons \underset{-2H}{\text{Hydroxybutyric acid}}$$

Ketone bodies are products of incomplete fat metabolism and their presence is indicative of acidosis.

Tests for Ketone Bodies

(Never heat urine specimen before performing the tests).

1. Rothera's Test

Saturate 5 mL of urine with ammonium sulfate, add a few crystals of sodium nitroprusside. Shake it. Add liquor ammonia from the side of the test tube, formation of a purple ring at the junction indicates a positive test.

Sensitivity > 1–5 mg% acetoacetic acid, or > 10–25 mg% of acetone.

2. Legal's Test

Take 10 mL of urine in a test tube and add a few crystals of sodium nitroprusside. Acidify with glacial acetic acid, invert to mix. Overlay with strong liquor ammonia, let stand for 5 minutes. A violet ring indicates a positive test. The degree of positivity depends upon the speed of the reaction.

3. Paper Strip/Tablet Methods (Ketur Test—Boehringer)

These contain sodium nitroprusside, aminoacetic acid and disodium phosphate. A positive test is indicated by development of a purple color.

4. Diacetic Acid Test (Gerhardt's Test)

Not a very sensitive test. Perform this test if the test for acetone was positive. Precipitate the phosphates in 5 mL of urine with 10% ferric chloride solution, drop-by-drop, filter and add more ferric chloride. If a purple-red color appears, it indicates presence of 0.05% or more of diacetic acid. False-positive test may appear with salicylates, sodium bicarbonate, etc.

Causes of Ketonuria

1. Diabetic

Ketonuria indicates ketoacidosis and if unchecked may go on to coma. Juvenile diabetics are more susceptible to develop this. Whenever glycosuria is more than 2+, always test for ketone bodies also.

2. Nondiabetic

In infants and in children:

- Acute febrile states
- Toxic states with vomiting, diarrhea, etc.
- Hyperemesis gravidarum
- Cachexia with vomiting
- Post-anesthesia vomiting
- Conditions where there is limited availability of glucose, e.g. glycogen storage disease
- Sometimes following exposure to cold or severe exercise.

Clinical Implications

a. Ketosis and ketonuria may occur whenever increased amounts of fat are metabolized, carbohydrate intake restricted, or the diet is fat rich.
b. Ketonuria occurs in association with:
   - Fever
   - Anorexia
   - Starvation
   - Prolonged vomiting
   - Gastrointestinal disturbances
   - Following anesthesias
   - Fasting.
c. In non-diabetics, ketonuria will frequently occur in acute illness. Fifteen percent of hospitalized cases will show ketone bodies in urine even though they are non-diabetics.
d. Children are particularly prone to developing ketonuria and ketosis.
e. Ketone bodies appear in urine before there is any significant increase of ketone bodies in the blood.

Interfering Factors

a. Carbohydrate free diets as well as high protein and fat will cause ketonuria.
b. Drugs that may cause false positive tests.
   - Levodopa
   - BSP or PSP
   - Isopropyl alcohol
   - Metformin
   - Paraldehyde
   - Ether
   - Pyridium
   - Insulin
   - Phenformin.

Clinical Relevance

1. Presence of ketone bodies in the urine is helpful in differentiating between a diabetic coma and an insulin shock (hypoglycemia).
2. Any stressful condition that distorts the normal regulation of a diabetic can be recognized at any early point by a positive urine ketone test.
3. Urine ketones indicate caution, not a crisis situation, in either a diabetic or a non-diabetic patient.
   - Appearance of ketones in a diabetic implies that the patient is not adequately controlled, and that adjustments of either the medication or the diet should be made immediately
   - In a non-diabetic, ketone bodies indicate a small amount of CHO metabolism and excessive fat metabolism.

Bile Salts

Bile salts when present decrease the surface tension of urine. When sulfur powder is added on the surface of urine, sulfur particles sink to the bottom of the test tube. In normal urine sample, sulfur particles float on the surface of the urine.

Method

1. Take about 10 mL urine in a test tube.
2. Sprinkle a little dry sulfur powder on the surface of urine.
3. Observe the sulfur particles.

Interpretation

1. Sulfur particles sink to the bottom: Bile salts present
2. Sulfur particles remain floating: Bile salts absent. Dipstick tests are available.

Bile Pigments (Table 5.4)

Bile pigments (always use fresh specimen). Normal level of bile pigments is urine in < 0.02 mg%.

1. Foam Test

Not very accurate as proteins can also form foam. Shake 5 mL of urine in a test tube, bile produces a yellowish foam which persists.

TABLE 5.4: Bile pigments: Reactivity of various methods

	Diazo method	*Harrison/Fouchet's test*
False positive	Chlorpromazine	Aspirin metabolites, urobilin or indican, urobilinogen
False negative	Ascorbic acid High levels of nitrites Oxidation of bilirubin, if examination is delayed by over 4 hours	Oxidation of bilirubin if examination is delayed

2. Iodine Ring Test

A sensitive cum reliable test. Layer a solution of 10% alcoholic iodine on urine in a test tube. A green ring indicates presence of bile.

3. Harrison Test

A sensitive test. To 5 mL of urine, add 5 mL of 10% barium chloride in a test tube. Shake it. Filter it off. Let the filter paper dry. When dry, add 1–2 drops of Fouchet's reagent to the dried precipitate. A green (disregard all other colors) color indicates bilirubinuria.

4. Diazo Test

p-nitrobenzene diazonium p-toluene sulfonate is the active reagent. Place 5 drops of the urine on the mat provided in the kit. Bilirubin, if present shall be absorbed onto the mat surface. Place a reagent tablet on it. Let 2 drops of water flow over the tablet. A positive test is indicated by the appearance of a blue to purple color within 30 seconds. Pink/red color is negative. Sensitivity > 0.1 to 0.05 mg% of bilirubin in urine.

5. Paper Strip Method

After dipping the strip in urine, match with the color chart provided by the manufacturers.

Causes of Hyperbilirubinuria

1. Moderate to severe hepatocellular damage.
2. Obstruction of bile ducts, extrahepatic or intrahepatic.

In early hepatocellular damage and in hemolysis, urine bilirubin may be negative.

Urobilinogen and Urobilin

Urobilinogen is colorless, and on standing, it gets oxidized to urobilin which has a brown color. It is best to perform tests for urobilinogen on fresh specimens. If delay is inevitable, collect the sample in a dark bottle, provide a surface layer of petroleum ether and add sodium carbonate (5 g for 24 hours volume) and refrigerate the sample.

Urobilinogen

Ehrlich's test: Nitrites and bilirubin interfere with this test. Sulfonamide and procaine cause yellowish color reactions. Pyridium, indole, porphobilinogen and PAS yield pink-red color not different from that produced by urobilinogen.

To 10 mL of fresh sample at room temperature add 1 mL of Ehrlich's reagent, invert several times and let stand for 5 minutes. A pink color is normal, cherry or darker red color indicate abnormal amounts of urobilinogen. Dilutions may be used. Color reactions are normal in dilutions up to 1:20 (sensitivity > 1.3 mg%).

Urobilin (Schlesinger's Test)

Convert urobilinogen to urobilin by adding a few drops of Lugol's solution. Mix 10 mL of urine with an equal quantity of saturated alcoholic solution of zinc acetate filter into a dry test tube.

Abnormal amounts of urobilin give the filtrate a green fluorescence, which is best seen against a dark background with a light source from the side, or in sunlight against a black background.

The filtrate obtained from Harrison's test for bilirubin can be used for urobilinogen or urobilin.

Paper strip method (Dipstick tests are available).

Estimated Urobilinogen

Normal values: 0.1–1 Ehrlich unit/dL

Urinary urobilinogen is an important tool in routine urinalysis since it serves as a guide in detecting and differentiating liver disease, hemolytic disease, and biliary obstruction. Sequential determination assists in evaluating progress of disease and response to treatment. Both urobilinogen and bilirubin in urine may be regarded as bile pigments, but the tests provide different information.

Increased values of urobilinogen occur in:

- Cirrhosis: Bilirubin in urine may or may not be present
- Hemolytic jaundice: Bilirubin does not appear in urine.

A number of drugs produce false positives or negatives.

Urobilinogen (Quantitative)

Normal values

2-hour specimen: 0.1–1.0 Ehrlich units/2 hours

24-hour specimen: 1–4 mg/24 hours.

This is one of the most sensitive tests employed to determine impaired liver function. Bilirubin, formed from the metabolism of hemoglobin entering the intestine in the bile, is transformed through the action of bacteria into urobilinogen. Part of the urobilinogen formed in intestine is excreted with the feces; another portion is absorbed into the portal bloodstream and carried to the liver where it is metabolized and excreted in bile. Traces of urobilinogen that escape removal from the blood by the liver are carried to the kidneys and excreted in the urine.

Clinical Relevance

Increase in Urinary Urobilinogen

This occurs in any condition that causes an increase in the production of bilirubin and by any disease that

prevents the liver from normally removing the reabsorbed urobilinogen from the portal circulation.

a. Increased urobilinogen is found whenever there is excessive destruction of RBCs as in:
   - Hemolytic anemias
   - Pernicious anemia
   - Malaria.
b. Values above normal also occur in:
   - Infectious and toxic hepatitis
   - Pulmonary infarction
   - Biliary disease
   - Cholangitis
   - Hemolytic jaundice and anemia
   - Chemical injury to liver due to chloroform and carbon tetrachloride poisoning
   - Cirrhosis
   - Congestive heart failure
   - Infectious mononucleosis.
c. An increased urobilinogen level is one of the earliest signs of acute liver cell damage.

Decrease in Urinary Urobilinogen

This occurs when normal amounts of bilirubin are not excreted into the intestinal tract. It usually indicates partial or complete obstruction of the bile ducts. As occurs in:

- Cholelithiasis
- Severe inflammatory disease
- Cancer of head of pancreas
- During antibiotic therapy. Suppression of normal gut flora may prevent breakdown of bilirubin to urobilinogen, leading to its absence in urine
- Decreased values are also associated with:
  - Severe diarrhea
  - Renal insufficiency.

Interfering Factors

a. Drugs and foods that may cause urobilinogen to be increased include:
   - Para-aminosalicylic acid (PAS)
   - Antipyrine
   - BSP
   - Cascara
   - Phenothiazines
   - Sulfonamides
   - Drugs causing hemolysis of RBCs
   - Bananas
   - Phenazopyridine.
b. Drugs that may cause decreased urobilinogen include those that cause cholestasis and those that reduce bacterial flora in the GI tract (e.g. antibiotics).
c. Peak excretion is said to occur from noon to 16:00 hours. The urinary urobilinogen is subject to diurnal variation.
d. Strongly alkaline urine will show higher value and strongly acid urine will show a lower level.

Porphyrins

Perform Ehrlich's test for urobilinogen by mixing equal parts of urine and Ehrlich's reagent. Add 2 parts of saturated sodium acetate solution and mix. If turbid, filter. Shake with a small quantity of chloroform. Urobilinogen is soluble in chloroform, while porphobilinogen is not. If after several extractions with chloroform the aqueous phase is still pink, the test is positive for porphobilinogen.

Causes

Conditions producing increased levels of any of the heme precursors are called porphyrias. The two rare major categories of genetically determined porphyria and erythropoietic porphyrias, in which the major diagnostic abnormalities occur in red cell chemistry, and hepatic porphyrias, in which heme precursors are found in urine or feces. In acquired disorders, precursors accumulate more in urine and feces than in red cells.

Normal Values

Porphobilinogens	:	2 mg/24 h or negative
Porphyrins	:	50–300 mg/24 h
DAL or ALA	:	1–710 mg/24 h
Fluorescent	:	Negative

Porphyrins are cyclic compounds formed from delta-aminolevulinic acid (DAL or ALA), which is important in the formation of hemoglobin and other hemoproteins that function as carriers of oxygen in the blood and tissues. In health, insignificant amounts of porphyrin are excreted in the urine. However, in conditions like porphyria (disturbance in metabolism of porphyrin), liver disease, lead poisoning, and pellagra, there is an increased level of porphyrins as well as DAL and ALA in the urine. Disorders of porphyrin metabolism also result in porphobilinogen. In acute attacks of porphyria, the patient may suffer skin lesions, abdominal pain, neuropathy, and mental disturbances. The urine of patients with this disease usually has a pinkish to reddish-black tinge and will become darker upon standing. In the laboratory, the urine is tested for the presence of porphyrins, porphobilinogen, and DAL or ALA. It is also given the black light screening test (porphyrins fluorescence when exposed to black or ultraviolet light).

A random sample or 24 hours sample may be submitted to the laboratory. Porphobilinogens are always done with porphyrin test. Should a single, fresh-voided specimen be ordered, only a porphobilinogen will be done. Protect specimen from light. The test must be performed within 60 minutes. Random sample should be obtained between 10:00 and 14:00 hours. Observe and record the color of urine. If porphyrins are present, the urine may have a grossly recognisable amber red or burgundy color. It may vary from pale pink to almost black. Some patients will excrete urine of normal color that turns dark after standing in the light.

Clinical Relevance

Porphyria

1. In the porphyrias, the urine contains increased amounts of porphyrins and porphobilinogens and may not contain increased amounts of DAL or ALA.
2. ALA and DAL excretion is elevated in acute intermittent porphyria, a hepatic porphyria that is aggravated by alcohol, barbiturates, and other drugs affecting the liver.

Lead Poisoning

1. ALA or DAL will be present in the urine
2. Porphyrins may or may not be present in the urine.

Other Conditions with Increased Levels of Porphyrins

- Cirrhosis
- Infectious hepatitis
- Hodgkin's disease
- Some cancers
- CNS disorders
- Heavy metal poisoning
- Carbon tetrachloride or benzene poisoning.

Interfering Factors

1. During menstruation and pregnancy, porphyrins may be normally increased
2. Drugs that can cause false-positive test are:
    - Acriflavine
    - Ethoxazene
    - Phenazopyridine
    - Sulfamethoxazole
    - Tetracyclines
    - Antipyretics
    - Barbiturates
    - Phenylhydrazine
    - Sulfonamides.

Blood in Urine (Hematuria)

Hematuria can be gross, urine appears reddish due to blood, it can also be microscopic, when it is not visible to the naked eye, here various tests are performed for confirmation.

1. Guaiac Test

In one test tube, mix 2 mL of 10% acetic acid, 5 mL of urine and 5 mL ether. In a second test tube, place 5 mL of 95% alcohol, 2 mL fresh hydrogen peroxide and a pinch of powdered guaiac. Now pour the guaiac solution slowly down the side of the first tube. Blood in the urine causes blue color to appear at the zone of contact between the guaiac and ether.

2. Benzidine Test

Saturate 2 mL of glacial acetic acid with benzidine and pour off the clear supernatant fluid. Add 1 mL of fresh hydrogen peroxide and 2 mL of urine. Development of blue color indicates a positive test (if the blue color develops before the addition of urine, the glassware is contaminated).

3. Paper Strips

(Sangur test – Boehringer). Blood reacts with the peroxide-orthotolidine reagent to produce a blue color.

Causes

a. Bleeding diathesis.
b. Local disorders of kidney and genitourinary tract.
    1. Trauma
    2. Cystitis
    3. Renal calculi
    4. Genitourinary tumors
    5. Heritable disorders
        - Hemoglobinopathies
        - Osler-Weber-Rendu disease
        - Polycystic kidney.

Diffuse Renal Lesions

1. Acute and chronic glomerulonephritis
2. Systemic lupus erythematosus
3. Polyarteritis
4. Goodpasture's syndrome
5. Tuberculous pyelonephritis
6. Allergic nephropathies (Henoch-Schonlein's purpura)
7. Thrombotic thrombocytopenic purpura
8. Focal embolic glomerulitis
9. Malignant hypertension
10. Chemical/Drug induced
    - Carbon tetrachloride
    - Sulfonamides
    - Dicoumarol, etc.

Nitrite/Bacteria

Normal values: Negative for bacteria.

Explanation of test: There are two methods that are used to detect bacteria in the urine during routine urinalysis—microscopic examination and clinical testing. The sediment when examined microscopically can reveal bacteria when present. Chemical dipstick testing is also commonly done. The nitrite area in the multiple reagent strip, is calibrated so that any shade of pink color that develops within 30 seconds indicates an amount of nitrite produced by 10^5 or more organisms per mL in the urine specimen.

Procedure

1. A first morning specimen is preferred because urine that has been in the bladder for several hours is more likely to yield a positive result. A clean catch or midstream urine is needed to avoid bacterial contamination.
2. Follow procedure as stated by the dipstick manufacturer.

Clinical Implications

1. The finding of 20 or more bacteria per high power field may indicate a urinary tract infection.
2. The presence of only a few bacteria should be interpreted with caution and suggests a urinary tract infection that cannot be confirmed or excluded until more definitive studies, such as cultures and sensitivity tests are performed.
3. A positive result from the nitrite test is a reliable indication of a significant bacteriuria and is an indication for urine culture.
4. A negative result should never be interpreted as indicating absence of bacteriuria because:
   a. If an overnight sample was not used, there may have been insufficient time for the conversion of nitrate to nitrite to have occurred.
   b. There may be a rare instance when nitrite does not appear in urine, and a person of this type could have significant bacteria without a positive test.
   c. Some strains of urinary pathogens do not produce enzymes necessary to change nitrate to nitrite and can cause a negative result.

Rapid Diagnostics

Rapidity of diagnosis is of utmost importance in today's context.

Product	*Determines*
a. Combur 9 test	Leukocytes, nitrite, pH, protein, glucose, ketone bodies, urobilinogen, bilirubin, blood
b. Combur 8 test	All of A except leukocytes
c. Combur 7 test	All of B except nitrite
d. Combur 6 test	All of C except bilirubin
e. Combur 4 test	Protein, glucose urobilinogen, blood
f. Ecur test	Protein, glucose, blood
g. Combur test	Glucose, protein, pH

Many manufacturers now provide rapid strip test (qualitative and semiquantitative). Important among them are as follows.

Strip Tests from Boehringer-Knoll Limited

Various other combinations or single test strips are also available, e.g. pertaining only to liver/kidney disorders.

Strip Tests from Roche Limited

Now with improved pad order and efficacy.

Product	*Deterimines*
a. Diastix	Glucose
b. Hemastix	Blood
c. Ictotest (tablets)	Bilirubin
d. Keto-diastix	Glucose and ketones pH,
e. Multistix	protein, glucose, ketones, bilirubin, blood, urobilinogen
f. Uristix	Glucose and protein
g. Combistix SG	Glucose, protein, pH and specific gravity
h. Multistix SG	All of (E) + Specific gravity
i. Neostix 3	Blood, glucose and protein

MULTIPLE® REAGENT STRIPS FOR URINALYSIS

Tests for glucose, bilirubin, ketone (acetoacetic acid), specific gravity, blood, pH, protein and urobilinogen in urine. Refer to the carton and bottle label for specific reagent areas on the product you are using.
(*Courtesy:* Roche)

Summary and Explanation/Intended Use

Bayer reagent strips for urinalysis are firm plastic strips to which are affixed several separate reagent areas. Depending on the product being used, Bayer reagent strips provide tests for glucose, bilirubin, ketone (acetoacetic acid) specific gravity, blood, pH, protein, and urobilinogen in urine. *Please refer to the carton and bottle label for*

specific reagent areas on the product you are using. Test results may provide information regarding the status of carbohydrate metabolism, kidney and liver function, and acid-base balance.

The reagent test areas on Bayer Reagent strips are ready to use upon removal from the bottle and the entire reagent strip is disposable. The strips may be read visually, requiring no additional laboratory equipment for testing. Certain configurations of strips may also be read instrumentally, using the Clinitek® family of urine chemistry analyzers and the appropriate program module or program card.

The directions must be followed exactly. Accurate timing is essential to provide optimal results. The reagent strips must be kept in the bottle with the cap tightly closed to maintain reagent reactivity. To obtain optimal results, it is necessary to use fresh, *well-mixed*, uncentrifuged urine.

Chemical Principles of the Procedure

Glucose

This test is based on a double sequential enzyme reaction. One enzyme, glucose oxidase, catalyzes the formation of gluconic acid and hydrogen peroxide from the oxidation of glucose. A second enzyme, peroxidase, catalyzes the reaction of hydrogen peroxide with a potassium iodide chromogen to oxidize the chromogen to colors ranging from green to brown.

Bilirubin

This test is based on the coupling of bilirubin with diazotized dichloroaniline in a strongly acid medium. The color ranges through various shades of tan.

Ketone

This test is based on the development of colors ranging from buff-pink, for a negative reading, to purple when acetoacetic acid reacts with nitroprusside.

Specific Gravity

This test is based on the apparent pKa change of certain pretreated polyelectrolytes in relation to ionic concentration. In the presence of an indicator, colors range from deep blue-green in urine of low ionic concentration through green and yellow-green in urines of increasing ionic concentration.

Blood

This test is based on the peroxidase-like activity of hemoglobin, which catalyzes the reaction of disopropylbenzene dihydroperoxide and 3.3′, 5, 5′-tetramethylbenzidine. The resulting color ranges from orange through green; very high levels of blood may cause the color development to continue to blue.

pH

The test is based on the double indicator principle that gives a broad range of color covering the entire urinary pH range. Colors range from orange through yellow and green to blue.

Protein

This test is based on the protein-error-of-indicators principle. At a constant pH, the development of any green color is due to the presence of protein. Colors range from yellow for "Negative" through yellow-green and green to green-blue for "Positive" reactions.

Urobilinogen

This test is based on a modified Ehrlich reaction, in which ρ-diethylaminobenzaldehyde in conjunction with a color enhancer reacts with urobilinogen in a strongly acid medium to produce a pink-red color.

Reagents

(Based on dry weight at time of impregnation):

Glucose

2.2% w/w glucose oxidase (microbial, 1.3 IU); 1.0% w/w peroxidase (horseradish, 3300 IU); 8.1% w/w potassium iodide; 69.8% w/w buffer; 18.9% w/w non-reactive ingredients.

Bilirubin

0.4% w/w 2, 4-dichloroaniline diazonium salt; 37.3% w/w buffer; 62.3% w/w non-reactive ingredients.

Ketone

7.1% w/w sodium nitroprusside; 92.9% w/w buffer.

Specific Gravity

2.8% w/w bromothymol blue; 68.8% w/w poly (methyl vinyl ether/maleic anhydride); 28.4% w/w sodium hydroxide.

Blood

6.8% w/w disopropylbenzene dihydroperoxide; 4.0% w/w 3.3′, 5, 5′-tetramethylbenzidine; 48.0% w/w buffer; 41.2% w/w non-reactive ingredients.

pH

0.2% w/w methyl red; 2.8% w/w bromothymol blue; 97.0% w/w non-reactive ingredients.

Protein

0.3% w/w tetrabromophenol blue; 97.3% w/w buffer; 2.4$ w/w non-reactive ingredients.

Urobilinogen

0.2% w/w ρ-diethylaminobenzaldehyde; 99.8% w/w non-reactive ingredients.

Warning and Precautions

Bayer reagent strips are for in vitro diagnostic use.

Storage

Storage below 30°C in a cool, dry place. Do not refrigerate. Keep out of direct sunlight. Do not use after expiration date.

Recommended Procedures for Handling Roche Reagent Strips

All unused strips must remain in the original bottle. Transfer to any other container may cause reagent strips to deteriorate and become unreactive. Do not remove desiccant (s) from bottle. **Do not remove strip from the bottle until immediately before it is to be used for testing. Replace cap immediately and tightly after removing reagent strip.** Do not touch test areas of the reagent strip. Work areas and specimen containers should be free of detergents and other contaminating substances.

Dip test areas in urine completely, but briefly, to avoid dissolving out the reagent. If using strips visually, read test results carefully at the time specified, in a good light (such as fluorescent) and with the test area held near the appropriate color chart on the bottle label. Do not read the strips in direct sunlight. If the strips are used instrumentally, carefully follow the directions given in the appropriate instrument-operating manual.

Important

Protection against ambient moisture, light and heat is essential to guard against altered reagent reactivity. Discoloration or darkening of reagent areas may indicate deterioration. If this is evident, or if test results are questionable or inconsistent with expected findings, the following steps are recommended:

1. Confirm that the product is within the expiration date shown on the label;
2. Check performance against known negative and positive control materials (e.g. Chek-stix® Control Strips);
3. Retest with fresh product. If proper results are not obtained, consult your local product representative for advice on testing technique and results.

Specimen Collection and Preparation

Collect urine in a clean container and test it as soon as possible. Do not centrifuge. The use of urine preservatives is not recommended. If testing cannot be done within an hour after voiding, refrigerate the specimen immediately and let it return to room temperature before testing.

It is especially important to use fresh urine to obtain optimal results with the tests for bilirubin and urobilinogen, as these compounds are very unstable when exposed to room temperature and light.

Prolonged exposure of urine to room temperature may result in microbial proliferation with resultant changes in pH. A shift to alkaline pH may cause false positive results with the protein test area. Urine containing glucose may decrease in pH as organisms metabolize the glucose. Bacterial growth from contaminating organisms may cause false positive blood reactions from the peroxidases produced.

Contamination of the urine specimen with skin cleansers containing chlorhexidine may affect protein (and to a lesser extent specific gravity and bilirubin) test results. The user should determine whether the use of such skin cleansers is warranted.

Procedure

Must be followed exactly to achieve reliable test results:

1. Collect fresh urine specimen in a clean, dry container. Mix well immediately before testing.
2. Remove one strip from bottle and replace cap. Completely immerse reagent areas of the strip in fresh urine and remove immediately to avoid dissolving out reagents.
3. While removing, run the edge of the entire length of the strip against the rim of the urine container to remove excess urine. Hold the strip in a horizontal position to prevent possible mixing of chemicals from adjacent reagent areas and/or contaminating the hands with urine.
4. a. If reading visually, compare reagent areas to corresponding color chart on the bottle label at the times specified. Hold strip close to color blocks

and match carefully. Avoid laying the strip directly on the Color Chart, as this will result in the urine soiling the chart.

b. If reading instrumentally, carefully follow the directions given in the appropriate instrument-operating manual.

Proper read time is critical for optimal results. If using strips visually, read the glucose and bilirubin tests at 30 seconds after dipping. Read the ketone test at 40 seconds; the specific gravity test at 45 seconds; pH, protein, urobilinogen and blood at 60 seconds. The pH and protein areas may also be read immediately or at any time up to 2 minutes after dipping.

After dipping the strip, check the pH area. If the color on the pad is not uniform, read the reagent area immediately, comparing the darkest color to the appropriate Color Chart. All reagent areas may be read between 1 and 2 minutes for identifying negative specimens and for determination of the pH and SG. Color changes that occur after 2 minutes are of no diagnostic value. If using strips instrumentally, the instrument will automatically read each reagent area at a specified time.

Quality Control

For best results, performance of reagent strips should be confirmed by testing known negative and positive specimens or controls whenever a new bottle is first opened. Negative and positive specimens or controls may also be randomly hidden in each batch of specimens tested. Water should not be used as a negative control. Each laboratory should establish its own goals for adequate standards of performance, and should question handling and testing procedures if these standards are not met. Chek-stix® Positive and Negative Control Strips, with positive, negative or defined results, provide a convenient basis for a urinalysis quality control program.

Because of the various constituents that are added to commercial controls other than Chek-stix Control Strips, or the way in which they are processed, specific gravity values determined using Bayer Reagent Strips may not always correspond with values given in the product inserts for these controls.

Due to its specificity for acetoacetic acid, the ketone reagent area may not react with commercial controls other than Chek-stix Positive Control Strips. If questionable results are obtained with the ketone reagent area, strip reactivity should be checked with Chek-stix Positive Control Strips or by testing negative and positive clinical specimens that have been identified as positive or negative with a reference test method.

Results

Results with Bayer Reagent Strips are obtained in clinically meaningful units directly from the Color Chart comparison when using strips visually. With instrumental use, the reagent pads are "read" by the instrument and the results are displayed or printed. The color blocks and instrumental display values represent nominal values; actual values will vary around the nominal values.

Limitations of Procedures

No laboratory tests, definitive diagnostic or therapeutic decisions should be based on any single result or method.

Substances that cause abnormal urine color, such as drugs containing azo dyes (e.g. Pyridium®, Azo Gantrisin®, Azo Gantanol®), nitrofurantoin (Macrodantin®, Furadantin®), and riboflavin, may affect the readability of the reagent areas on urinalysis reagent strips. The color development on the reagent pad may be masked, or a color reaction may be produced on the pad that could be interpreted visually and/or instrumentally as a false positive.

Glucose

Ascorbic acid concentrations of 50 mg/dL or greater may cause false negatives for specimens containing small amounts of glucose (75–125 mg/dL). Ketone bodies reduce the sensitivity of the test; moderately high ketone levels (40 mg/dL) may cause false negatives for specimens containing small amounts of glucose (75–125 mg/dL) but the combination of such ketone levels and low glucose levels is metabolically improbable in screening. The reactivity of the glucose test decreases as the SG of the urine increases. Reactivity may also vary with temperature.

Bilirubin

Indican (Indoxyl sulfate) can produce a yellow-orange to red color response that may interfere with the interpretation of a negative or a positive bilirubin reading. Metabolites of Lodine® (etodolac) may cause false positive or atypical results; ascorbic acid concentrations of 25 mg/dL or greater may cause false negatives. Since very small amounts of bilirubin may be found in the earliest phases of liver disease, the user must consider whether the sensitivity of Bayer Reagent Strips to bilirubin is sufficient for the intended use.

Ketone

False positive results (Trace or less) may occur with highly pigmented urine specimens or those containing large amounts of levodopa metabolites. Compounds such as

mesna (2-mercaptoethane sulfonic acid) that contain sulfhydryl groups may cause false positive results or an atypical color reaction.

Specific Gravity

The chemical nature of the Bayer SG test may cause slightly different results from those obtained with other specific gravity methods when elevated amounts of certain urine constituents are present. Highly buffered alkaline urines may cause low readings relative to other methods. Elevated specific gravity readings may be obtained in the presence of moderate quantities (100–750 mg/dL) of protein.

Blood

Elevated specific gravity may reduce the reactivity of the blood test. Clapoten® (captopril) may also cause decreased reactivity. Certain oxidizing contaminants, such as hypochlorite, may produce false positive results. Microbial peroxidase associated with urinary tract infection may cause a false positive reaction. Levels of ascorbic acid normally found in urine do not interfere with this test.

pH

If proper procedure is not followed and excess urine remains on the strip, a phenomenon known as "runover" may occur, in which the acid buffer from the protein reagent will run onto the pH area, causing a false lowering of the pH result.

Protein

False positive results may be obtained with highly buffered or alkaline urines. Contamination of the urine specimen with quaternary ammonium compounds (e.g. from some antiseptics and detergents) or with skin cleansers containing chlorhexidine may also produce false positive results.

Urobilinogen

The reagent area may react with interfering substances known to react with Ehrlich's reagent, such as p-aminosalicylic acid and sulfonamides. Atypical color reactions may be obtained in the presence of high concentrations of ρ-aminobenzoic acid. False negative results may be obtained if formalin is present. Strip reactivity increases with temperature; the optimum temperature is 22–26°C. The test is not a reliable method for the detection of porphobilinogen. The absence of urobilinogen cannot be determined with this test.

Expected Values

Expected values for the typical "normal" healthy population and the abnormal population are listed below for each reagent. Exact agreement between visual results and instrumental results might not be found because of the inherent differences between the perception of the human eye and the optical systems of the instruments.

Glucose

The kidney normally excretes small amount of glucose. These amounts are usually below the sensitivity of this test but on occasion may produce a color between the negative and the 100 mg/dL color blocks, and that is interpreted by the instrument as a positive result. Results at the first positive level may be significantly abnormal if found consistently.

Bilirubin

Normally no bilirubin is detectable in urine by even the most sensitive methods. Even trace amounts of bilirubin are sufficiently abnormal to require further investigation. Atypical colors (colors that are unlike the negative or positive color blocks shown on the Color Chart) may indicate that bilirubin derived bile pigments are present in the urine sample and may be masking the bilirubin reaction. These colors may indicate bile pigment abnormalities and the urine specimen should be tested further.

Ketone

Normal urine specimens ordinarily yield negative results with this reagent. Detectable levels of ketone may occur in urine during physiological stress conditions such as fasting, pregnancy and frequent strenuous exercise. In ketoacidosis, starvation or with other abnormalities of carbohydrate or lipid metabolism, ketones may appear in urine in large amounts before serum ketone concentrations are elevated.

Specific Gravity

Random urines may vary in specific gravity from 1.001–1.035. Twenty-four hour urines from normal adults with normal diets and normal fluid intake will have a specific gravity of 1.016–1.022.

Blood

The significance of the Trace reaction may vary among patients, and clinical judgment is required for assessment in an individual case. Development of green spots (intact

erythrocytes) or green color (free hemoglobin/myoglobin) on the reagent area within 60 seconds indicates the need for further investigation. Blood is often, but not always, found in the urine of menstruating females. This test is highly sensitive to hemoglobin and thus complements the microscopic examination.

pH

Both the normal and abnormal urinary pH range is from 5 to 9.

Protein

Normally, no protein is detectable in urine, although the normal kidney excretes a minute amount. A color matching any block greater than Trace indicates significant proteinuria. For urine of high specific gravity, the test area may most closely match the Trace color block even though only normal concentrations of protein are present. Clinical judgment is needed to evaluate the significance of Trace results.

Urobilinogen

The normal urobilinogen range obtained with this test is 0.2 to 1.0 mg/dL. A result of 2.0 mg/dL represents the transition from normal to abnormal, and the patient and/or urine specimen should be evaluated further.

Specific Performance Characteristics

Specific performance characteristics are based on clinical and analytical studies. In clinical specimens, the sensitivity depends upon several factors: the variability of color perception, the presence or absence of inhibitory factors typically found in urine, the specific gravity, and the pH (see Limitations of Procedures section); and the lighting conditions when the product is read visually. Because the color of each reagent area changes as the analyte concentration increases, the percentage of specimens detected as positive will increase with the analyte concentration.

Each color block or instrumental display value represents a range of values. Because of specimen and reading variability/specimens with analyte concentrations that fall between nominal levels may give results at either level. Results at levels greater than the second positive level for the glucose, ketone, protein and urobilinogen tests will usually be within one 'level of the true concentration. Exact agreement between visual results and instrumental results might not be found because of the inherent differences between the perception of the human eye and the optical systems of the instruments.

The following table lists the generally detectable levels of analytes in contrived urine; however, because of the inherent variability of clinical urines, lesser concentrations may be detected under certain conditions.

Reagent area	*Sensitivity*
Glucose	75–125 mg/dL glucose
Bilirubin	0.4–0.8 mg/dL bilirubin
Ketone	5–10 mg/dL acetocetic acid
Blood	0.015–0.062 mg/dL hemoglobin
Protein	15–30 mg/dL albumin

Glucose

The test is specific for glucose; no substance excreted in urine other than glucose is known to give a positive result. The reagent area does not react with lactose, galactose, fructose nor reducing metabolites of drugs (e.g. salicylates and nalidixic acid. This test may be used to determine whether the reducing substance found in urine is glucose. Reactivity may be influenced by urine specific gravity and temperature. In dilute urines containing less than 5 mg/dL ascorbic acid, as little as 40 mg/dL glucose may produce a color change that might be interpreted as positive. The test is more sensitive than the copper reduction test (e.g. Clinitest® Reagent Tablets). If the color appears somewhat mottled at the higher glucose concentrations, match the darkest color to the color blocks.

Bilirubin

The test has a sensitivity of 0.4–0.8 mg/dL bilirubin.

Ketone

The test reacts with acetoacetic acid in urine. It does not react with acetone or (3-hydroxybutyric acid. Some high specific gravity/low pH urines may give reactions up to and including Trace. Clinical judgment is needed to determine the significance of reactions up to and including Trace.

Specific Gravity

The specific gravity test permits determination of urine specific gravity between 1.000 and 1.030. In general, it correlates within 0.005 with values obtained with the refractive index method. For increased accuracy, 0.005 may be added to readings from urines with pH equal to or greater than 6.5. Strips read instrumentally are automatically adjusted for pH by the instrument. The Bayer SG test is not affected by certain nonionic urine constituents such as glucose nor by the presence of radiopaque dye.

Blood

The sensitivity of this test may be reduced in urines with high specific gravity. The test is equally sensitive to myoglobin as to hemoglobin. The appearance of green spots on the reacted reagent area indicates the presence of intact erythrocytes in the urine. The color chart includes examples of trace and moderate nonhemolyzed color blocks. Reactions ranging from trace to large, with proportionately more numerous spots, may be observed, (A hemoglobin concentration of 0.015-0.062 mg/dL is approximately equivalent to 5–20 intact red blood cells per microliter). Because of the optical systems of urine chemistry instruments, the sensitivity to intact erythrocytes is lower than that perceived visually.

pH

The pH test area measures pH values generally to within 1 unit in the range of 5–8.5 visually and 5–9 instrumentally. pH readings are not affected by variations in the urinary buffer concentration.

Protein

The reagent area is more sensitive to albumin than to globulins, hemoglobin, Bence-Jones protein, and muco-protein; a negative result does not rule out the presence of these other proteins.

Urobilinogen

This test area will detect urobilinogen in concentrations as low as 0.2 mg/dL (approximately 0.2 EU/dL) in urine. The absence of urobilinogen in the specimen cannot be determined.

Multistix® Urinalysis Strips (Fig. 5.3)

FIG. 5.3: Presentation

Dependable Results When and Where You Need Them

Bayer's Multistix strips lead the market in providing a range of rapid urine testing results. When read visually or automatically on either the Clinitek 50 or Clinitek 500 readers they enable on the spot clinical decisions to be made with confidence (Table 5.5).

Easy to Use (Figs 5.4 to 5.6)

Rapid Results (Fig. 5.6)

- Fast, reliable results available in 1–2 minutes
- Automated reading provided in 1 minute using.

TABLE 5.5: Multistix configurations

	Leuc	*Nitrite*	*Urobil*	*Prot*	*pH*	*Blood*	*SG*	*Ket*	*Bill*	*Gluc*
Multistix 10 SG	√	√	√	√	√	√	√	√	√	√
Multistix 8 SG	√	√		√	√	√	√	√		√
Multistix GP	√	√		√	√	√	√	√		√
N-Multistix SG		√	√	√	√	√	√	√	√	√
Multistix SG			√	√	√	√	√	√	√	√
Labstix SG				√	√	√	√	√		√
N-Labstix		√		√	√	√		√		√
Bili-Labstix				√	√	√		√	√	√
Labstix				√	√	√		√		√
Hema-Combistix				√	√	√				√
Uristix				√						√
Albustix				√						

FIG. 5.4: Dip

FIG. 5.6: Read at correct time

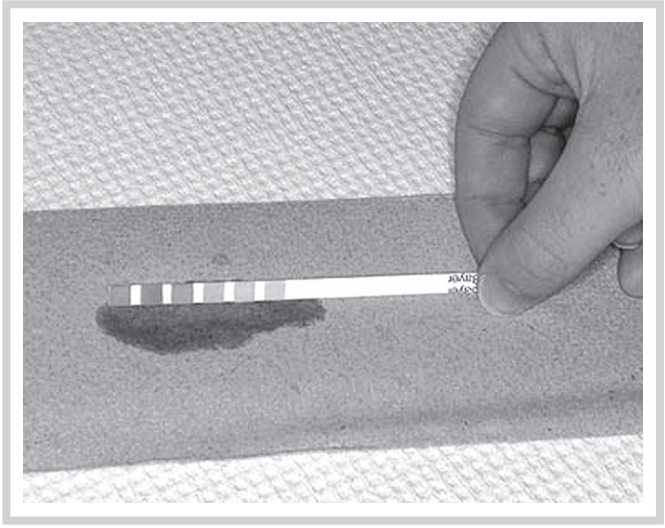


FIG. 5.5: Blot

First Line Health Screen for a Variety of Settings

- *Menu:* Glucose, ketones, bilirubin, urobilinogen, specific gravity, blood , pH, protein, nitrite, leukocytes.

Improved Use of Resources

- **Diabetes Management/Renal checks using micro-albumin (Albumin : Creatinine Ratio) while the patient waits**
- Screens out non-infected urine samples so that only the positives need to be referred for laboratory follow-up in cases of urinary tract infection.

AUTOMATION IN URINALYSIS

Clinitek Status® Urine Analyzer (Fig. 5.7)

Dependable Results in Any Patient Setting

Introducing the new Clinitek Status. Providing simple, accurate results for higher standards in urinalysis; Urinalysis has long been an essential tool in health care, but visually read results may be less than ideal today's world. The Clinitek Status can help (Fig. 5.8):

- New levels of precision and reliability
- Unprecedented convenience

FIG. 5.7: Clinitek status—the instrument

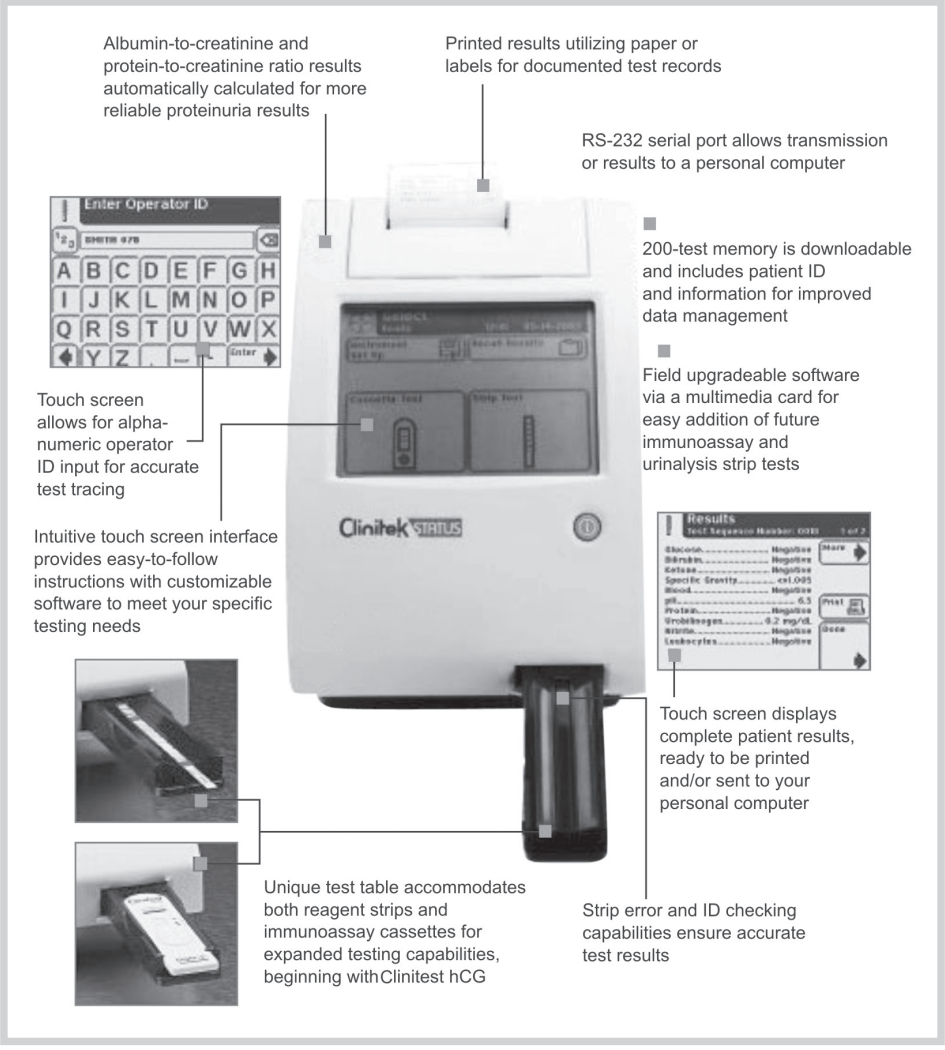


FIG. 5.8: Clinitek Status instrument (***Courtesy:*** Siemens Medical Solutions)

TABLE 5.6: Specifications

Mains/Battery (optional)	*Dimensions*	*Weight*	*Instrument memory*	*Computer powered interface*
240 V Transformer (supplied)/6 AA non-rechargeable alkaline batteries (not supplied)	Depth–272 mm (10.7 inches) Width-171 mm (6.7 inches) Height–158 mm (6.2 inches)	Clinitek status® instrument only (without batteries or power supply –1.66 kg (3.65 lbs)	200 patient test results 200 patient details (patient's name and/or patient identification)	Via RS 232 serial port

- Time saving simplicity
- Reassuringly proven performance
- Minimal training required
- Audit compliant
- Pregnancy test capability using the NEW CIinitest hCG
- Automation reduces the risk of errors.

A Wide Range of Test Parameters

Clinitek status is suitable for use with a wide range of Multistix tests including:

- Multistix 10SG
- Multistix 8SG

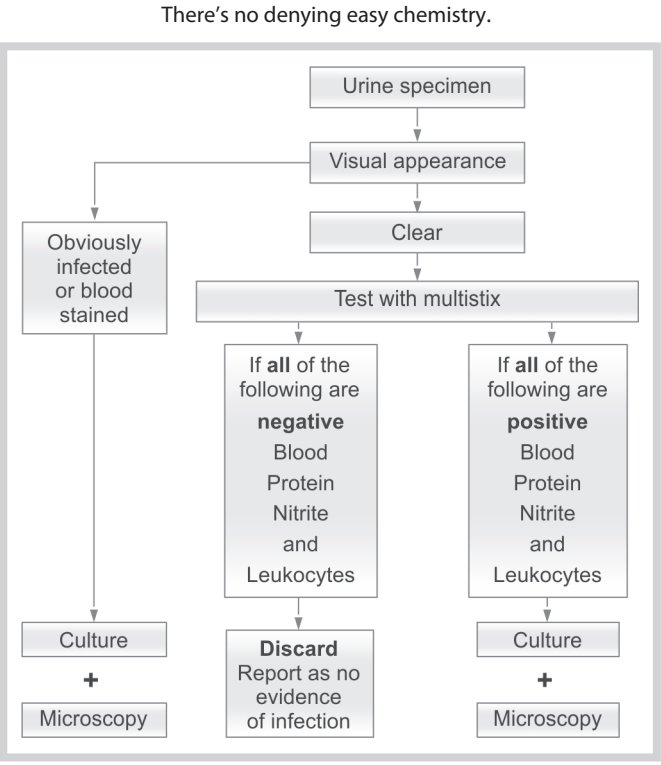


FIG. 5.9: Schematic flow through diagram for ascertaining UTI

Leukocytes	A passive result may indicate renal disease or urinary tract infection
Nitrite	A positive result may indicate urinary tract infection
Urobilinogen (10SG only)	Normally present in urine: elevated levels may indicate liver abnormalities or excessive destruction of RBC's, e.g. in homolytic anemia. urobilinogen should be considered alongside billirubin as a differential diagnosis
Protein	A positive result indicates renal disease, raised blood pressure or urinary tract infection
pH	Normal range 4 to 6. A pH above 7 suggests states urine unsuitable for testing
Blood	Presence in urine suggests serious renal or urological disease, or renal tract infection
Specific gravity	Monitors the concentrating and diluting power of the kidney. Assists in the interpretation of other tests
Ketone	May indicate uncontrolled diabetes or a reduced carbohydrate diet
Bilirubin (10SG only)	Indicative of hepatic or biliary disease. Bilirubin may appear in urine before other signs of abnormality are apparent
Glucose	The most important cause of glucose in urine is diabetes mellitus

FIG. 5.10: Etiological basis of positive test results

- Multistix GP
- Clinitek microalbumin
- Clinitest hCG.

Clinitek® 50 Urine Analyzer (Fig. 5.11)

Dependable Results in Any Patient Setting

Combined with the Bayer market leading urinalysis strips, Multistix®, the Bayer Clinitek 50 provides the complete urinalysis solution. Suitable for use in a wide range patient settings, the Clinitek 50 provides on the spot, accurate results that allowing on the spot clinical decisions.

Easy to Use

1. Dip reasgent strip into sample and press *start* button (Fig. 5.12)
2. Blot side of reagent strip and place strip on instrument feed table (Fig. 5.13)
3. Instrument analyzes, displays abnormally and prints results at the rate of one test per minute (Fig. 5.14).

A Helping Hand

- The Clinitek 50 requires only 10 seconds of operators time, meaning you can get on with caring for your patient whilst the instrument does the test.

User Friendly

- Display prompts make the Clinitek 50 intuitive and easy to use.

FIG. 5.11: Clinitek 50: The instrument (*Courtesy:* Siemens Medical Solutions)

FIG. 5.12: Dip the reagent strip

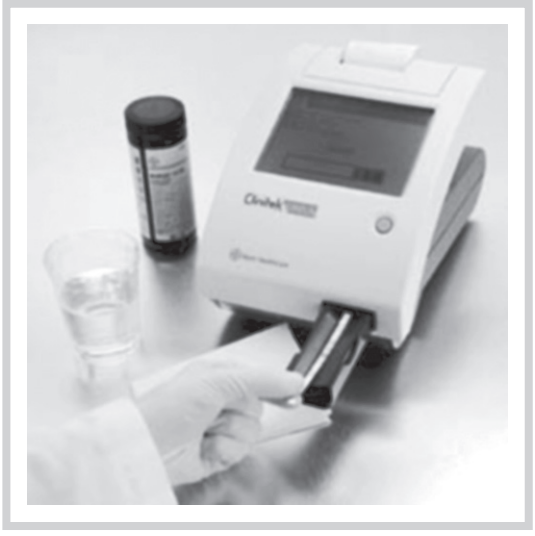


FIG. 5.13: Place reagent strip on the instrument feed table

Rapid Results

- Fast, reliable results available in 1 minute, giving a printed record of the patients results.

Improved Use of Resources

Screens out non-infected urine samples so that only the positives need to be referred for laboratory follow-up (Fig. 5.9).

A Wide Range of Test Parameters

Clinitek 50 is suitable for use with;

- Multistix lOSG Multistix 8SG
- Multistix GP

FIG. 5.14: The instrument with printout

- Clinitek Microalbumin
- See Figure 5.10 for etiological basis of positive results obtained.

Clinitek® 500 Urinalysis Instrument

In combination with the Bayer market leading, Multistix® range, the Clinitek® 500 enables automated reading of strips in high throughout settings (Fig. 5.15). Complimented by a user friendly interface and comprehensive data management it provides a discrete platform offering accuracy of results and efficiency in workflow.

FIG. 5.15: Clinitek® 500 urinalysis instrument

Efficiency in Processing

- 1 strip processed every 7 seconds
- 1 result every minute
- Strip needs only to be placed in analyzer platform. Continuous "load and capture"mechanism draws strip into reader
- Strip automatically discarded into waste tray after processing
- Barcode reader for data entry.

User Friendly

- Easy to read touch screen display
- Operator screen guidance for processing
- Barcode reader for data entry.

Intelligent Data Management

- Accurate identification and flagging of abnormal data
- Two screening functions—confirmatory and microscopic
- Customizable testing and reporting to meet local needs
- Memory storage of 500 patient results and 200 control results.
- Flexible reporting options
  - Internal data storage
  - Transfer via RS232 port
  - Print using on board printer
  - Send to external printer.
- Operator and patient ID facility.

A Wide Range of Test Parameters

Clinitek® 500 is suitable for use with:

- Multistix 1OSG
- Multistix 8SG
- Multistix GP.

SPECIAL URINE TESTS

Calcium in Urine (Sulkowitch Test)

Fasting or random samples may be tested. Before the test, the patient should be on neutral low-calcium diet for 3 days. Collect 24 hours urine specimen. Mix equal parts of urine and Sulkowitch reagent, let stand for 2–3 minutes and read as under.

0	=	No precipitate, no urine calcium; serum calcium level 5–7.5 mg%.
1+	=	Fine white cloud, normal urine and blood calcium level.
2+ and 3+	=	Thicker, coarser precipitate, raised urinary calcium.
4+	=	Precipitate like milk, strongly positive.

Normal Values

- 24 hours levels
- 100–250 mg/24 hours on average diet
- < 150 mg/24 hours on low calcium diet.

Most of the calcium discharged by the body is excreted via stool. However, there is a small quantity of calcium that is normally excreted in the urine, this varies with the variation in dietary calcium. The 24 hours test is most often required to determine the function of the parathyroid gland, which maintains a balance between calcium and phosphorus by means of parathormone.

Calcium in urine can also be estimated by using regular serum biochemistry tests—OCPC or Arsenazo method.

Clinical Relevance

Increased Levels

1. Caused by:
   - Hyperparathyroidism (results in constant 3 + to 4 + Sulkowitch test).
   - Sarcoidosis
   - Primary cancers of breast and lung
   - Metastatic malignancies
   - Myeloma with bone metastasis
   - Wilson's disease
   - Renal tubular acidosis
   - Glucocorticoid excess.
2. Increased urinary calcium usually accompanies elevated blood calcium levels.
3. Calcium excretion greater than intake is always excessive, and excretion above 400–500 mg/24 h is reliably abnormal.
4. Increased levels of calcium occur whenever calcium is mobilized from the bone, as in metastatic cancer and prolonged skeletal mobilization.
5. When calcium is excreted in increasing amounts, a potential for nephrolithiasis or nephrocalcinosis is created.

Decreased Levels

Caused by:

1. Hypoparathyroidism (hypocalcemia caused by hypoparathyroidism is usually associated with a negative reaction).
2. Vitamin D deficiency (vitamin D is essential for absorption of calcium).
3. Malabsorption syndrome.

Interfering Factors

a. Falsely high values are seen in:
   - High sodium and magnesium intake
   - Very high milk intake

- Levels are often high immediately after meals
- Drugs:
  - Androgens
  - Cholestyramine
  - Vitamin D
  - Parathyroid injection
  - Nandrolone, in some cancer patients.

B. False negative (lowered) values are seen in
- Increased dietary phosphate
- Alkaline urine
- Drugs:
  - Sodium phytate
  - Thiazides
  - Viomycin.

Be Careful

1. Urine calcium test is not a substitute for serum calcium, it can, however, be done in an emergency. Hypercalcemia can be life threatening.
2. Low urinary calcium patients should be observed for tetany.
3. The first sign of calcium imbalance may be the occurrence of pathological fractures that can be related to calcium excess.

Serotonin (5-Hydroxytryptamine)

Carcinoids

Carcinoids: (Argentaffinomas) may produce serotonin, which is metabolized to 5-hydroxy-indole acetic acid (5-HIAA). Presence of this compound in urine in more than traces indicates *malignant carcinoid metastatic to the liver.*

Test

Acidify 2 mL of filtered urine with 2 drops of 10% HCl and extract twice with 20–25 mL of ether. Evaporate the dry residue in 1 mL of 0.1 N HCl. Add 1 mL Ehrlich's reagent. Boil for 2–3 minutes. A distinct blue color indicates the presence of 5-HIAA in abnormal amounts in urine.

Normal Values

Qualitative— Negative

Quantitative— 2–10 mg/24 h.

60–100 mEq/24 h.

For screening purposes, a random test may be enough. Serotonin is a vasoconstricting hormone produced normally by argentaffin cells of the GI tract. The principal function of the cells is to regulate smooth muscle contraction and peristalsis. In carcinoid tumor (tumor of the argentaffin cells), there is rise in levels of 5-hydroxy-indoleacetic acid (5-HIAA), which happens to be a denatured product of serotonin.

Method

1. No bananas, pineapples, tomatoes, eggplants, or avocados to be consumed during the 24 hours test because they contain serotonin.
2. A 24 hours urine container with preservative is labeled with the name of patient, test and date.
3. General instructions for 24 hours sample collection are observed.

Clinical Relevance

1. Levels in excess of 100 mg per 24 hours are indicative of large carcinoid tumor, especially when metastatic. However, this increase is found only in 5–7% cases of carcinoid tumors.
2. Levels between 10 mg and 100 mg per 24 hours may be seen in:
   - Hemorrhage
   - Thrombosis
   - Nontropical sprue
   - Severe pain of sciatica or skeletal and smooth muscle spasm.

Interfering Factors

False positives:

1. Bananas, pineapples, plums, walnut, and avocados may increase 5-HIAA levels, for all of them contain serotonin.
2. Drugs that may lead to false-positive result
   - Acetanilide
   - Acetophenetidin
   - Caffeine
   - Glyceryl guaiacolate
   - Fluorouracil (5 FU)
   - Mephenesin
   - Melphalan
   - Methocarbamol
   - Methamphetamine
   - Reserpine
   - Phenacetin solution
   - Lugol's iodine
   - Phenmetrazine
   - Methysergide maleate.

False negatives:

Drugs that may falsely decrease 5-HIAA levels:

- ACTH
- Chlorpromazine

- Heparin
- Imipramine
- Isoniazid
- MAO inhibitor
- Methenamine mandelate
- Methyldopa
- Phenothiazines
- Promethazine
- P-chlorophenylaniline.

Ideally, the patient should take no drugs for 72 hours prior to test if possible.

Cystine

To 5 mL of urine, add 2 mL of 5% sodium cyanide solution and let them react for 10 minutes. Add 5 drops of 5% sodium nitroprusside solution and mix thoroughly. Cystine produces a magenta color. If no cystine is present, a pale brown or pale pink color results. All solutions should be freshly prepared. Also, examine the urinary sediment for cystine crystals. Urinary cystine is raised in cystinurias.

Normal values

Qualitative	:	Negative
Quantitative	:	Children under 8 years: 2–13 mg/24 h.
Individuals above 8 years	:	7–28 mg/h.

These tests of urine are useful in the differential diagnosis of cystinuria, an inherited disease from cystinosis. Cystinuria is a hereditary disease, characterized by bladder calculi. In cystinosis, cystine is deposited in lung tissues.

Clinical Relevance

Values are Increased in

1. Cystinuria (up to 20 times normal) in which there is excessive urinary excretion of lysine, ornithine, arginine, and cystine.
2. Cystinosis (no excess of lysine, arginine or ornithine).

Fat in Urine

Take equal parts of urine and ether, cloudiness due to fat disappears, decant ether onto a watch glass, evaporate, fat leaves a greasy deposit. Fat may be seen microscopically.

Hereditary Metabolic Disorders

Errors of Carbohydrate Metabolism

1. *Galactosuria:* Positive test for reducing substance (Benedict's qualitative). Negative glucose-oxidase test. Positive phloroglucinol test.
2. *Pentosuria:* Positive Benedict's qualitative test. Negative glucose-oxidase test. Positive orcinyl-HCl test.
3. *Fructosuria:* Positive Benedict's test. Negative glucose-oxidase test. Positive resorcinol-HCl test (Seliwanoff).

Errors of Amino Acid Metabolism

1. *Cystinuria*: Positive cyanide-nitroprusside test. Cystine crystals in urine.
2. *Fanconi's syndrome:* Positive glucose-oxidase test. Paper chromatography for amino acids.
3. *Wilson's disease*: Positive glucose-oxidase test. Paper chromatography for amino acids.
4. *Phenylketonuria*: Positive ferric chloride test.
5. *Hartnup disease*: Paper chromatography.
6. *Alkaptonuria (Homogentisic acid):* Positive Benedict's test. Urine darkens on standing. Negative glucose-oxidase test. Urine reduces silver on sensitized plate.
7. *Tyrosinosis*: Paper chromatography. Positive Millon test.
8. *Maple syrup disease*: Maple syrup odor of urine. Paper chromatography.

Abnormal Porphyrin Metabolism

1. *Acute porphyria*: Urine darkens on exposure to sunlight. Positive porphobilinogen test. Spectroscopic and fluorimetric identification.
2. *Cutaneous porphyria*: Red urine. Spectroscopic and fluorimetric tests.

Ferric Chloride Testing

Many amino acids react with ferric chloride to give distinctive colors. Ferric chloride testing is a screening test not only for aminoacidurias but also for many abnormal metabolites and drug excretion products. Definitive diagnosis requires specific identification and measurement of the relevant materials in blood or urine.

Method: Add 10% (w/v) of Ferric chloride solution to 1–2 mL of freshly voided urine. Document color change.

Substance	*Color alteration*
Amino acids	
α-Ketobutyric acid	Purple, fading to red brown
Homogentisic acid (alkaptonuria)	Rapidly fading blue or green
p-Hydroxyphenylpyruvic acid (tyrosinosis)	Rapidly fading green
Valine, leucine, and isoleucine (maple syrup disease)	Blue

Contd...

Contd...

Phenyl pyruvic acid (phenylketonuria)	Stable green or blue green
Other metabolites	
Acetoacetic acid	Red or red-brown
Melanin	Gray, changing to black
Indican (Hartnup disease, intestinal stasis, malabsorption)	Violet or blue
Drugs	
Aspirin, salicylates	Stable red-wine color
Phenothiazine derivatives	Immediate purple pink
p-Aminosalicylic acid (PAS)	Red-brown
Phenol derivatives	Violet

Uric Acid

Normal Values

0.4—1.0 g/24 h on normal diet
0.2—0.5 g/24 h on purine free diet up to 2.0 g/24 h on high purine diet.

Uric acid formation occurs as a result of the metabolic breakdown of nucleic acids, purines are the main sources of this breakdown. The test is required in the investigation of metabolic disturbances to identify gout and diagnose kidney disease. It also reflects the effect of uricosuric agents when these drugs are used, by indicating the total amount of uric acid excreted. A 24-hour sample is needed.

Method: Use routine serum biochemical methods for estimation of uric acid.

Clinical Relevance

Increased Levels (uricosuria)

- Found in:
  - Gout
  - Chronic myeloid leukemia
  - Polycythemia vera
  - Liver disease
  - Febrile illness
  - Toxemias of pregnancy
  - Fanconi's syndrome.
- Cytotoxic drugs used to treat lymphoma and leukemia often cause greatly increased urinary uric acid levels
- High uric acid concentration plus low urine pH may lead to uric acid stones in the urinary tract.

Decreased Levels

Found in kidney disease (chronic glomerulonephritis) because hampered renal function diminishes uric acid excretion.

Interfering Factors

1. Drugs
   - Salicylates
   - Thiazide diuretics
   - Chronic alcohol consumption.
2. X-ray contrast media can markedly increase uric acid levels.
3. Many other drugs can also influence these results.

Vanillylmandelic Acid (VMA)

(Catecholamines or 3-Methoxy-4-Hydroxymandelic acid)

Normal values

VMA up to 9 mg/24 h

Catecholamines

Epinephrine	100–230 mg/24 h
Norepinephrine	100–230 mg/24 h
Metanephrine	24–96 mg/24 h
Normetanephrine	12–288 mg/24 h

These investigations for adrenal medullary function are usually needed for a person with hypertension suspected to be having pheochromocytoma (a tumor of chromaffin cells of the adrenal medulla). Incidence is about 1% among hypertensives. The compounds mentioned above contain a catechol nucleus and an amine group and are, therefore, called catecholamines. The major portion of the hormones is changed into metabolites, mainly 3-methoxy-4-hydroxy mandelic acid or VMA.

Method/Principle

Catecholamines are adsorbed from untreated urine on to a column of Amberlite IRC 50, eluted and condensed in alkaline solution with ethylenediamine and the resulting fluorescence read. However, spectrophotometric methods are less subject to drug interference than fluorimetry. Samples should be collected in 10 mL hydrochloric acid and refrigerated.

Clinical Relevance

Elevated VMA Levels

1. High levels found in pheochromocytoma
2. Mild to moderate elevations seen in:
   - Neuroblastomas
   - Ganglioneuromas
   - Ganglioblastomas.

Elevated Catecholamines

Found in:

- Pheochromocytoma
- Neuroblastomas

- Ganglioneuromas
- Ganglioneuroblastomas
- Progressive muscular dystrophy
- Myasthenia gravis.

Interfering Factors

Increased VMA Levels are Caused by

1. Starvation (patients on nil orally: Therapy should not undergo this test).
2. Foods: Tea, coffee, cocoa, vanilla, gelatin foods, fruit juice, chocolate, fruit, especially bananas, cider vinegar, salad dresssing, carbonated drinks, jelly and jam, candy gum, artificially flavored or colored foods, foods containing liquorice.
3. *Drugs causing increased VMA levels:*
   - Aspirin
   - BSP
   - Glyceryl guaiacolate
   - Phenazopyridine
   - PSP
   - Sulfonamides
   - Levodopa
   - Lithium
   - Nitroglycerin
   - Mephenesin
   - Chlorpromazine
   - Para-aminosalicylic acid (PAS)
   - Methocarbamol
   - Methylene blue
   - Nalidixic acid
   - Oxytetracycline
   - Penicillin.

False Decreased Levels of VMA are Caused by

- Alkaline urine
- Uremia (impairs VMA excretion)
- Radiographic contrast agents
- Drugs:
  - Clofibrate
  - Guanethidine drugs
  - Imipramine
  - Methyldopa
  - MAO inhibitors
  - Clonidine
  - Reserpine
  - Imipramine.

Interfering Factors in Determining Catecholamine Levels

- Vigorous exercise may increase catecholamine levels
- Drugs:
  - Ampicillin
  - Ascorbic acid
  - Chloral hydrate
  - Epinephrine
  - Erythromycin
  - Hydralazine
  - Methenamine
  - Methyldopa
  - Nicotinic acid
  - Quinine
  - Tetracycline
  - Vitamin B complex.

Be Careful

- Explain that it is a 24-hour collection test
- Explain diet and drug restrictions
- Exclude all restricted foods for at least 3 days before test date
- Exclude all drug intake for 3 to 7 days before the test date
- Rest and adequate food and fluids are encouraged, and stress is to be avoided during the test
- Patients can resume all restricted foods, drugs, and activity as soon as test is completed.

17-ketosteroids (17-KS)17-Ketogenic Steroids (17–KGS) 17-Hydroxycorticosteroids (17-OHCS)

Normal Values

17-ketosteroids
Men : 8-18 mg/24 h
Women : 5-15 mg/24 h
17-ketogenic steroids
Men : 5.5–23 mg/24 h
Women : 3–15 mg/24 h.
17-hydroxycorticosteroids: Up to 10 mg/24 h.

The above mentioned substances are urinary steroids and their estimation is indicated in investigation of endocrine disturbances of the adrenals and testes.

17-ketosteroids have 19 carbon atoms with a ketone group at C-17. These steroids are composed of adrenal hormones and metabolites of testicular androgens. In men, the adrenals produce 2/3rd of these hormones, while the testes produce the remainder. In women, the adrenals produce all of the hormones.

17-ketogenic steroids are composed of glucocorticoid derivatives and pregnanediol, have 21 carbon atoms and a hydroxyl group at C-17. Their estimation gives a good reflection of adrenal cortex activity.

17-hydroxycorticosteroids have 21 carbons with hydroxy groups at C-17 and C-21 and a ketone at C-20. These are also known as Porter-Silber chromogens.

Method/Principle 17 KS

Their assessment is a colorimetric assay. Urine is subjected to acid hydrolysis and the steroids are extracted with ethylene dichloride. A solvent aliquot is evaporated to dryness under a nitrogen stream and the resultant residue is reacted with m-dinitrobenzene (Zimmerman reaction), which in the presence of alkali gives a red color with compounds containing an active methylene group. This color obtained has an absorption maximum at 520 μ.

Method/Principle 17-OHCS

Porter-Silber reaction: The glucuronide conjugates of urinary corticosteroids are hydrolyzed with β-glucuronidase. The "freed" steroids and free steroids (i.e. tetra and dihydro derivatives) normally present in the urine are extracted in methylene chloride. This extract is washed with a dilute aqueous alkali to remove a considerable amount of blank material which consists of estrogens, bile acids and other interfering chromogens. A portion of methylene chloride is shaken with a phenylhydrazine hydrochloride—sulfuric acid—ethanol reagent. For correction of the residual blank material, another portion of the extract is shaken with just the ethanol sulfuric acid reagent. The upper layer of methylene chloride is removed, and the lower phase after color development is measured spectrophotometrically at 410 nm. No need to add preservative for 24 hours urine collection for 17-OHCS, but stop all medication 2–3 days prior to test day.

Other methods available are based upon ELISA techniques.

Clinical Relevance

- There is decrease in 17-KGS and 17-KS excretion in Addison's disease, hypopituitarism, Simmond's diseases and cretinism.
- There is an increase in 17-KGS excretion in precocious puberty because of adrenal hyperplasia, surgery, excessive burns and infection.
- Increased 17-OHCS and 17-KGS usually imply hyperplasia of the adrenal cortex, tumor, cancer, or some variation of the adrenogenital syndrome.
- Steroid levels are also enhanced in Cushing's syndrome, eclampsia, acute pancreatitis, and ACTH therapy. If the beta-alpha ratio is >0.4, it is indicative of adrenal carcinoma. Unless the 17-KS are increased, the beta-alpha ratio is not likely to be abnormal.

Interfering Factors

a. Severe stress will cause increased levels of KS and KGS
b. KS levels are often increased in third trimester of pregnancy.
c. Drugs:
    1. Increasing 17-KS levels:
        - Chloramphenicol
        - Meprobamate
        - Spironolactone
        - Chlorpromazine
        - Nalidixic acid
        - Phenaglycodol
        - Cloxacillin
        - Penicillin
        - Erythromycin
        - Quinidine
        - Ethinamate
        - Secobarbital
        - Oleandomycin
        - Phenazopyridine
        - Spironolactone.
    2. Decreasing 17-KS levels:
        - Chlordiazepoxide
        - Probenecid
        - Estrogen
        - Meprobamate
        - Promazine
        - Metyrapone
        - Reserpine.
    3. Increasing 17-OHCS levels:
        - Acetazolamide
        - Ascorbic acid
        - Chloral hydrate
        - Chloramphenicol
        - Chlordiazepoxide
        - Chlormerodrin
        - Chlorpromazine
        - Chlorthalidone
        - Colchicine
        - Cloxacillin
        - Erythromycin
        - Spironolactone
        - Digitoxin
        - Digoxin
        - Cortisone
        - Ethinamate
        - Etryptamine
        - Glutethimide
        - Meprobamate
        - Hydralazine
        - Oleandomycin
        - Paraldehyde
        - Quinine
        - Quinidine.

4. Decreasing 17-OHCS levels:
   - Aminoglutethimide
   - Diphenylhydantoin
   - Estrogen
   - Dexamethasone
   - Calcium gluconate
   - Phenothiazines
   - Oral contraceptives
   - Reserpine
   - Corticosteroids
   - Mitotane.

Chlorides

Normal Values

- 110–250 mEq/24 hours
- 10–20 g NaCl/24 hours.

Vary widely with intake amount and perspiration. The test findings have meaning only in relation to salt intake and output.

Method

Use routine serum biochemistry assay technique for urine chloride estimation.

Clinical Relevance

Results are meaningful only when considered in relation to other data such as state of health/illness, salt intake and urine volume.

1. Normal findings: Urinary excretion of chloride decreases to a very low level whenever the serum level is much below 100 mEq/liter.
2. Decreased levels:
   a. In some conditions, urinary excretion of chloride increases even when the serum level is as low as 85 mEq/liter or less. Occurs in Addison's disease when there is a deficiency of adrenal hormones that controls the excretion of sodium and chloride.
   b. Decreased levels are also associated with:
      - Malabsorption syndrome
      - Pyloric obstruction
      - Prolonged gastric suction
      - Diarrhea
      - Diaphoresis
      - Congestive heart failure
      - Emphysema.
3. Increased levels are associated with:
   - Dehydration
   - Starvation
   - Salicylate toxicity
   - Mercurial and chlorothiazide diuretics.

Interfering Factors

1. Urinary chloride concentration varies with dietary salt intake, perspiration and to some extent, with urine volume.
2. False elevations may occur if the patient has taken bromides.

Sodium

Normal Values

130–200 mEq/24 h.

The test is indicated in the study of renal and adrenal disturbances and of water and acid-base imbalances.

Method

Use colorimetric, flame photometry or ISE method.

Clinical Relevance

Results have significance only when considered in relation to other data, such as a state of health/illness, salt intake, and urine volume.

1. Increased Levels

Caused by:

- Dehydration
- Starvation
- Salicylate toxicity
- Adrenal cortical insufficiency
- Mercurial and chlorothiazide diuretics
- Chronic renal failure
- Diabetic acidosis.

2. Decreased Levels of Sodium Associated with

- Malabsorption syndrome
- Congestive heart failure
- Pyloric obstruction
- Diarrhea
- Diaphoresis
- Acute renal failure
- Pulmonary emphysema
- Aldosteronism
- Cushing's disease.

3. Decreased Levels

Often accompanied by an equivalent loss of chloride.

Interfering Factors

1. Dietary salt intake
2. Altered renal function.

Potassium

Normal Values

40–80 mEq/24 h.

Test is required to assess electrolyte balance of the body by measuring the amount of potassium excreted in 24 hours. This measurement is useful in the study of renal and adrenal disorders and of water and acid-base imbalances.

Method

Use colorimetric, flame photometry or ISE method.

Clinical Relevance

1. **Increased Levels**
   - Chronic renal failure
   - Diabetic and renal tubular acidosis
   - Dehydration
   - Starvation
   - Primary aldosteronism
   - Cushing's disease
   - Salicylate toxicity
   - Mercurial chlorothiazide, ammonium chloride, and Diamox diuretics.
2. **Decreased Levels**
   - Malabsorption syndrome
   - Diarrhea
   - Acute renal failure
   - Adrenal cortical insufficiency (in some cases)
   - Excessive mineralocorticoid activity (aldosterone)
   - In patients with potassium deficiency, regardless of the cause.
3. **Cautionary Finding**
   - In excessive vomiting or stomach suctioning, the accompanying alkalosis maintains urinary potassium excretion at levels inappropriately high for the degree of actual potassium depletion
   - In diabetes insipidus, urinary potassium is normal.

Interfering Factors

Varies with dietary intake.

Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)

Normal Values

FSH

Men	:	4–25 IU/mL
Women	:	4–30 IU/mL
Postmenopausal	:	40–250 mu/mL
Midcycle	:	Two times baseline

LH

Men	:	7–24 IU/mL
Women	:	6–30 IU/mL
Postmenopausal	:	Over 30 IU/mL
Midcycle	:	Over three times baseline.

These investigations are required in determining whether a gonadal insufficiency is primary or due to deficient stimulation by the pituitary hormones. The production of these hormones is under control of pituitary gland. In women, FSH promotes maturation of the ovarian follicle, and the maturing follicle produces estrogens. As the levels of estrogen rise, luteinizing hormones are produced. Together, FSH and LH induce ovulation. In men, FSH produces spermatogenesis, and LH induces the secretion of androgens.

FSH is an aid in studying various causes of hypothyroidism in women as well as endocrine dysfunction in men. In primary ovarian failure or testicular failure, FSH is increased.

Method

Use regular ELISA/CLIA/RIA based methods for estimation.

Clinical Relevance

Blood and urine estimation are used.

Decreased FSH Levels Occur in

- Feminizing and masculinizing ovarian tumors when production is inhibited as a result of increased estrogen
- Failure of pituitary or hypothalamus
- Anorexia nervosa
- Neoplasm of testes or adrenal glands that secrete estrogens or androgens.

Increased FSH Levels Occur in

- Turner's syndrome (ovarian dysgenesis). Approximately, 50% of patients with primary amenorrhea have Turner's syndrome
- Hypogonadism and primary gonadal failure
- Complete testicular feminization syndrome
- Precocious puberty, either idiopathic or secondary to a central nervous system lesion
- Klinefelter's syndrome.

Pregnanediol

Normal Values

Standardization extremely difficult:

Proliferative phase	:	0.5–1.5 mg/24 h
Luteal phase	:	2–7 mg/24 h

Postmenopausal : 0.2–1.0 mg/24 h
Pregnancy : 5–63 mg/24 h.

The test helps in assessing ovarian and placental function. It is indicated when a deficiency of progesterone is suspected. Combined deficiency of estrogen and progesterone is hinted by menstrual irregularities and difficulty in conceiving and maintaining a pregnancy. To be specific, it measures the hormone progesterone and its principal excreted metabolite, pregnanediol. Progesterone has its main effect on the endometrium by causing the endometrium to enter the secretory phase and become ready for implantation of the blastocyte if fertilization has taken place. Pregnanediol excretion is high in pregnancy and low in luteal deficiency or placental failure. A 24-hours urine sample collection is needed.

Method

Use regular ELISA/CLIA/RIA based methods for esti mation.

Clinical Relevance

Increased Levels
Associated with:

- Luteal cysts of ovary
- Arrhenoblastomas of the ovary
- Hyperadrenocorticism.

Decreased Levels
Associated with:

- Amenorrhea
- Threatened abortion (not always)
- Fetal death
- Toxemia.

Pregnanetriol

Normal Values

Adult : Up to 2 mg/24 h
Children : Up to 1.0 mg/24 h
Infants : Up to 0.2 mg/24 h.

Do not confuse this with pregnanediol. Pregnanetriol reflects one segment of adrenocortical activity. This is a precursor in adrenal corticoid synthesis and arises from 17-hydroxyprogesterone, not from progesterone. The 24-hour urine test is conducted to diagnose adrenogenital syndrome, a defect in 21-hydroxylation.

The diagnosis of adrenogenital syndrome is considered in:

- Adult women who show signs and symptoms of excessive androgen production with or without hypertension
- Craving for salt
- Sexual precocity in boys
- Infants who exhibit signs of failure to thrive
- External genitalia in women (pseudoher-maphroditism). In boys, differentiation must be made between a virilizing tumor of the adrenal gland, neurogenic and constitutional types of sexual precocity, and interstitial cell tumor of the testes.

Method

Use regular ELISA/CLIA/RIA based methods for estimation.

Clinical Relevance

Elevated pregnanetriol levels occur in:

- Congenital adrenocortical hyperplasia
- Stein-Leventhal syndrome.

Estrogen Fractions

Normal Values

Women - Total: 4–60 mg/24 h.
Estrone (E): 2–25 mg/24 h.
Estradiol (E2) : 0–10 mg/24 h.
Pregnancy - Estriol (E3): 2–30 mg/24 h.
Men: 4–24 mg/24 h.

To evaluate ovarian function and gynecologic problems, estradiol, estron, and estriol are routinely measured. Estrogens will be normally increased in pregnancy and in some tumors of the ovary and adrenal cortex.

Estrogens are decreased in the absence of deficiency of ovarian hormones. Estriol levels are used in the management of high-risk pregnancies as a method of assessing placental function. A fall in estriol should be judged by at least two different serial measurements. A falling estriol excretion signifies impending fetal death.

Method

Use regular ELISA/CLIA/RIA based methods for estimation.

Clinical Relevance

1. **Decreased Estrogen Values are Seen with**
   - Hypo or dysfunction of pituitary and adrenal glands
   - Primary ovarian malfunction
   - Agenesis of the ovaries.
2. **Increased Estrogen Levels are Found in**
   - Solid ovarian tumors, granulosa/theca cell
   - Tumor/hyperplasia of the adrenal cortex.

3. **Decreasing Estriol Levels**
   - More than 40% of previous values is associated with placental insufficiency. An abrupt drop of 40% or more is associated with fetal distress.
4. **Miscellaneous Causes of Estriol Level's Decline are**
   - Anemia
   - Malnutrition
   - Pyelonephritis
   - Intestinal disease
   - Hemoglobinopathies.

Interfering Factors

Drugs interfering are:

- Ampicillin
- Hydrochlorothiazide
- Exogenous corticosteroids
- Meprobamate
- Meth. mandelate
- Cascara
- Phenazopyridine
- Diethylstilbestrol
- Prochlorperazine
- Hexamine
- Senna
- Tetracyclines.

Heavy Metals and Trace Elements in Blood/ Urine

Description

Heavy metals include antimony, arsenic, bismuth, cadmium, cobalt, copper, lead, mercury, selenium, thallium, and zinc.

Antimony exposure occurs in miners, smelters, and ore refinery workers.

Arsenic is found naturally in food and the environment as well as in pesticides.

Bismuth exposure occurs in workers in cosmetic, disinfectant, and pigment industries. It may also occur as a result of treatment for syphilis.

Cadmium accumulates in the lungs, liver and kidneys via exposure to food, water, air, and cigarette smoke.

Cobalt, a component of vitamin B_{12}, is found in most foods. It is also used to treat some resistant anemias and some radiosensitive malignancies.

Copper is a trace element found in normal diets. It is one of the few heavy metals that are potentially harmful at low levels as well as at toxic levels. Toxic levels may be caused by the use of copper IUDs, ingestion of contaminated substances, or fungicide exposure.

Lead is absorbed into the body through the ingestion of lead containing paint or through industrial exposure.

Mercury is found in fungicides, industrial processes, and in fish (polluted water). It can also be ingested in the form of mercury salts. High mercury levels have been noted among dental workers.

Selenium is a metal used for the activity of human glutathione peroxidase. Exposure occurs as a result of the manufacture of glass, paints, dyes, electronic equipment, fungicides, rubber, and semiconductors.

Thallium is present in cosmetics, pesticides, and in some medications. It is absorbed through intact skin and mucous membranes.

Zinc is a trace metal important for cellular growth and metabolism. Toxicity can occur from industrial exposure and consumption of acidic food or beverages from galvanized containers.

Normal values are given below:

	Blood	*SI units*
Antimony	0.052 ± 0.019 µg/dL	4.35 ± 1.6 nmol/L
Arsenic	2–23 µg/L	0.03–0.31 µmol/L
Chronic poisoning	100–500 µg/L	1.33–6.65 µmol/L
Acute poisoning	600–9300 µg/L	7.98–124 µmol/L
Bismuth	0.1–3.5 µg/L	0.5–16.7 nmol/L
Cadmium		
Smokers	0.6–3.9 µg/L	5.3–34.7 nmol/L
Non-smokers	0.3–1.2 µg/l	2.7–10.7 nmol/L
Toxic	100–3000 µg/L	0.9–26.7 µmol/L
Cobalt	0.11–0.45 µg/L	1.9–7.6 nmol/L
Copper		
Infants	20–70 µg/dL	3.1–11 µmol/L
Child 6 years	90–190 µg/dL	14.1–29.8 µmol/L
Child 12 years	80–160 µg/dL	12.6–25.1 µmol/L
Adult male	70–140 µg/dL	11–22 µmol/L
Adult female	80–155 µg/dL	12.6–24.3 µmol/L
Pregnant	118–302 µg/dL	18.5–47.4 µmol/L
Lead		
Child	<25 µg/dL	<1.21 µmol/L
Adult	<40 µg/dL	<1.93 µmol/L
Industry exposure	<60 µg/dL	<2.90 µmol/L
Toxic concentration	>100 µg/dL	>4.83 µmol/L
Toxic concentration in children	>25 µg/dL	1.21 µmol/L
Mercury	0 6–59 µg/L	3–294 µmol/L
Non-fish eaters	<5 µg/L	<25 nmol/L
Selenium	58–234 µg/L	0.74–2.97 µmol/L

Contd...

	Blood	*SI units*
Thallium	<0.5 µg/dL	<24.5 nmol/L
Toxic concentration	10–800 µg/dL	0.5–39.1 µmol/L
Zinc	70–150 µg/dL	10.7–23 µmol/L
Antimony	<10 µg/L	<82.1 µmol/L
Toxic concentration	>10 µg/L	>82.1 µmol/L
Arsenic	5-50 µg/L	0.067–0.665 µmol/L
Chronic poison	50–5000 µg/L	0.67–66.5 µmol/L
Acute poison	1000–20,000 µg/L	13.3–266 µmol/L
Bismuth	0.3–4.6 µg/L	1.4–22 nmol/L
Cadmium	0.5–4.7 µg/L	4.4–41.8 nmol/L
Industrial exposure	10–580 µg/L	0.09–5.16 µmol/L
Cobalt	1–2 µg/L	17–34 nmol/L
Copper	2–80 µg/L	0.03–1.26 µmol/L
Lead	<80 µg/L	<0.39 µmol/L
Industrial exposure	<120 µg/L	<0.58 µmol/L
Mercury		
Adult	<20 µg/L	<0.10 µmol/L
Toxic concentration	>150 µg/L	>0.75 µmol/L
Lethal concentration	>800 µg/L	>4 µmol/L
Selenium	7–160 µg/L	0.09–2.03 µmol/L
Toxic concentration	>400 µg/L	5.08 µmol/L
Thallium	<2 µg/L	<9.8 nmol/L
Toxic concentration	1–20 µg/L	4.9–97.8 µmol/L
Zinc	150–1200 µg/L	2.3–18.4 µmol/L
Toxic concentration	>1200 µg/L	18.4 µmol/L

Toxic/Poisoning Symptoms and Treatment

Symptoms

Antimony: Vomiting.

Arsenic: Gastric pain, vomiting, diarrhea, convulsions, coma, and death in acute poisoning; and diarrhea, scaling and pigmentation of skin, hair loss, and peripheral neuropathy in chronic poisoning.

Bismuth: Weakness, decreased appetite, fever, halitosis, black gum line, rheumatic type pain, and renal damage.
Cadmium: Pneumonia, pulmonary edema, and cardiovascular collapse from inhalation, violent gastrointestinal symptoms from acute ingestion, and osteomalacia and renal dysfunction from chronic ingestion.

Cobalt: Thyroid gland hyperplasia, cardiomyopathy, nerve damage, and myxedema.

Copper: Nausea, vomiting, headache, diarrhea, and abdominal pain.

Lead: Anorexia, abdominal pain, vomiting, irritability, and apathy.

Mercury: Fatigue, headache, loss of memory, apathy, emotional instability, paresthesia, ataxia, deafness, dysarthria, visual deterioration, dysphagia, coma, and death.

Selenium: Garlic smell in breath and urine, metallic taste, headaches, nausea, vomiting, pneumonia and pulmonary edema.

Thallium: Ataxia, pulmonary edema, vomiting, constipation, restlessness, delirium and coma.

Zinc: Cough, chest discomfort, tachycardia, hypertension, gastrointestinal irritation, nausea, vomiting, diarrhea, and metallic taste in mouth.

Treatment

Antidotes for heavy metal poisoning include BAL (British anti-Lewisite), deferoxamine, dimercaprol, and EDTA. Heavy metals respond to hemodialysis and/or hemoperfusion in varying degrees (poor to well).

Usage

Screening for heavy-metal toxicity from overexposure, ingestion, or occupational exposure. Disorders for individual metals found under test listings for individual metals. Drugs that may further increase some values include carbamazepine, estrogens, oral contraceptives, penicillamine, phenobarbital, phenytoin, and sodium salts.

MICROSCOPY OF THE URINARY SEDIMENT

Use a clean, fresh morning specimen. Obtain urinary sediment by centrifuging urine at 3000 rpm for 5 minutes. Draw off the clear supernatant fluid, place a drop of the sediment on a glass slide and cover it with a coverslip. Examine first under low power, then under high power, vary the light intensity for seeing casts. If protein is present, look for casts, RBCs, pus cells and epithelial cells.

In most instances, an unstained sediment is sufficient. However, should a difficulty arise or the examiner is inexperienced, staining can be done with Sternheimer and Malbin stain.

A drop of methylene blue solution can be added to the sediment and would help in identifying cellular structure and bacteria. A crystal violet safranin stain is used to identify cellular elements; a peroxidase stain will diffe-

rentiate renal tubular cells that are peroxidase negative and neutrophils (pus cells) that are peroxidase positive. In most cases, qualitative or semiquantitative examination of the urine is enough. For following the progress of active renal disease, Addis' count may be used. Cell counts can be expressed as occasional, 1+, 2+, 3+ or full field. Count in at least 10 high power fields for cells and express the average as the number of cells per high power field.

Addis count: A method of quantitative enumeration of red blood cells, white cells, and casts in a 12-hour urine specimen is known as the Addis count (Addis, 1948). The chief value of the Addis count is in following the progress of known renal disease, e.g. acute glomerulonephritis. (For diagnostic purposes, careful examination of the sediment from a random fresh urine sample is usually sufficient).

An accurately time 12 hour urine specimen should be collected, with attention to the factors which contribute toward preservation of the formed elements, which are to be counted A 6 to 9 hour specimen may be used. A concentrated specimen of low pH is desirable; this is most easily obtained by collection of the specimen overnight while the patient is not normally eating or drinking. Intake of fluids should be restricted during the collection period as the patient's condition permits. Particular attention should be paid to avoiding contamination of the specimen with vaginal discharge or feces.

Formalin is the preservative of choice for preservation of cells and casts; it also inhibits bacterial growth. Sufficient formalin is introduced by rinsing the collection bottle with a solution of 10% formaldehyde in water and discarding the excess solution. It is advisable to keep the specimen at room temperature during and after collection in order to prevent precipitation of dissolved materials, for precipitation obscures the cells, casts, and makes counting difficult. The specimen should be examined as soon as possible after collection.

Procedure

1. Mix the specimen well and measure the volume carefully.
2. A preliminary microscopic examination of the urinary sediment should be performed with a 10:1 concentration of the sediment (centrifuge 10 mL of urine, resuspend the sediment in 1 mL of urine and examine). From the results of this examination, the volume in which to resuspend the sediment in step 5 can be determined.
3. Transfer 10 mL of urine to a special Addis graduated centrifuge tube and centrifuge for 5 minutes at 2000 rpm.
4. Pour off the supernatant urine and save for protein determination. Adjust the volume of the remainder to 1 mL. When the amount of sediment is large, adjust the volume to 2 to 5 mL after the calculations appropriately.
5. Mix well to resuspend the sediment, and with a capillary pipette, mount the resuspended sediment on both sides of *two* Levy-Hausser counting chambers with improved Neubauer rulings.
6. Under low power, count the number of casts in the four rule areas (4 × 9 = 36 sq mm) on the two sides of the two counting chambers. Using the high-power objective, count the red blood cells and white blood cells and epithelial cells in 4 sq mm (usually 1 sq mm from each side of each chamber). Squamous epithelial cells are not countered.

The number of cells and casts excreted in 12 hours or 24 hours may be reported. This number is determined as follows:

Number counted per sq mm × 1/10 = number/sq mm corrected for concentration of specimen.

Number/sq mm × 1 mm/0.1 mm = number/cu mm.

Number/cu mm × 1000 = number/mL

Number/mL × 12 h vol in mL = number/12 h.

Interpretation

Normal values (see the following Table). *Red blood cells,* 0 to 500,000 per 12 h. *Non squamous white cells,* 0 to 1,000,000 per 12 h. *Casts*, 0 to 5000 hyaline casts per 12 h.

In children, the number of erythrocytes and leukocytes may be lower and the number of casts greater (Lyttle, 1933).

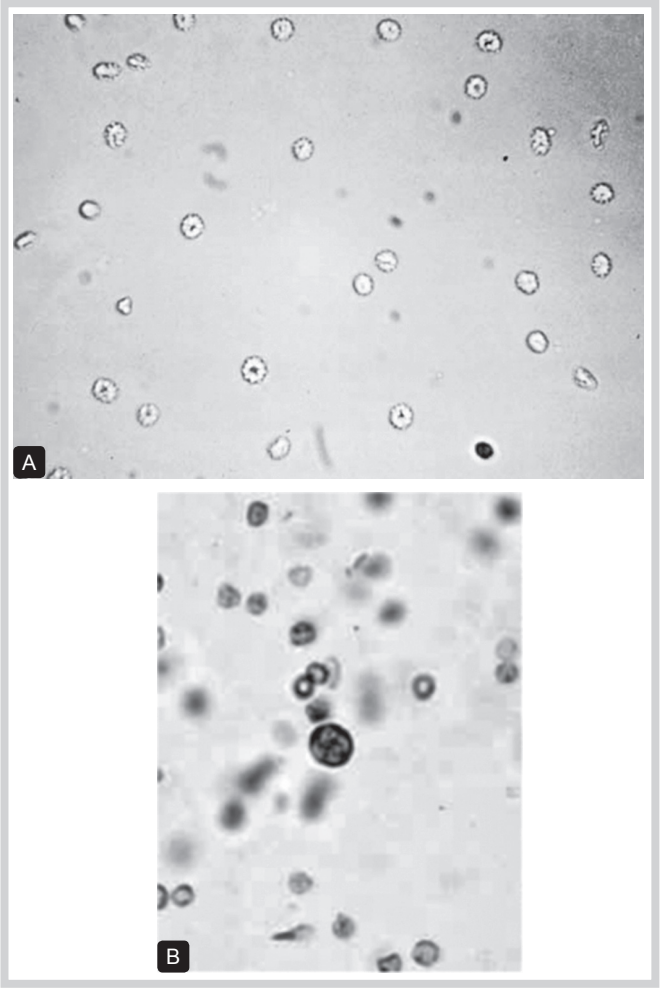
Addis has given the following average counts per 12 hours in cases of glomerulonephritis.

	Casts	*Erythrocytes*	*White cells*
Acute	690,000	405,000,000	48,000,000
Chronic active	1,850,000	34,000,000	14,000,000
Chronic latent	48,000	16,000,000	2,400,000
Chronic terminal	398,000	26,400,000	10,000,000

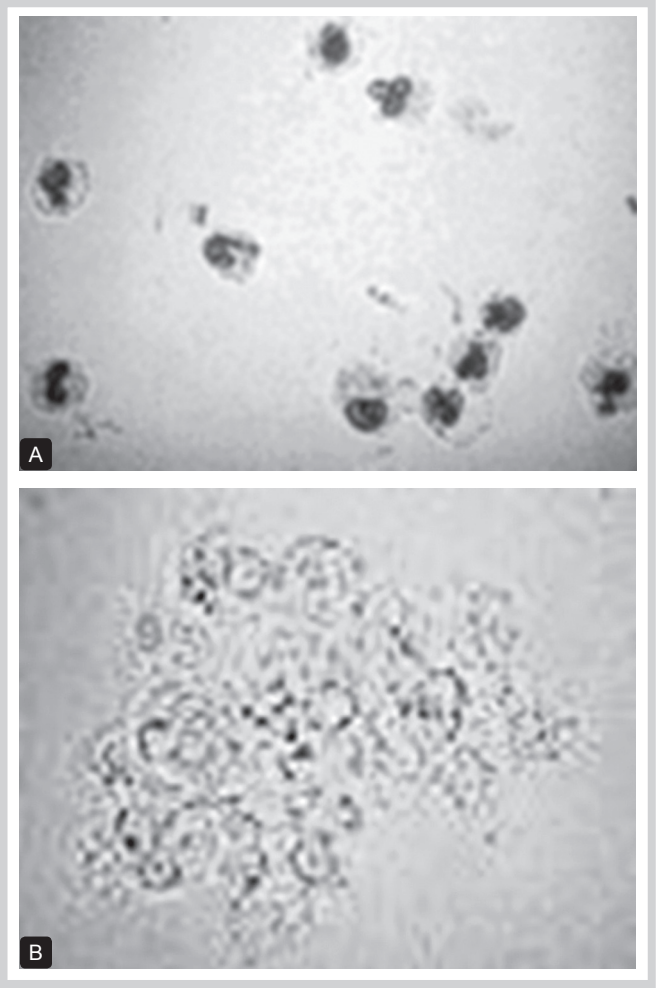
Red Blood Cells (Figs 5.16A and B)

Under high power, they appear as pale discs. If the specimen is stale, because of dissolution of hemoglobin, these cells will appear as ghost cells. These red cells may show crenated margins.

RBCs may be confused with oil droplets or yeast cells. Oil droplets are variable in size and are refractile. Yeast cells usually show budding. Alkaline hematin stains dark purple in alkaline urine.



FIGS 5.16A AND B: Red blood cells in urine



FIGS 5.17A AND B: (A) White Blood Cells in urine; (B) Pus cells in urine

Neutrophilic Leukocytes (Pus Cells) (Figs 5.17A and B)

Unstained neutrophilic leukocytes appear as round granular 12 μ spheres, larger than RBC. These may look like small epithelial cells—let a drop of glacial acetic acid flow under the coverslip—the segmented nucleus of a leukocyte becomes clearer.

Epithelial cells have a single, rounded nucleus. Glitter cells are larger neutrophils, cytoplasmic granules may show brownian movement.

Renal Tubular Epithelial Cells (Figs 5.18A and B)

Unstained cells are almost the same size as that of a neutrophil but contain a large round nucleus. Oval fat bodies are those cells containing fat globules, the nucleus, then, are not visible.

Bladder Epithelial Cells (Figs 5.19A and B)

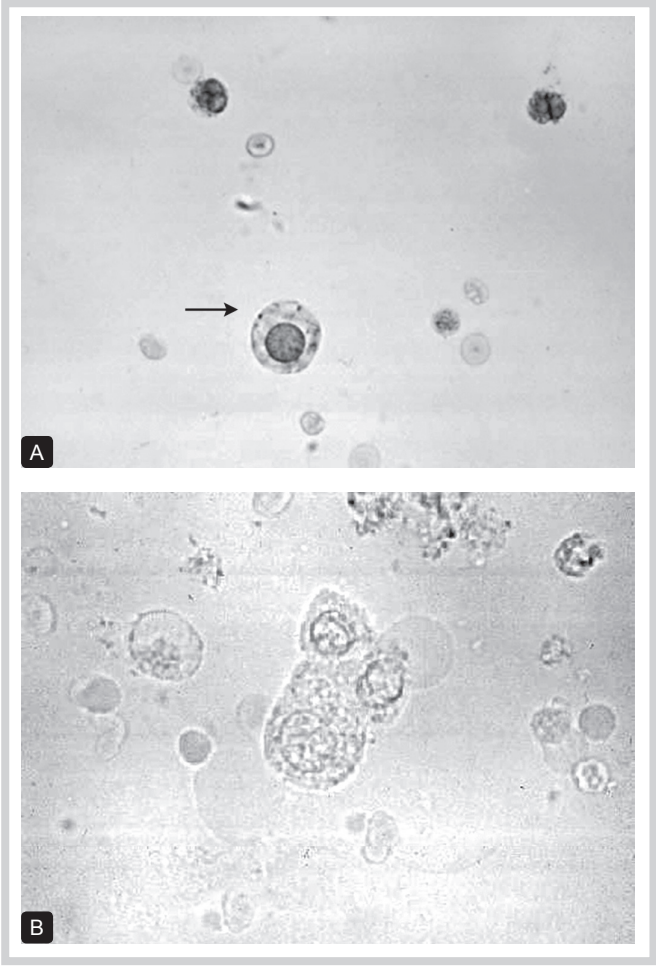
Unstained cells are larger than renal tubular cells, have a round nucleus and vary in size depending on depth of origin in transitional epithelium. Superficial cells are large and flat with small nucleus.

Squamous Epithelial Cells (Fig 5.20)

Unstained, these are large, flattened cells with abundant cytoplasm and a small round nucleus. The cell may be folded or rolled.

Casts (Fig. 5.21)

These are cylindrical; diameter varies according to the size of the renal tubule or duct of their origin. The ends are usually rounded but may be flat, irregular or tapered.



FIGS 5.18A AND B: Renal tubular epithelial cells

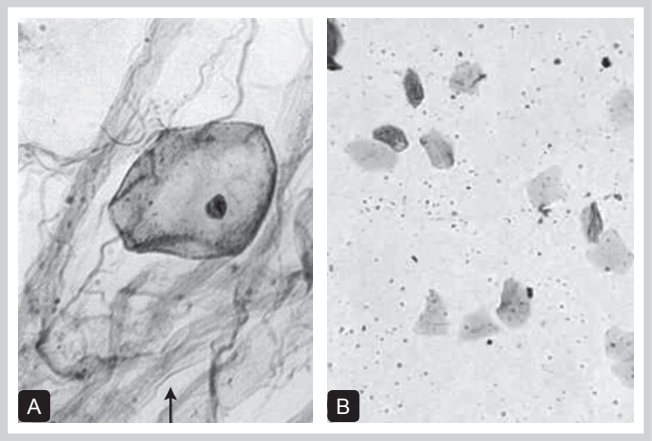


FIG. 5.20A AND B: (A) Squamous cells in urine; (B) Urinary squamous cells

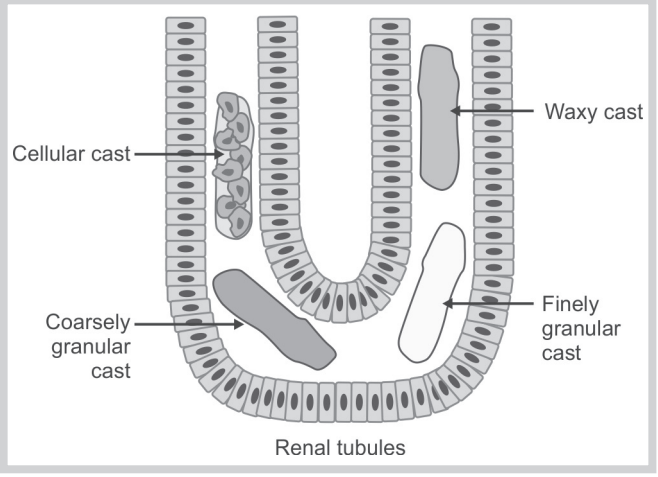
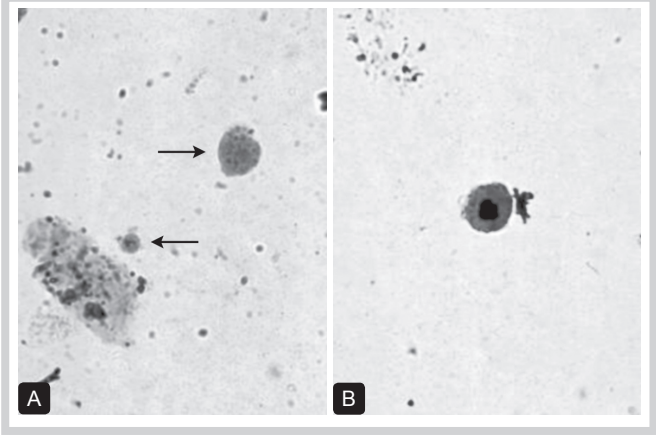


FIG. 5.21: Casts seen in the renal tubule



Transitional cell (white) and a leukocyte (black), 400 x

Transitional cell and bilirubin crystals

FIGS 5.19A AND B: Transitional epithelial cells in urine

Hyaline (Fig. 5.22)

These are colorless, homogeneous, transparent.

Coarse Granular Casts

These contain fat, degenerated cell or protein aggregates which appear as dark granules (Figs 5.18 to 5.20).

Finely Granular Casts

These contain fine granules in all or in part of the cast (Figs 5.21 to 5.23).

Fatty Casts (Fig. 5.24)

These contain highly refractile globules of varying size. Fat droplets will stain bright orange with Sudan III.

Red Cell Casts (Fig. 5.25)

Yellow under LP objective. If many cells are present in each cast, the matrix will not be visible.

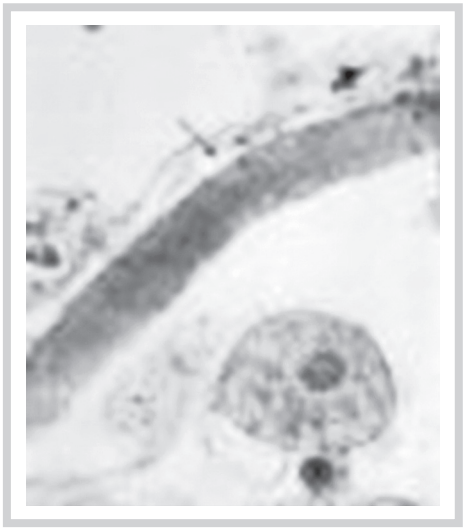


FIG. 5.22: Hyaline cast in urine

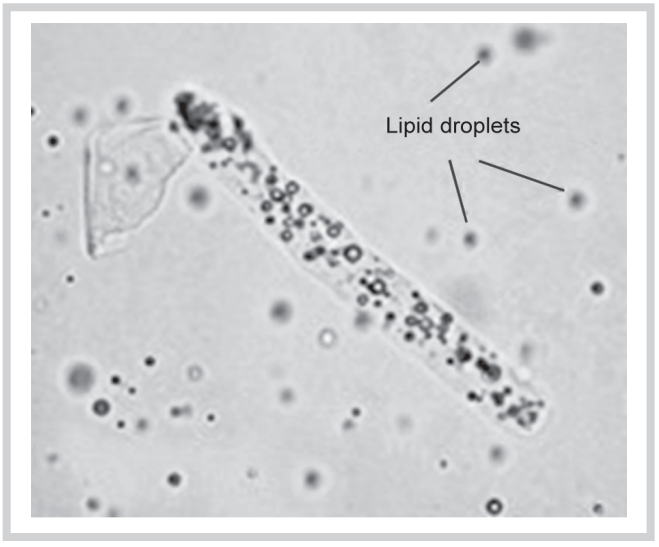
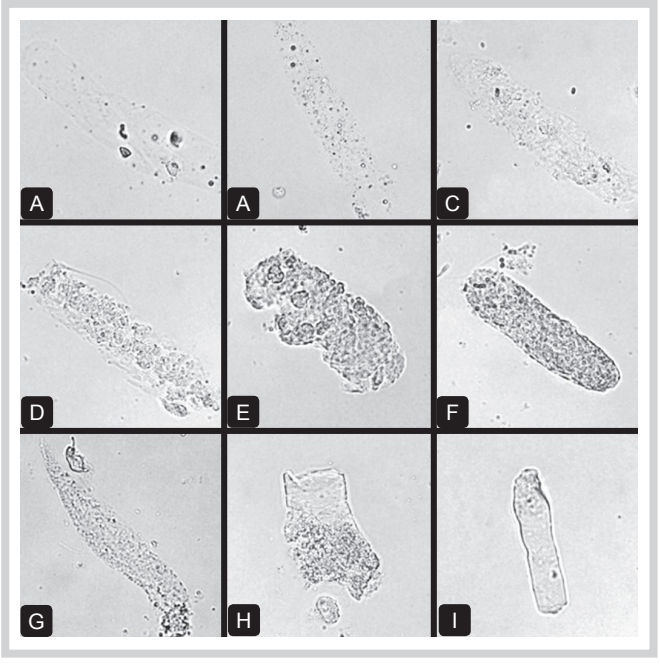


FIG. 5.24: Fatty cast



FIGS 5.23A TO I: Array of casts observed in urine (A) Hyaline cast; (B) Fatty cast; (C) Hyaline to finely granular cyst; (D) Cellular cast; (E) Cellular to coarsely granular cast; (F) Coarsely granular cast; (G) Finely granular cast; (H) Granular to waxy cast; (I) Waxy cast

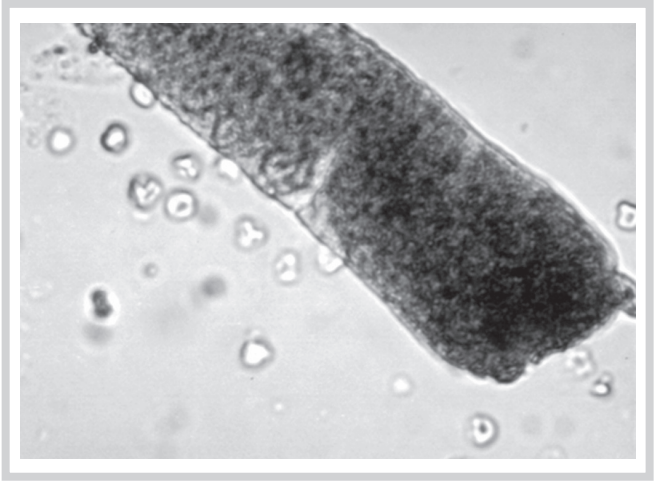


FIG. 5.25: RBC cast

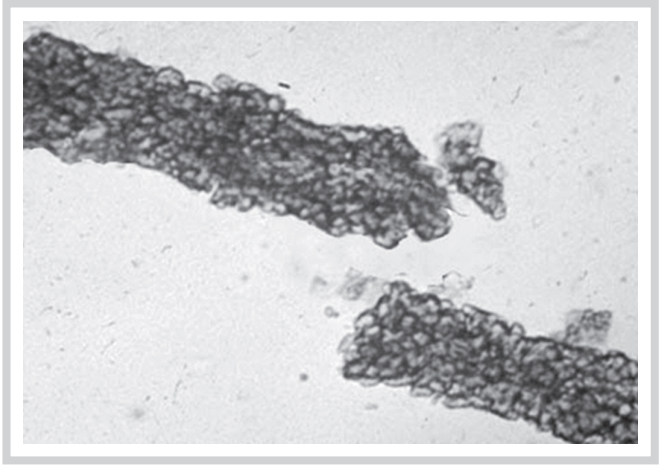


FIG. 5.26: RBC casts in urine

Blood Casts (Fig. 5.26)

These contain hemogobin from degenerated RBCs. Are yellow to orange in color, best seen with LP objective.

Leukocyte Casts (Fig. 5.27)

These contain small granular cells in a clear matrix. The leukocytes may be admixed with red cells or epithelial cells. Clumps of leukocytes may sometimes look like casts.

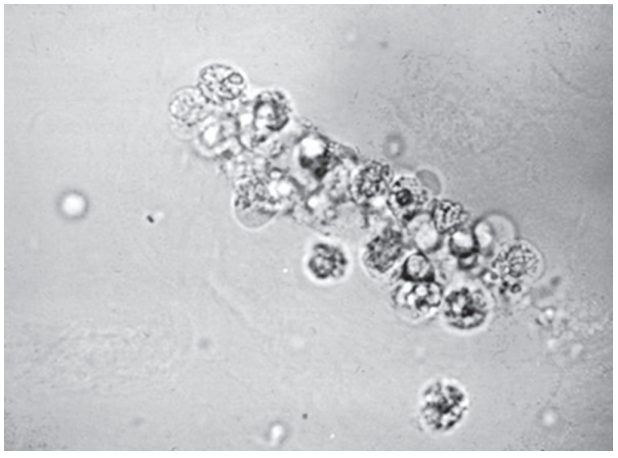


FIG. 5.27: Leukocyte casts

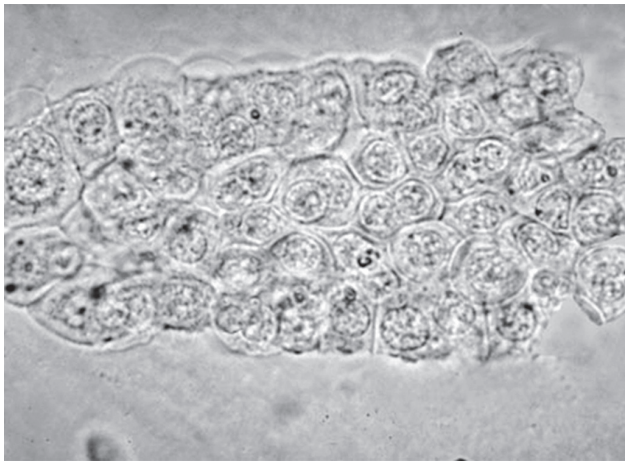


FIG. 5.28: Renal tubular epithelial cell casts in urine

Tubular Epithelial Casts (Fig. 5.28)
These resemble leukocyte or mixed cell casts. They often appear as two rows of cells in a narrow cast.

Waxy Casts (Figs 5.21 to 5.23)
These are yellow and homogeneous, have sharper outlines than hyaline casts with irregular ends and cracks.

Structures commonly confused with casts are mucous threads and rolled, cigar-shaped squamous epithelial cells. Mucous threads are long, ribbon-like strands with poorly defined edges and have pointed or split ends. Often, they appear to have longitudinal striations.

Fat: Free globules are seen in grape-like clusters. They vary in size more than the yeast cells or red cells (Fig. 5.29).

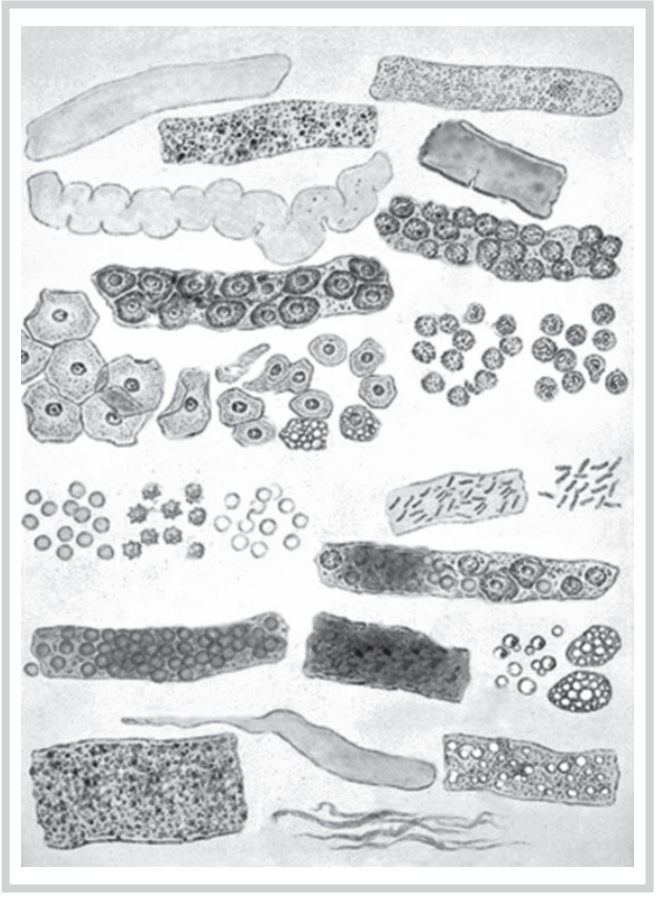


FIG. 5.29: Casts as seen in urine—diagrammatic presentation

Detailed Study of Important Urinary Microscopy Constituents

Red Cells and Red Cell Casts

Normal Values of RBCs
1-2/LPF (low powered field)
0-1/HPF (high powered field)
Red cell casts Nil (zero)/LPF.

In a healthy subject, red cells are only occasionally found in the urine, but persistent finding needs to be investigated. Examine sediment under low and high power. RBCs are studied under high power.

Clinical Relevance

Red cell casts :

- Casts composed largely of RBCs are rarely found normally and indicate hemorrhage or desquamative conditions of the nephron
- RBC casts imply acute inflammatory or vascular disorder in the glomerulus

- They may be the only manifestation of:
  - Acute glemerulonephritis
  - Renal infarction
  - Collagen disease
  - Kidney involvement in subacute bacterial endocarditis.
- The usual finding in SLE is RBC casts and epithelial cell casts.

Red Blood Cells

The finding of more than one to two RBCs per HPF is an abnormal condition that can indicate:

- Renal or systemic disease
- Trauma to kidney.

Increased Red Cells are found in

- Pyelonephritis
- SLE
- Renal stones
- Cystitis
- Hemophilia
- Prostatitis
- Tuberculosis of urinary tract
- Malignancies of urinary tract.

Red cells in excess of WBCs: Imply bleeding into the urinary tract as may occur in:

- Trauma
- Tumors
- Aspirin consumption
- Anticoagulant therapy
- Thrombocytopenia.

Interfering Factors

- Increased numbers of RBCs can be found following violent exercise, a traumatic catheterization, passage of stones, or contamination by menstrual fluid
- Alkaline urine hemolysis RBCs and dissolves casts
- Many drugs can cause RBC appearance in urine
- Red cell casts may occur after strenuous physical activity and contact sports.

White Cells and White Cell Casts

Normal Values

WBCs : 0–5/high powered field (HPF)

WBC casts : none (zero)/LPF.

WBCs may come from anywhere in the genitourinary field. While white cell casts always come from renal tubules.

Clinical Relevance

Leukocytes:

- Large numbers of WBCs indicate bacterial infection of urinary tract
- If infection is in the kidney, WBCs may be associated with cellular and granular casts, bacteria, epithelial cells and relatively few red cells
- Usually, presence of abnormal numbers of WBCs in urine necessitates urine culture
- WBC casts
- White cell casts indicate renal parenchymal infection
- May be found in:
  - Pyelonephritis most common cause
  - Acute glomerulonephritis
  - Interstitial inflammation of the kidney
- It is difficult to differentiate between WBC and epithelial cell casts
- As pyelonephritis may remain completely asymptomatic even though renal tissue is being progressively destroyed, careful examination (using low power) of urinary sediment for leukocyte casts is mandatory.

Interfering Factors

Vaginal discharge can contaminate the sample. Either a "clean catch" (midstream sample) or a catheterized specimen should be taken to rule out contamination.

Epithelial Cells and Epithelial Cell Casts

Normal Values

Occasional renal epithelial cell may be found.

Renal epithelial cell casts are formed by cast-off tubular cells. Since tubular cells are being replaced, it is of little importance, therefore, to find an occasional epithelial cells or clumps.

Clinical Relevance

Large numbers of epithelial cells are abnormal.

May be seen in:

- Nephrosis
- Amyloidosis
- Poisoning from heavy metals and toxins.

Squamous epithelial cells (squames) are usually seen when urine is contaminated with vaginal discharge.

Hyaline Casts

Normal Value

Occasional hyaline cast/LPF may be found.

These are clear, colorless casts and are formed when protein (Tamm-Horsfall) within the tubules precipitates and gels. Their appearance in the urine depends on the rate of urine flow, urine pH, and the degree of proteinuria. Examine under low power.

Clinical Relevance

- Hyaline casts imply possible damage to the glomerular capillary membrane, which is permitting leakage of proteins through the glomerular filter

- Hyaline casts may be temporarily seen in:
  - Fever
  - Postural strain
  - Emotional fatigue
  - Strenuous exercise
  - Palpation of kidney.
- When large numbers of hyaline casts appear in the urine along with heavy proteinuria, fine granular casts, fatty casts, or oval fat bodies or fat droplets, nephrotic syndrome should be considered
- Casts may not be found even if proteinuria is heavy because of dilute urine or because pH is alkaline
- In cylindruria, large numbers of casts are seen, but there may not be any protein in the urine.

Granular Casts

Normal Value
Occasional granular cast may be seen.

Granular casts result from the disintegration of the cellular material of WBCs and epithelial cells into coarse and fine particles.

Clinical Relevance
- Acute tubular necrosis
- Advanced glomerulonephritis
- Pyelonephritis
- Malignant nephrosclerosis
- Chronic lead poisoning.

Waxy Cysts

Never seen in healthy subjects. Seen in terminal diseases of kidney.
- Chronic renal disease
- Tubular inflammation and degeneration.

Oval Fat Bodies and Fatty Casts (Fig. 5.30)

Never seen in urines of healthy individuals. In nephrotic syndrome, fat accumulates in the tubular cells and eventually sloughs off, forming oval fat bodies. This fat is probably a cholesterol ester. Fatty casts usually composed of individual droplets. The presence of fat droplets, oval fat bodies, or fatty casts is the hallmark of the nephrotic syndrome.

Clinical Relevance
Fatty casts are found in chronic renal disease and indicate tubular inflammation and degeneration.

Crystals

Crystals Seen in Normal Acid Urine (Fig. 5.31)

1. Amorphous urates: Yellow-red granules.
2. Uric acid: Yellow or red-brown irregular but usually whetstone crystals or rhomboids.
3. Calcium oxalate: Refractile, octahedral "envelopes".

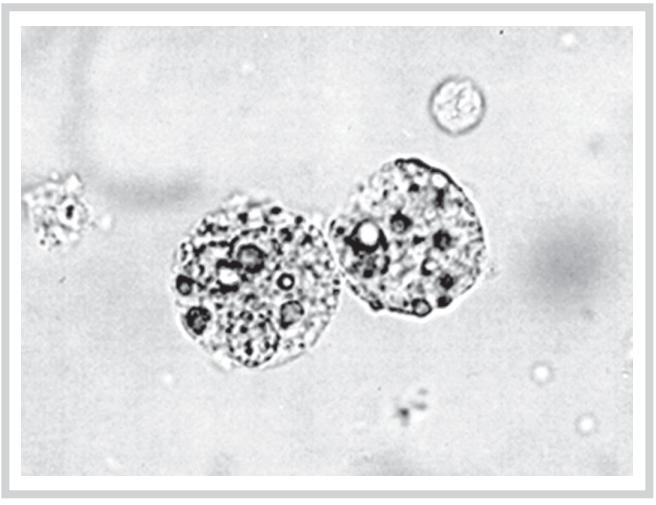


FIG. 5.30: Oval fat body

Crystals Seen in Normal Alkaline Urine (Fig. 5.32)

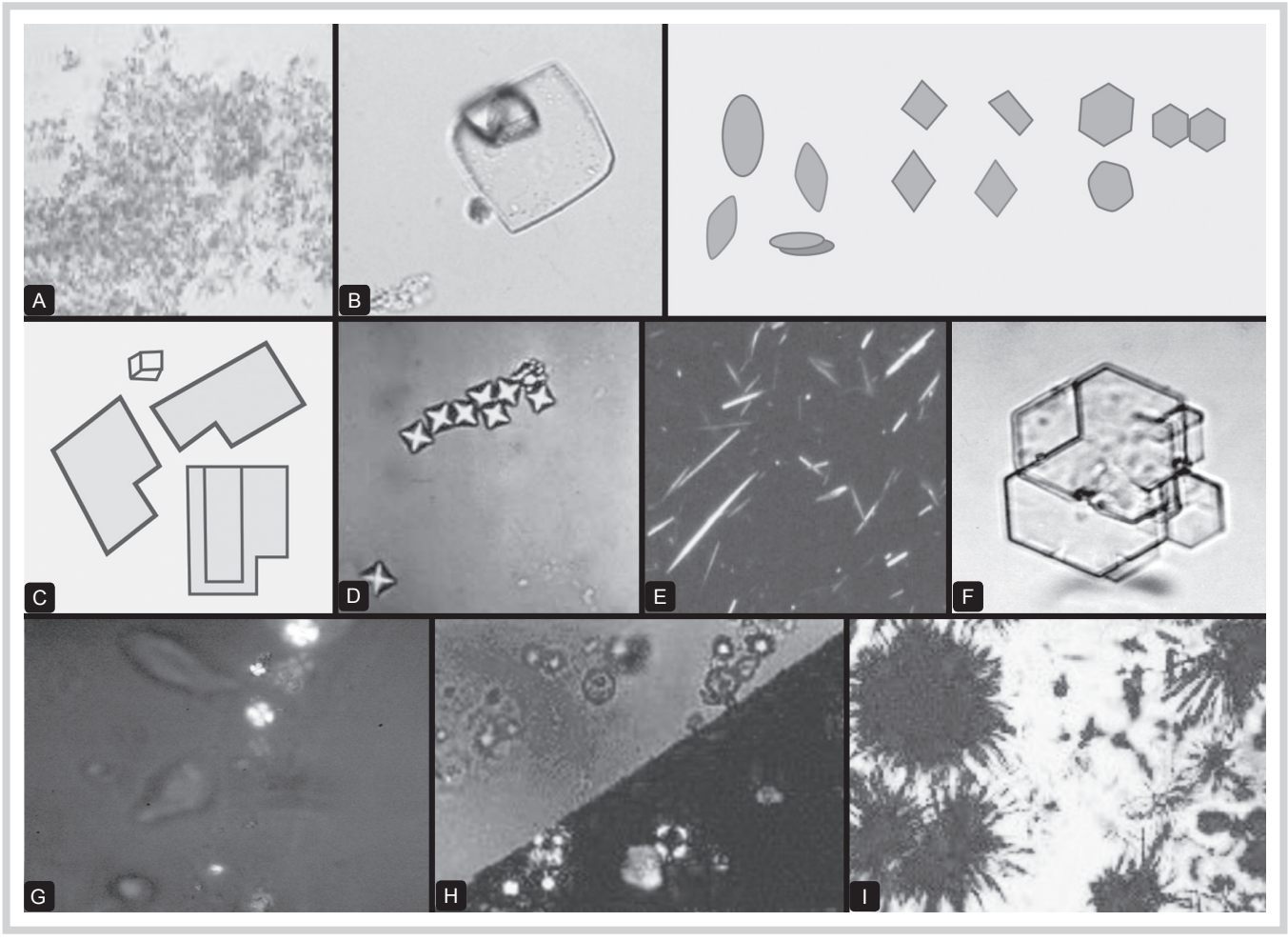
1. Amorphous phosphates: Fine precipitate.
2. Triple phosphate: Colorless, three to six-sided prisms. Occasionally fern leaf.
3. Ammonium biurate: Yellow brown spheres "thorn apple".
4. Calcium phosphate: Stellate prisms.
5. Calcium carbonate: Colorless spheres or dumb-bells, tiny.

Crystals Seen in Abnormal Urine

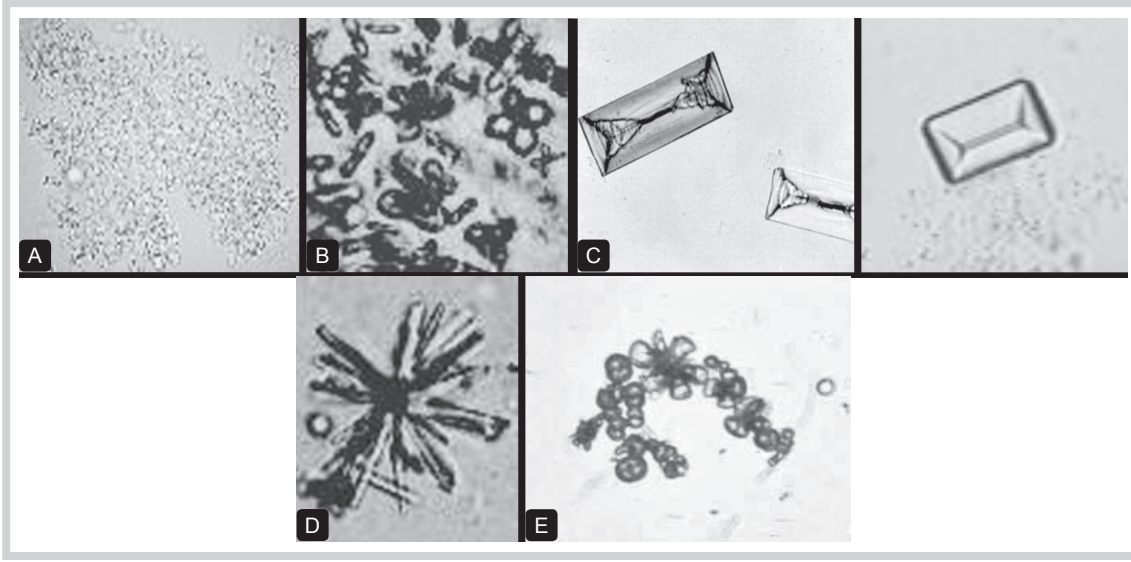
1. Cystine: Colorless, refractile, hexagonal plate.
2. Tyrosine: Fine needles arranged in sheaves or clumps, usually yellow, silky.
3. Leucine: Yellow, oily appearing spheres with radial and concentric striations.
4. Sulfonamide crystals (sulfadiazine): Yellow-brown asymmetrical, striated sheaves and round forms with radial striations.

Cholesterol appears as flat notched plates in acid urine, calcium oxalate and calcium hydrogen phosphate crystals are found in neutral urine. Uric acid and urates redissolve on warming at 60°C. Ampicillin is occasionally seen as masses of long, tiny colorless crystals in acid urine when given parenterally.

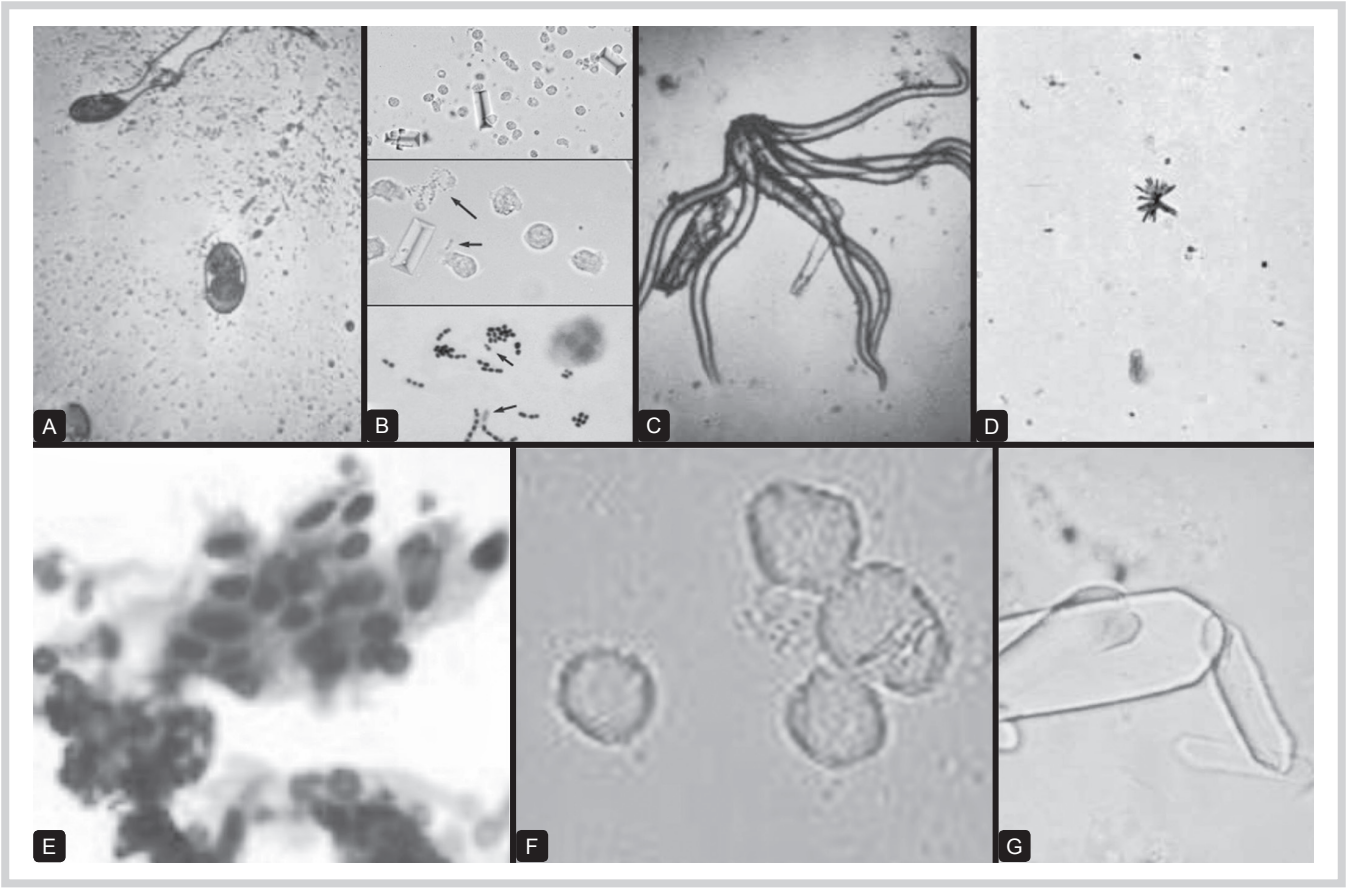
Other miscellaneous incidental findings observed on microscopic examination of urinary sediment are shown in Figure 5.33.



FIGS 5.31A TO I: Crystals, etc. usually found in acidic urine: (A) Amorphous urates; (B) Uric acid; (C) Cholesterol; (D) Calcium oxalate crystal; (E) Sodium urate; (F) Cystine crystal; (G) Fat droplets as seen in polarising light; (H) Leucin spheres; and (I) Tyrosine needles



FIGS 5.32A TO E: Crystals usually found in alkaline urine: (A) Amorphous phosphate; (B) Calcium carbonate; (C) Triple phosphate in urine; (D) Calcium phosphate; and (E) Ammonium urate crystals in urine



FIGS 5.33A TO G: (A) Pollen; (B) Bacteria in urine; (C) Cloth fiber in urine; (D) Bilirubin crystals; (E) Bladder epithelial cells (these cells are atypical); (F) Starch; (G) Calcium oxalate monohydrate crystals

Bacteria, Fungus and Parasites

Bacteria may or may not (contaminated, overgrown) be important. A dry film may be made by spreading a drop or two of the urine sediment on a glass slide, fixed and stained with Gram's stain. If bacteria are identified in an uncentrifuged specimen under an oil immersion lens, it suggests that more than 100,000 organisms/mL are present, i.e. significant bacteriuria.

Acid-fast bacilli may be seen but urine should always be cultured as smegma also contains some acid-fast bacilli.

Yeast cells may be seen in UTI (e.g. in diabetes mellitus) but yeasts are also common contaminants (Fig. 5.34).

Parasites and Parasitic Ova

These may be seen as fecal or vaginal contaminants. In *Schistosoma haematobium*, typical ova may be seen in urine accompanied by RBCs from urinary bladder. *Trichomonas vaginalis* may come from vagina when urethral or bladder infection is suspected, the protozoa should be searched for immediately in a wet preparation. Spermatozoa are generally present in the urine of men after nocturnal emissions (Fig. 5.35).

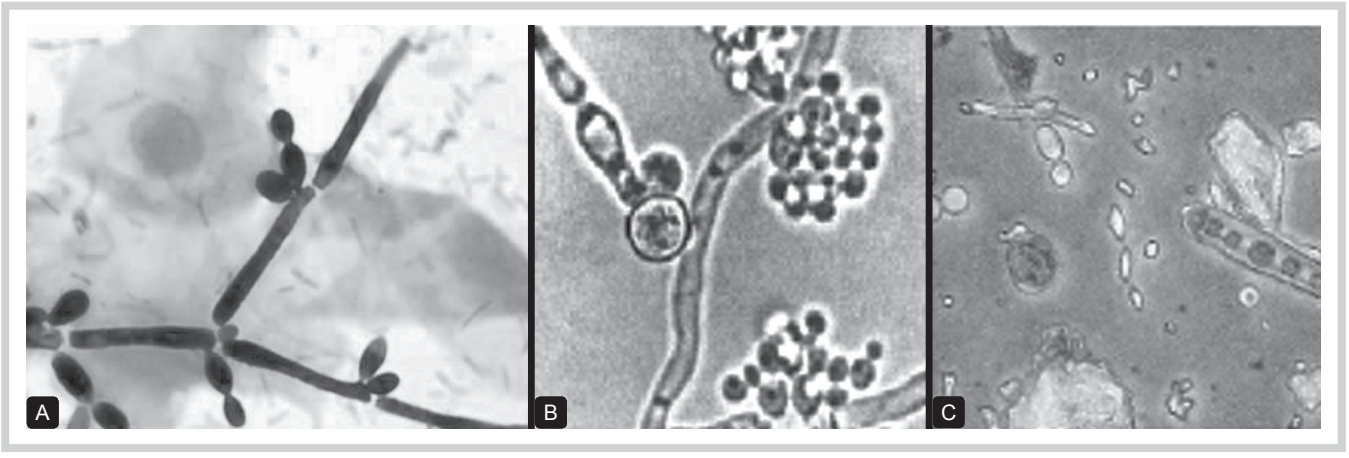
Casts in Urine: Common Causes

Hyaline

- Normal people after strenuous exercise
- Congestive heart failure
- Diabetic nephropathy
- Chronic renal failure
- Glomerulonephritis and pyelonephritis.

Red Cell

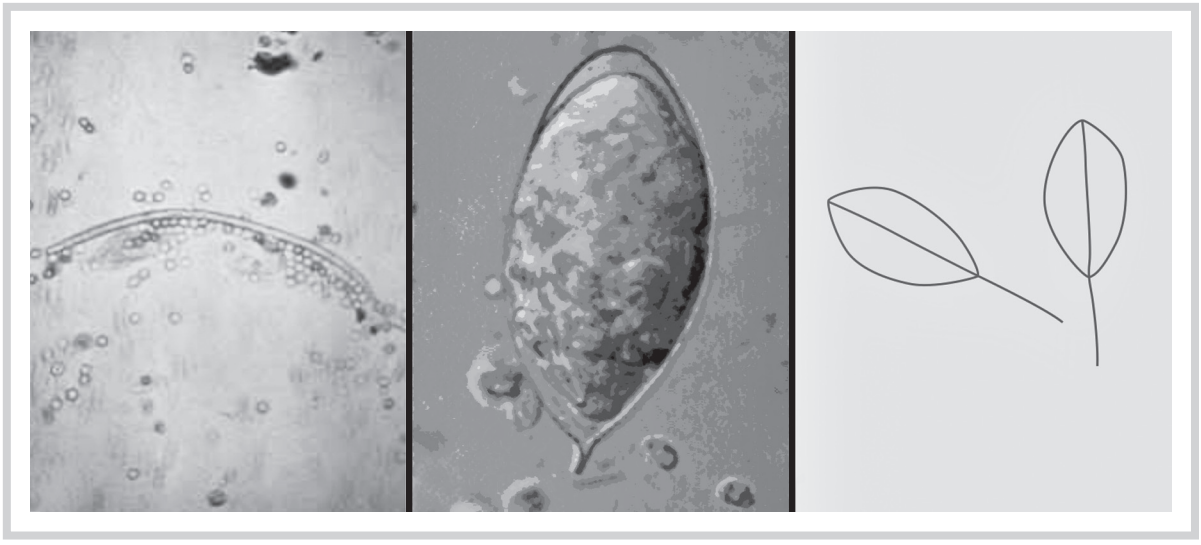
- Acute glomerulonephritis
- Lupus nephritis



FIGS 5.34A TO C: (A) Fungi seen in urine candida; (B) Fungi seen in another photomicrograph; (C) Fungal contaminants in urine

TABLE 5.7: Common patterns of abnormal urine composition in disease

Disease	*Daily volume*	*Color*	*Sp Gr*	*Protein*	*Red cell*	*Casts*	*Microscopic and other findings*
Normal	600–2500	Yellow amber	1.003 1.030	0-trace	0 to Occ	0 to Occ	Hyaline, casts, must be acid and fresh or preserved
Hyperpyrexia	Decreased	Amber	Increased	Trace to+	0 to +	None to few	Hyaline casts, tubular cells
Congestive heart failure	↓	Amber	↑-varies with renal function	1–2+	None to +	+	Hyaline and granular casts
Eclampsia	↓	Amber	↑	3–4+	None to +	3-4+	Hyaline casts
Diabetic coma	↓ or ↑	Light	↑	+	0	None to +	Hyaline casts, glucose, ketonuria
Acute glomerulonephritis (Ac. GN)	↓	Smoky red	↑	2–4+	1–4+	2–4+	Blood, cellular, granular, hyaline casts, renal, tubular epithelium
Degenerative phase (De. GN)	Normal or ↓	Light	Normal or ↑	4 +	1–2 +	4+	Granular, waxy, fatty casts, broad casts
Lipoid nephrosis	↓	Light to dark	Very high	4+	0 to trace	4+	Hyaline, granular, fatty, waxy casts, fatty tubule cells
Collagen disease	Normal, ↓ or ↑	Light to dark	Normal or ↓	1–4+	1–4+	1–4+	Blood, cellular, granular, hyaline, waxy, fatty, broad casts, fatty tubule cells
Pyelonephritis	Normal or ↑	Cloudy, dark	Normal or ↓	1–2+	None to+	None to+	Pus casts and hyaline casts. Many pus cells, bacteria
Benign hypertension	Normal or ↑	Normal or Light	Normal or ↓	None to+	0 to trace	None to+	Hyaline and granular casts
Malignant hypertension	Normal ↓ or ↑	Light	Low, fixed	1–2+	Trace to+	1–2+	Hyaline and granular casts
Occ = Occasional	↓ = Reduced or decreased		↑ = Increased or raised			0= Nil/Zero	



FIGS 5.35A TO C: (A) Microfilaria; (B) *Schistosoma haematobium*; (C) Trichomonas vaginalis

- Goodpasture's syndrome
- Subacute bacterial endocarditis
- Renal infarction.

White Cell

- Acute pyelonephritis
- Interstitial nephritis
- Lupus nephritis.

Epithelial Cell

- Tubular necrosis
- Cytomegalovirus infection
- Toxicity from heavy metals, salicylates
- Transplant rejection.

Granular

- Nephrotic syndrome
- Pyelonephritis
- Glomerulonephritis
- Transplant rejection
- Lead toxicity.

Waxy Casts

- Severe tubular atrophy
- Renal failure.♥

CHAPTER 6

Renal Function and its Evaluation

Assessment of renal function involves urine analysis; both routine and microscopic, blood chemistry, urography and special renal function tests.

RENAL PHYSIOLOGY IN BRIEF

Each kidney contains about 1 million nephrons. A nephron has a glomerulus and a long tubule that has three parts: (1) the proximal convoluted tubule (PCT), (2) the thin loop of Henle (LH), and (3) the distal convoluted tubule (DCT).

The glomeruli are the ultrafilter and the filtrate produced is like plasma except that it has almost no protein, 180 liters of this filtrate is produced in 24 hours, of which 178 liters of water and most of the organic and inorganic solutes are reabsorbed. Normally, some components of the filtered solutes are actively absorbed (completely or almost so)—glucose, phosphate and amino acids, sodium, etc. For some solutes, such as glucose, phosphate and amino acids, the maximum reabsorptive capacity of the tubule is limited and filtered material in excess of this limit is passed on in the urine. Normal renal threshold for glucose is 180 mg%, if excess is presented to the nephron, it would result in glycosuria. Other solutes are not reabsorbed, or are only passively and partially reabsorbed or are actively secreted by the tubule. Inulin (a carbohydrate) used for renal function studies is not at all reabsorbed by the tubules. Some urea is passively reabsorbed, but most of the filtered urea escapes reabsorption. Exogenous creatinine, H^+, K^+, phenol red (PSP), iodopyracet (Diodrast), para-aminohippurate and penicillin are actively secreted by the tubule cells, thus increasing excretion over the amount filtered.

FUNCTIONS OF THE KIDNEY

1. Removal in solution of solid waste substances (e.g. end products of protein metabolism and foreign substances like dyes).
2. Regulation of water balance.
3. Regulation of acid-base equilibrium and electrolyte excretion. This includes secretion of H^+ and production of ammonia from amino acids, principally glutamine. The H^+ and NH^+ produced are exchanged for Na^+ 4 in the DCT, thus providing for conservation of this essential cation.

Urinalysis

This has already been dealt in depth in previous chapter

Impaired Renal Function and Blood Chemistry

1. Reduction in glomerular filtration rate or renal blood flow is accompanied by a rise in blood urea nitrogen (BUN), creatinine and non-protein nitrogen (NPN). Phosphate and sulfate retention is common. These days newer markers like cystatin c are available that reflect kidney function better than the previously available tools.
2. Low serum protein concentrations occur commonly. Edema may occur if serum albumin drops below 2.5 gm% or total serum proteins become less than 5.5 gm%.
3. Acid-base equilibrium is disturbed in nephritis. Renal acidosis is partly due to failure to conserve sodium during excretion of anions (e.g. chlorides and phosphates).
4. Anemia accompanies chronic renal disease.

CONCENTRATION: DILUTION TESTS

If the patient's routine urine specimens contain no sugar or protein and have a specific gravity of 1.025 or higher, a concentration test is unnecessary.

Principle

Urine specific gravity is a measure of capacity of the tubules to reabsorb water from glomerular filtrate, thus concentrating the urine. Determination of osmolality is better but equipment needed for this is generally not available.

Concentration Test

It is contraindicated in uremia and is unreliable in a case of heart failure with edema.

1. No fluids for 24 hours after the morning meal (uremic patients are not to be dehydrated; they may have a large obligatory renal water loss).
2. Collect urine specimens during the last 12 hours of the period and determine specific gravity of each.
3. Specific gravity should reach 1.025 or more.

In some patients with edema, nocturnal diuresis will invalidate the test.

Dilution Test (Water Test)

It is contraindicated in patients with renal/cardiac edema. The test may be modified for use in the diagnosis of adrenal insufficiency.

1. Evening meal as desired. Nothing orally after 8.00 pm.
2. At 8.00 am empty bladder and drink 1500 mL water within 45 minutes.
3. Void every half an hour until noon (Save 8 specimens).
4. Specific gravity should be 1.003 in at least one of the specimens.
5. Total quantity of voided specimens should be over 80% of intake (i.e. over 1200 mL).

Interpret as in concentration test above. This vasopressin test gives reliable results in the presence of cardiac edema or ascites. A further, application of the test is in diabetes insipidus, where urine concentration is normal after giving vasopressin but not after fluid restriction.

Conditions that Impair Concentrating Ability

Renal Diseases:

- Pyelonephritis
- Acute or chronic glomerular failure
- Nephrogenic diabetes insipidus
- Renal tubular acidosis.

Metabolic Disturbances:

- Osmotic diuresis (especially diabetes mellitus)
- Hypokalemia
- Hypercalcemia (especially hyperparathyroidism)
- Lithium use
- Ethanol use
- Prolonged overhydration
- Severe hypoproteinemia.

Systemic Diseases Affecting Renal Medulla:

- Multiple myeloma
- Amyloidosis
- Sickle cell anemia or trait.

PHENOL RED TEST

Principle

This is a measure of tubular excretion. Phenol red test (PSP) is removed from peritubular capillary blood by the renal tubules and excreted into the urine.

Intravenous Method

Make sure that no residual volume remains in the bladder and evacuation is complete. At the beginning of the test, the bladder should not be empty for it is necessary that the patient be able to void for the 15 minutes collection.

1. The patient should drink 2 glasses of water and additional water during the test to ensure a urine volume sufficient to permit collection of urine specimen at the stated time. Larger urine volumes reduce error resulting from incomplete bladder emptying.
2. Inject 1 mL of dye (6 mg) intravenously. Collect the total volume of urine voided at 15, 30 and 60 minutes after injection of the dye. Determine the PSP content of each specimen.

Interpretation

	% PSP excretion			
	15 min	*30 min*	*60 min*	*Total*
Normal	15–27+	12–20	13–20	55–60
Renal insufficiency	< 15	< 12	< 12	< 40

Ureteral Catheterization Method (Cystoscopy)

The method can be used to study function of a single kidney at a time. Have the 2 ureteral catheters dripping into separate test tubes containing dilute NaOH and inject

1 mL of PSP IV. Normally, the dye appears within 3 minutes, as indicated by pink coloration in the tubes.

Phenol Red Test for Residual Urine

(Modified PSP test to serve as a measure of residual urine).

Let the patient empty his bladder. Give IV 1 mL of PSP (6 mg of dye). The water intake here is limited to 200 mL or less during each of the 2 subsequent half-hour periods; this is necessary because the rapid filling of the bladder in the presence of uretheral obstruction may result in a loss of bladder tone and a consequent increase in residual urine. Collect all urine that the patient can pass half an hour and 1 hour after the injection of the dye.

Normally, the PSP excretion is 50–60% in the first half-hour, plus another 10–15% during the second half-hour.

Interpretation

	First specimen (½ hour)	*Second specimen (1 hour)*
Normal	50–60%	10–15%
Residual (a)	15%	25%
Urine (b)	35%	25%
Present (c)	25%	25%

If the initial half-hour excretion is less than, equal to, or only slightly more than the second half-hour excretion, residual urine must be present. In case the excretion curve is flat and the morning specific gravity low, catheterization should be done after collection of the second half-hour specimen to confirm the presence or absence of residual urine, since under these circumstances one cannot distinguish between severely depressed renal function and a large residual volume of urine. If the specific gravity of the morning urine is high, renal insufficiency is unlikely and a flat excretion curve is a reliable index of residual urine volume.

A rough estimate of residual urine volume can be calculated from the following formula:

$$\text{Vol}^{(½\ \text{h})} \frac{(60 - \text{PSP})^{(\%\text{dh})}}{\text{PSP}^{(\%\text{dh})}} = \text{mL residual urine}$$

$\text{Vol}^{(½\ \text{h})}$ = Volume of first half-hour specimen.
$\text{PSP}^{(½\ \text{h})}$ = % of PSP recovered in first specimen.
60 = Expected normal PSP excretion in the first half-hour.

(Values of the second half-hour are not used in this calculation).

CLEARANCE TESTS

By these tests, the capacity of the kidneys to clear waste products or foreign materials (inulin, etc.) from the blood into the urine is found. From the determination of blood concentration of the test material and the quantity eliminated in the urine, "clearance" can be calculated in terms of millimeters of blood cleared per unit time.

Creatinine Clearance

Creatinine is filtered through the glomerulus. Under ordinary circumstances, the clearance of endogenous creatinine approximates the glomerular filtration rate. The clearance formula is:

$$\text{Clearance} = \frac{UV}{P}\ UV$$

where, U = mg% creatinine in urine
P = mg% creatinine in plasma
V = mL of urine excreted per minute or per 24 hours.

Methods

1. Twenty-four hours endogenous creatinine clearance. The entire volume of 24 hours period is collected. A blood sample is withdrawn during the forenoon of the day of the test. Creatinine concentrations in plasma/serum and urine are found and the volume of the urine is measured and the clearance estimated as per the formula given.
2. Two to six hours clearance periods may be used. Fasting state is preferred for the brief clearance period. A 2 to 6 hours urine collection is completed and a blood sample withdrawn at about the midpoint of the urine collection period. Creatinine concentrations are estimated in urine and plasma and the urine volume is found and clearance estimated.

Interpretation

Normal values for men and women corrected to 1.73 sq m body surface area range from 140–180 liters/24 hours (100–150 mL/minute). To correct clearance to standard 1.73 sq m body surface area

$$\text{Clearance observed} \times \frac{1.73\,\text{sq m}}{\text{Estimated surface area}}$$

= Corrected clearance.

(This test is superior to urea clearance).

Creatinine Clearance Test

Normal Range

110–150 mL/min (males)
105–132 mL/min (females)

Effect of Age on Normal Function

Ages 50–75, subtract 5 mL for each 5-year interval.
Age 75 and above, subtract 8 mL for each 5-year interval.

Artefacts that Lower Calculated Figure

- Incomplete urine collection
- Bacterial multiplication in collecting vessel
- Ketones, barbiturates, PSP in urine at higher levels than is plasma.

Causes for Reduced Creatinine Clearance

Acute: Shock, hypovolemia, nephrotoxic chemicals, acute glomerulonephritis, malignant hypertension, eclampsia.
Chronic: Glomerulonephritis, pyelonephritis, hypertensive nephrosclerosis, polycystic kidneys.

PRINCIPLES OF PRECISE TESTS OF RENAL FUNCTION (TABLE 6.1)

Clearance of inulin or endogenous creatinine and of iodopyracet (Diodrast) or PAH helps differentiate diseases of glomeruli and tubules.

Glomerular Filtration Rate (GFR)

Inulin, a polysaccharide is eliminated exclusively through the glomeruli, is neither excreted nor absorbed by the tubules. Inulin clearance is therefore, a measure of the glomerular filtration rate. Other radioactive labeled substances can be used. Normal in adults is 100–130 mL/minute per 1.73 sqm of body surface.

Renal Plasma Flow (RPF)

Para-aminohippurate (PAH), iodopyracet and ^{131}I or ^{125}I labeled sodium iodohippurate at low concentration in plasma are cleared almost completely by filtration and tubular secretion as the plasma flows through kidney.

If, for example, at a plasma PAH concentration of 1 mg%, 6 mg PAH appear, in urine per minute, 600 mL of plasma must be passing the kidneys per minute. The normal range is 500–800 mL of plasma per minute per 1.73 sq m of body surface. Varying with the hematocrit, this indicates a flow through the kidneys of 1000–1500 mL of whole blood per minute or almost one-third of the cardiac output at rest.

Filtration Fraction

Ratio of volume of glomerular filtration to the volume of plasma from which the filtrate was obtained is expressed

TABLE 6.1: Renal function tests at a glance

Determination		*Normal values*
Phenolsulfonphthalein (PSP, Phenol red)	1 mL IV	15 min 35% (28–51)\ 30 min 17% (13–24) 55–60% 60 min 12% (9–17) 120 min 6% (3–10)
Clearance tests Inulin clearance Iothalamate ^{131}I Endogenous creatinine clearance	Glomerular filtration rate -do- -do-	Corrected to 1.73 sq m SA Male: 110–150 mL/min Female: 105–132 mL/min
Iodohippurate ^{131}I PAH clearance	Renal plasma flow	Male: 560–830 mL/min Female: 490–700 mL/min
Filtration fraction	GFR FF PRF	Male: 17–21% Female: 17–23%
Urea clearance (Cu)		Standard: 40–65 mL/min Maximal: 60–100 mL/min
Maximal glucose reabsorptive capacity	TmG	Male: 300–450 mg/min Female: 250–350 mg/min
Maximal iodopyracet capacity	TmD	Male: 43–59 mg/min Female: 33–51 mg/min
Maximal PAH excretory capacity	Tm PAH	80–90 mg/min.

as GFR/RPF. This ratio is called the filtration fraction. The normal filtration fraction is:

$$\frac{120 \text{ mL/min}}{600 \text{ mL}} = 0.2$$

MAXIMAL TUBULAR CAPACITY (TM)

At high concentrations of iodopyracet, iodohippurate, or PAH in plasma, the excretory capacity of the renal tubule is exceeded. By measuring the amount of test material excreted under these conditions and correcting for the amount of test material simultaneously filtered through the glomerulus, the maximal excretory capacity of the tubule is obtained. Tm for reabsorption of glucose, amino acids, etc. can be determined similarly, although in this instance the amount filtered minus the amount excreted per unit of time equals the maximal reabsorptive capacity.

Cystatin C

Quantitative Turbidimetric Immunoassay for Estimation of Cystatin C in Human Serum

(Turbidimetry as a technology is given in Turbidimetric Assays in the one of the following/subsequent chapters)

Summary

Cystatin C, a non-glycosylated, low molecular weight (13 kDa) protein belongs to the family of cysteine protease inhibitors. Cystatin C is produced at a constant rate by nearly every nucleated cell in the human body. Its biochemical characteristics allow its free filtration in the glomerulus. Subsequently it is reabsorbed and almost completely catabolized in the proximal tubule. Practically no Cystatin C returns to the blood. Therefore, Cystatin C concentration in human blood is closely related to Glomerular filtration rate (GFR). An increased Cystatin C concentration in human blood may indicate a reduced GFR, which may be due to renal diseases. The production of Cystatin C in the body is not influenced by renal condition, increased protein catabolism or dietetic factors. Moreover, it does not change with age or muscle mass like creatinine does. Serum Cystatin C is therefore proposed to be a ideal endogenous marker of glomerular filtration rate (GFR) especially in patients with moderate to severe renal impairment. Studies have demonstrated that Cystatin C is the most suitable marker of moderately impaired renal function.

Quantia-Cystatin C® is an immunoturbidimetric assay useful for quantitative measurement of Cystatin C in human serum/plasma.

Reagents

1. Quantia-Cystatin C® Activation Buffer (R1): Ready to use buffer solution.
2. Quantia-Cystatin C® Latex Reagent (R2): Purified immunoglobulin fraction that is directed against Cystatin C which is covalently linked to uniform suspension of polystyrene latex particles.
3. Quantia-Cystatin C® Calibrator: Ready to use human pool serum containing Cystatin C equivalent to the stated amount on mg/L basis. An International Cystatin C calibrator is being prepared by a working group formed by the IFCC/EU. Quantia-Cystatin C® calibrator is traceable to a standard that has been validated against the coming IFCC standard, by the university of Lund, a leading partner in the working group, The Quantia-Cystatin C® calibrator is already standardized against the International Cystatin C calibrator.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity, and performance.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. **Do not freeze.**
2. The shelf life of the reagent, activation buffer and the calibrator is as per the expiry date mentioned on the respective vial labels.

Onboard Stability on Automated Analyzer

In appropriate bottles the Quantia-Cystatin C® activation buffer and Quantia-Cystatin C® latex reagent are stable for 9 weeks when stored at 2–8°C onboard the analyzer.

Principle

Quantia-Cystatin C® is a turbidimetric immunoassay for the quantitative determination of Cystatin C in human serum and is based on the principle of agglutination reaction. The test specimen is mixed with Cystatin C latex reagent (R2) and activation buffer (R1) and allowed to react. Presence of Cystatin C in the test specimen results in the formation of an insoluble complex producing a turbidity, which is measured at wavelength of 546 nm. The extent of turbidity corresponds to the concentration of Cystatin C in the specimen.

Note:

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the reagents derived from human source have been tested for HBsAg and HIV antibodies and are found to be non-reactive. However, handle the material as if infectious.

3. Reagent contains sodium azide as preservative in concentrations that is not characterized as dangerous. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagent be periodically verified with Quantia-Cystatin C® control set (Cat. No. 108202005) .
5. Gently mix the Quantia-Cystatin C® latex reagent (R2) well before use to disperse the latex particles uniformly to improve test performance.
6. As the reagents within lots have been matched, reagents from different lots must not be interchanged.
7. Calibrators of different manufacturers must not be used with Quantia-Cystatin C® reagents.
8. The *Quantia-Cystatin C®* reagents are not adaptable for Nephelometric analyzers.

Specimen Collection and Preparation

No special preparation of the patient is required prior to specimen collection by approved techniques.

EDTA/Heparinized plasma or serum should be used for testing. Should a delay in testing occur, store the samples at 2–8°C.

Interference

No interference was observed with hemoglobin 8 g/L, bilirubin 420 mg/L, and triglycerides 12.5 mmol/mL. Interference of RF does not take place with Quantia-Cystatin C® assay as it uses avian antibodies.

Reference Values

The reference values for Cystatin C (architect ci8200) was determined to be 0.51–1.05 mg/L.

It is recommended that each laboratory must define its own reference range for relevant population taking into account all affecting factors.

Remarks

1. Usage of well-calibrated equipment and accessories and procedures is critical for achieving correct results.
2. Markedly lipemic, hemolyzed, and contaminated serum samples could produce nonspecific values.
3. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
4. Several Cystatin C based prediction equations for calculation of GFR for adults and children have been published. It should be noted that these formulas were evaluated with different Cystatin C assays and may reveal inaccurate GFR.
5. In contrast to creatinine concentration, Cystatin C levels are lower in hypothyroid and higher in hyperthyroid state as compared with the euthyroid state. Therefore, thyroid function has to be considered when Cystatin C is used as a marker of kidney function.

Warranty

This product is designed to perform as described on the label and package insert. The manufacturer disclaims any implied warranty of use and sale for any other purpose.

Samples can be stored for up to one week at 2–8°C, provided they are not contaminated. Do not use hemolyzed, icteric, or highly turbid sera. Turbid or particulate samples must be clarified by centrifugation at 2000 rpm for 15 minutes prior to testing. Use the clear supernatant for testing.

Additional Material Required

Analyzer, well-calibrated micropipettes, disposable tips, isotonic saline, test-tube rack, Optically clean disposable/glass semi-microcuvettes (for cuvette mode semiauto-analyzers).

Test Procedure

Method for preparation of Cystatin C calibration curve on semiautomatic and automatic analyzer.

Bring reagents and samples to room temperature before use.

The *Quantia-Cystatin C®* calibrator is ready to use. The concentration (S) of Cystatin C is mentioned on the calibrator vial label and at the end of the package insert. Use saline for preparing dilutions of the calibrator. In analyzers, where onboard dilution of calibrator is possible, follow instructions provided in the instrument manual. In analyzers where onboard calibrator dilution is not possible follow the procedure mentioned below:

Dilute the *Quantia-Cystatin C®* calibrator serially as mentioned below for the preparation of the calibration curve.

Test Tube No.	1	2	3	4	5
Calibrator dilution No.	D1	D2	D3	D4	D5
Saline volume	-	300 µL	300 µL	300 µL	300 µL
Calibrator volume	300 µL	300 µL	300 µL	300 µL	300 µL
Concentration volume (S) of Cystatin C in mg/L	S	S/2	S/4	S/8	S/16

- At least five dilutions of the calibrator (D1-D5) covering the measuring range (0.5–8.0 mg/L) must be used for preparing the calibration curve

- During calibration on instrument with programming facilities, increasing concentration of the standard must be used for preparing the calibration curve.
- Check that sufficient amount of calibrator is present in sample cup as per the requirement of the instrument protocol during testing in automated analyzer.

Note: The calibrator values are lot dependent. Always check the calibrator dilution concentrations when a new calibration is performed. The calibration curve is usually stable for 4 weeks.

Semiautomated Analyzer

Assay conditions:

Wavelength	546 nm
Reaction temperature	37°C
Cuvette	1 cm path length

Test Procedure for Preparation of Calibration Curve

1. Zero the instrument with distilled water.
2. Pipette 400 µL of Quantia-Cystatin C® Activation buffer (R1) and 75 µL of Quantia-Cystatin C® Latex reagent (R2) in the measuring cuvette. Mix well and incubate for 5 minutes at 37°C.
3. Add 5 µL of calibrator (D1), mix gently and start the stopwatch simultaneously.
4. Read absorbance (A1), exactly at 10 seconds, and absorbance (A2) again at the end of exactly 280 seconds.
5. Repeat steps No. 2–4 for each selected diluted calibrator (D2 - D5) for preparing calibration curve.
6. Calculate ΔA (A2 - A1) for each selected calibrator (D1 - D5). Plot a graph of ΔA versus concentration of Cystatin C on the graph paper provided with the kit.
7. The calibration curve so obtained is valid only for the same lot of *Quantia-Cystatin C*® reagents.

Test Procedure for the Determination of Cystatin C in Specimen:

1. Follow steps 2 – 4 as mentioned in the above procedure for calibration curve on Semiautomatic analyzers using the test specimen in place of the calibrator.
2. Calculate ΔA (A2-A1) for the test specimen.

Calculations

Cystatin C concentration in mg/L can be obtained as mentioned below:

The concentration of Cystatin C (mg/L) can be determined directly by interpolating ΔA of the test specimen from the calibration curve. If the ΔA of the test specimen is greater than ΔA of the highest standard concentration (D1) then the test has to be rerun by carrying our dilution of test specimen such as 1:2, 1:4, etc. Interpolate the ΔA of the diluted test specimen on the calibration curve and obtain the cystatin C concentration. Multiply the obtained Cystatin C concentration "C" with the dilution factor of the test specimen to determine the Cystatin C concentration in neat specimen.

Automated Analyzer

General Application Parameter Set up (Table 6.2)

A defined application for the *Quantia-Cystatin C*® immunoturbidimetric assay must be installed in accordance with the general instrument settings given below. For instructions refer the respective instrument manual.

TABLE 6.2: Suggested instrument applications

Parameters	*Suggested applications*
Filter	546
Quantia-Cystatin C® activation buffer (R1) volume	220 µL
Sample volume	3 µL
Incubation time before addition of R2	120 seconds
Quantia-Cystatin C® latex reagent (R2) volume	45 µL
Read absorbance A1 immediately after mixing reagents and sample and absorbance A2 at the end of 5 minutes.	

Note: Applications suitable for Olympus AU 400/600/2700, Synchron LX 20, Hitachi 902/911/917/Vitros 5.1/ Modular P/Architect/Advia 1650 &1800/Cobas c501/Pentra 400 can be made available on request.

Test Procedure for Preparation of Calibration Curve

Perform the calibration as per the instrument protocol given by the manufacturer.

Test Procedure for the Determination of Cystatin C in Specimen:

When a valid calibration has been performed and the controls are within the expected range (provided in assay value sheet), specimens can be measured. Check that sufficient amount of sample is present as per the requirement of the instrument protocol.

Calculations

The results are automatically calculated by the analyzer and presented in mg/L.

For GFR Prediction Calculation:

For calculation of GFR from Cystatin C values measured with Quantia-Cystatin C® assay the following prediction equation is recommended using mg/L as the unit factor.

$$\text{GFR [mL/min/1.73 m}^2\text{]} = \frac{79.901}{\text{Cystatin C (mg/L)}} \; 1.4389$$

GFR can be calculated with the GFR calculator available on our website www.tulipgroup.com.

Quality Control

The calibration of Quantia-Cystatin C® must be validated with Quantia-Cystatin C® Control set.

Specific Performance Characteristics

The performance characteristics mentioned further have been validated using Quantia-Cystatin C on Abbott Architect ci8200 and Quantiamate.

Measuring Range

The Quantia-Cystatin C® assay has been designed to measure Cystatin C concentration in the range of 0.5–8.0 mg/L. The exact range is dependent on the calibrator value, which is lot specific. The Quantia-Cystatin C® assay is linear within the measuring range.

Detection Limit

0.33 mg/L. The detection limit represents the lowest measurable Cystatin C concentration that can be distinguished from zero.

Prozone Limit

No prozone effect was observed in concentration less than 16 mg/L of Cystatin C.

CHAPTER 7

Stool Examination

Average healthy adults defecate from three times a day to three times a week. Common pattern is once a day. The stool tends to be soft and bulky on a diet high in vegetables and small and dry on a diet high in meat. Two thirds of the stool weight is attributable to its water content. The normal brown color is of still undetermined origin. The odor results from indole and skatole, produced by bacteria from tryptophan.

Feces are composed of:

1. Waste residue of indigestible material in food.
2. Bile (pigments and salts).
3. Intestinal secretions, including mucus.
4. Leukocytes that migrate from the bloodstream.
5. Shed epithelial cells.
6. Large numbers of bacteria that make up to one-third of total solids.
7. Inorganic material (10–20%) that is chiefly calcium and phosphates.
8. Digested food (present in very small quantities).

SPECIMEN COLLECTION

A wide mouthed jar with a screw cap is good enough, provided it is neat, clean, and without any extraneous material in it. It should, however, never be overfilled and should be opened slowly to release the gas that accumulates frequently in it (if not done so, the contents may be released explosively). Since, rectal evacuation is not completely at will and feces passed correlate very poorly with the food consumed; hence, collection should be done over a period of 3 days. The accuracy of this method can be enhanced somewhat by having the patient ingest carmine dye (0.3 g) and charcoal (1 g) at the beginning and the end of a collecting period, respectively, collecting the stools from the beginning of the appearance of the dye to the beginning of the appearance of the charcoal.

Be Careful

- Feces should be urine free when collected. Collect the entire stool and transfer to another container by a tongue blade. Deliver to the laboratory immediately after collection
- Warm stools are best for detecting ova and parasites. Do not refrigerate for ova and parasites
- Some coliform bacilli produce antibiotic substances that destroy enteric pathogens
- Refrigerate stool if it cannot be examined immediately. Never place a stool in an incubator
- A diarrheal stool will usually give good results
- A freshly passed stool is the specimen of choice
- Preferably, stool specimens should be collected before antibiotic therapy is initiated and as early in the course of disease as possible
- Only a small amount of stool is needed; the size of a walnut. If mucus and blood are present, they should be included in part of the specimen to be examined
- Do not use a stool that has been passed into the toilet bowl or that has been contaminated with barium or other X-ray medium
- Label all stool specimens with patient's name, date, and reason for examination/testing.

Interfering Factors

- Meat interferes with some tests and should usually be omitted from the diet for 3 days before a test for blood (not necessary for the guaiac method)

- Stool specimens from patients receiving barium, bismuth, oil, or antibiotics are not satisfactory
- Bismuth from paper towels and toilet tissues interferes with tests.

Normal Values in Stool Analysis

These are listed in Table 7.1.

TABLE 7.1: Normal values in stool analysis

Macroscopic	*Normal examination*
Color	Brown
Odor	Varies with pH stool and depends upon bacterial fermentation and putrefaction
Consistency	Plastic; not unusual to see seeds and vegetable skins; soft and bulky in a high vegetable diet; small and dry in a high meat diet
Size and shape	Formed
Gross blood	Absent
Mucus	Absent
Pus	Absent
Parasites	Absent
Fat	Colorless, neutral fat (18%) and fatty acid crystals and soaps
Undigested	None to small amount food, meat fibers, starch, trypsin
Eggs and segments of parasites	Absent
Yeasts	Absent
Leukocytes	Absent
Chemical examination	*Normal*
pH	Neutral to weakly alkaline
Adult	7.0–7.5
Newborn	5.0–7.0
Bottle-fed infants	Neutral to slightly alkaline pH of 7.0–8.0
Breastfed infants	Slightly acidic
Occult blood	Negative
Urobilinogen	50–300 mg/24 hr
Porphyrins	Coproporphyrins < 200 µg/24 h Protoporphyrins < 1500 µg/24 h Uroporphyrins < 100 µg/24 h
Nitrogen	1–2 g/24 h
Bile	Negative in adults, positive in children
Trypsin	Positive in small amounts in adults, in greater amounts in normal children

INSPECTION OF FECES

A simple inspection of feces may lead to a diagnosis of parasitic infection, obstructive jaundice, diarrhea, malabsorption, rectosigmoidal obstruction, dysentery or ulcerative colitis or gastrointestinal tract bleeding.

Note the quantity, form, consistency and color of the stool (Table 7.2).

Interfering Factors

1. Stool darkens on standing.
2. Color is influenced by diet, food dyes, certain foods, and drugs.
   a. Yellow to yellow green color occurs in the stool of breastfed infants who lack normal intestinal flora. It also occurs in sterilization of bowel by antibiotic.
   b. Green color occurs in diets high in chlorophyll-rich vegetables and with use of the drug calomel.
   c. Black or very dark brown color may be due to drugs such as iron, charcoal, and bismuth, to foods such as cherries, or to an unusually high proportion of meat in the diet.
   d. Light-colored stool with little odor may be due to diets high in milk and low in meat.

TABLE 7.2: Inspection of feces

Type of stool	*Likely reason*
Watery stool	Diarrhea
Large amount of mushy, foul smelling, grey stool that floats on water	Steatorrhea
Little firm, spherical masses	Constipation (irritable colon syndrome, over use of laxatives)
Narrow ribbon-like stool	Spastic bowel or rectal narrowing or stricture
Clay colored	Obstructive jaundice or presence of Barium sulphate
Reddish stool	Blood from lower gastrointestinal tract, beets consumption or BSP use
Black, tarry stool	Bleeding from upper GIT, Iron, bismuth or charcoal consumption
Green stool	Ingestion of spinach, etc. calomel, presence of biliverdin, seen in patients taking antibiotics orally
Parasites	Parasitic infestation (discussed later)

e. Claylike color may be due to a diet with excessive fat intake or barium used in X-ray examination.
f. Red color may be due to a diet high in beet or use of drugs such as BSP.
g. Drug-induced color changes are given below:
   - Black—iron salts, bismuth salts, charcoal
   - Green—mercurous chloride, indomethacin, calomel
   - Green to blue—dithiazanine
   - Brown staining—anthraquinones
   - Red—phenolphthalein, pyrvinium pamoate, tetracyclines in syrup, BSP
   - Yellow—santonin
   - Yellow to brown—senna
   - Light—sitosterols
   - Whitish discoloration—antacids
   - Orange red—phenazopyridine
   - Pink to red to black—anticoagulants (excessive dose) salicylates causing internal bleeding.

Pus

Patients with chronic ulcerative colitis and chronic bacillary dysentery frequently pass large quantities of pus with the stool that has to be examined microscopically.

It may also occur in localized abscesses or fistulas communicating with sigmoid rectum or anus. Large amounts of pus never accompany amebic colitis. No inflammatory exudate is seen in the watery stools of patients with viral gastroenteritis.

Mucus

Even in slightest quantity is abnormal (Table 7.3).

TABLE 7.3: Mucus in stool—causes

Remarks	*Causes*
Translucent gelatinous mucus clinging to the surface of the formed stool	Spastic constipation or mucous colitis. In emotionally disturbed patients and may result from excessive straining
Bloody mucus clinging to stool mass	Neoplasm, inflammation of rectal canal
Mucus with pus and blood	Ulcerative colitis, bacillary dysentery, ulcerating carcinoma of the colon, and more rarely, acute diverticulitis or intestinal tuberculosis
Copious mucus, up to 3–4 liters of mucus per day	Villous adenoma of the colon (may lead to dehydration and hypokalemia)

Odor and pH

Normal Values

Characteristic odor varies with the pH of stool; normal pH is neutral or weakly alkaline.

The pH is dependent on bacterial fermentation and putrefaction in the bowel. Substances called indole and skatole, formed by intestinal putrefaction and fermentation, are mainly responsible for the odor of normal stools.

Interfering Substances

Carbohydrate fermentation changes pH to acidic. Protein breakdown changes the pH to alkaline.

Blood

Blood in stools should never be ignored, however, slight the quantity may be. Bleeding in the upper GIT may give black-tarry appearance to stools while that arising from lower GIT may give red color or be seen as frank blood.

Causes

Upper GI Tract
- Peptic ulcer—gastric or duodenal
- Erosive gastritis
- Atrophic gastritis
- Esophageal varices
- Mallory-Weiss syndrome
- Hiatus hernia
- Esophagitis.

Small and Large Bowel
- Meckel's diverticulum
- Polyps
- Infectious diarrheas
- Inflammatory bowel disease (Crohn's disease, ulcerative colitis)
- Diverticular disease
- Vascular malformations
- Carcinoma.

Rectum and Anus
- Hemorrhoids
- Anorectal fissure.

Drugs

Associated with increased GIT blood loss:
- Salicylates
- Steroids
- Rauwolfia derivatives
- Indomethacin
- Colchicine.

Loss of more than 50–75 mL of blood from the upper GIT generally imparts a dark red to black color and a tarry consistency to the stool. Persistence of tarry appearance for 2 or 3 days suggests loss of at least 1000 mL of blood. Smaller increases in blood content may not alter appearance of the stool. Such stools are said to contain "Occult blood" (usually associated with GIT neoplasm).

Interfering Factors

Drugs such as salicylates, steroids, indomethacin, colchicine, iron (used in massive therapy), and Rauwolfia derivatives are associated with increased gastrointestinal bleeding in normal persons and with even more pronounced bleeding when disease is present. Gastrointestinal bleeding tests may be falsely positive in the undermentioned circumstances:

- Meat in diet contains hemoglobin and enzymes that can give false positive tests for up to 4 days after eating. The guaiac method does not require meat-free diet due to lesser sensitivity
- Vitamin C taken in quantities greater than 500 mg per day may cause false negative test for occult blood in stool
- Drugs that may cause a false positive test for occult blood include:
  - Boric acid—Iodine
  - Bromides—Inorganic iron
  - Colchicine—Oxidizing agents
- Testing method must be followed exactly or the results are not reliable
- Use an aliquot from center of formed stool
- Time reaction exactly
- Liquid stools may cause false negatives with filter paper methods.

Tests for Occult Blood

These tests are based upon a little understood chemical reaction in which the reagent is oxidized by hydrogen peroxide at low pH (acid added) and catalyzed by the presence of heme—the intact iron containing porphyrin ring.

All iron heme derivatives are active. Free iron and free porphyrin rings are not active. The most important substance, which contains the active 'heme' besides hemoglobin, is myoglobin contained in muscle fibers.

The ideal test for screening should be sensitive enough to react to a significant amount of blood without reacting to the minute amounts of blood present in the feces of normal people on a normal diet (especially if non-vegetarian). It should also be specific enough not to react with substances in diet or in common medicines and at the same time be simple, easy, rapid and inexpensive to perform.

Commonly used reagents

1. Gum guaicum
2. Orthotolidine
3. Benzidine (carcinogenic)
4. Phenolphthalein.

Benzidine Test

Benzidine test is an extremely sensitive test and can give false positives in people on abundant meat diet. Only 1–2% people with significant bleeding will show a negative test (false negative). False positives may be overcome in some cases by boiling the emulsion of feces for 1–2 minutes and then repeating the test.

Method

Benzidine reagent consists of 4 g benzidine base/100 mL of glacial acetic acid. It is stable for about 4 months. Emulsify peasized bit of feces in 5 mL of water. Mix 1 mL emulsion and 1 mL of reagent in test tube and add several drops of 3% H_2O_2. Positive reaction is indicated by the appearance of a blue color in the mixture and is reported as follows:

Trace —Faint blue color after 1 minute
1+ —Definite blue-green slowly
2+ —Green-blue rapidly
3+ —Blue almost immediately
4+ —Dark blue immediately.

Guaiac Test

This is less sensitive. Has 5% false positive in patients on nonvegetarian diet and 3–5% false negatives. It is a better screening test. With loss of 20–30 mL of blood, all tests will be positive.

Method

Guaiac reagent consists of 1 g guaiac in 5 mL of 95% ethanol (stable in brown bottle for a month in a refrigerator).

To an emulsion of feces—or better yet, a small smear of feces on a piece of filter paper add 2–3 drops of gum guaiac solution, 2–3 drops of glacial acetic acid and 2–3 drops of 3% HO_2.

Positive tests are reported as:

Trace — Faint blue-green in 1 minute
1+ — Light blue slowly
2+ — Clear blue rapidly
3+ — Deep blue almost immediately
4+ — Deep blue immediately.

Orthotolidine

This test has an intermediate sensitivity and is replacing other tests, though, not in India.

Reagents

Mix: Orthotolidine barium peroxide 200 mg. Glacial acetic acid 5 mL (Stable only for one day).

Method

- Using a clean applicator stick, smear the stool on a small square of filter paper
- Pipette a few drops of the reagent on to the filter paper
- After exactly 30 seconds, examine for a blue color.

Result

A blue green color appearing within 30 seconds means a positive test.

Occult Blood (Hemospot®)

Courtesy: Tulip Group

Summary

Fecal occult blood is a term used to describe the presence of blood in the feces. Blood is present in the feces due to bleeding from the gastrointestinal tract. Small increases in blood content may not alter the appearance of the stool and such stools are said to contain "occult blood", detection of which can be most useful in uncovering and localizing disease. Hemoglobin levels of 5 mg/dL or more are diagnostically significant.

Screening for occult blood is especially important because over one half of all cancers (excluding skin) are those of the gastrointestinal tract. Early diagnosis and treatment of patients with colonic cancer results in a relative good prognosis for survival.

Hemospot test is useful in the detection of bleeding caused by gastrointestinal disorders such as colitis, polyps, diverticulitis, colorectal cancer and hookworm infestation. Fecal occult blood tests are recommended for use in:

1. As an aid to routine physical examinations.
2. Routine hospital testing.
3. Screening for gastrointestinal bleeding from any source including colorectal cancer.

Reagents

1. Hemospot test cards consisting of a filter paper impregnated with the guaiac resin (reactive surface).
2. Developer solution consists of stabilized hydrogen peroxide solution, which is ready to use.
3. Positive control.

Reagent Storage and Stability

Store the reagent at 20–30°C, in a cool place away from direct sunlight, fluorescent light, UV rays and moisture. Do not refrigerate.

The shelf life of the reagents and test cards is mentioned on the kit/developer solution label.

Principle

If blood is present in the stool sample, the hematin in the hemoglobin molecule catalyzes the release of oxygen from the hydrogen peroxide, which in turn oxidizes the colorless phenolic components of gum guaiac to colored quinones. During test, after the addition of the developer solution to the reactive surfaces of the result window, the reaction area turns blue if occult blood is present in the sample. If the reaction area does not change color, then it indicates that there is no occult blood present in the sample.

Note

1. In vitro diagnostic reagent for laboratory or professional use only. Not for medicinal use.
2. The kit contains hydrogen peroxide solution, which may be irritating. Avoid contact with eyes, skin and clothing. In case of contact, flush with large quantities of water.
3. Do not expose the test cards and developer solution to direct sunlight, fluorescent light and UV rays.

Quality Control

Positive control provided with the kit should be run occasionally to validate the performance of the test cards and reagent.

Preparation and Sample Collection

Preparation of the Patient

1. As for all occult blood tests, certain medications such as aspirin, indomethacin, phenylbutazone, reserpine, corticosteroids and nonsteroidal anti-inflammatory drugs can induce gastrointestinal bleeding and cause false positive results. These medications should be temporarily discontinued with the consent of the physician for 7 days prior to testing and during the test period.
2. Vitamin C when taken in amounts greater than 250 mg per day has been shown to induce false negative results. Rectal medications (suppositories) and iron containing medications may also interfere with these tests and should be discontinued 2 days before and during the test period with the consent of the physician.
3. For at least 2 days before and during the test period, all raw meat and red meat should be avoided. Raw broccoli, cauliflower, radishes and turnips may cause false positive results, hence should be avoided.

Sample Collection

1. A clean dry detergent free glass or plastic container of a suitable size is ideal for collection of the specimen.

Urine should not be passed simultaneously into the collection container. Clean pieces of plastic are convenient for transferring stool from the collection container to the transport vessel.

2. The stool samples should be collected from different areas of the formed stool (samples from the outside of stool are most likely to reflect the condition of the lower colon, while specimens taken from inside of the stool are more likely to reflect conditions of the upper gastrointestinal tract) and also provides a more representative sample to be tested.
3. The two test fields provided in Hemospot facilitate detection and localizing the source of bleeding. Because bleeding may be intermittent, it is preferable to collect specimens from different bowel movements, preferably consecutive ones.

Material Provided with the Kit

1. Hemospot test cards
2. Dropper bottle containing developer solution
3. Sample applicators
4. Positive control.

Additional Material Required

Gloves, stopwatch, rust free needle/pin.

Test Procedure

1. Pierce the nozzle of the developer solution with a rust free sharp pin or needle.
2. Retrieve the required number of test cards to perform the desired number of tests.
3. Label the cards with correct patient identity.
4. Open the sample application windows labeled A and B respectively, to expose the reactive surfaces of the test card.
5. By using the sample applicator provided in the kit, spread a very thin layer of stool on the reactive surfaces on the window A; similarly, on window B; from a different part of the stool.
6. Wait until the smeared sample has dried completely.
7. Turn over the test card.
8. Open the result window and add one drop of developer to fields RA and RB (the reverse side of the sample smeared on the sample application windows) respectively.
9. Observe for color change exactly at 2 minutes.
10. Even if one of the field's has a blue color, the test is positive for occult blood.

Interpretation of Results

- No blue color indicates absence of occult blood in the stool.
- Trace blue coloration indicates presence of approximately 5 mg/dL of occult blood in the stool.
- Strong blue coloration indicates significantly more than 5 mg/dL of occult blood in the stool (Figs 7.1 to 7.4).

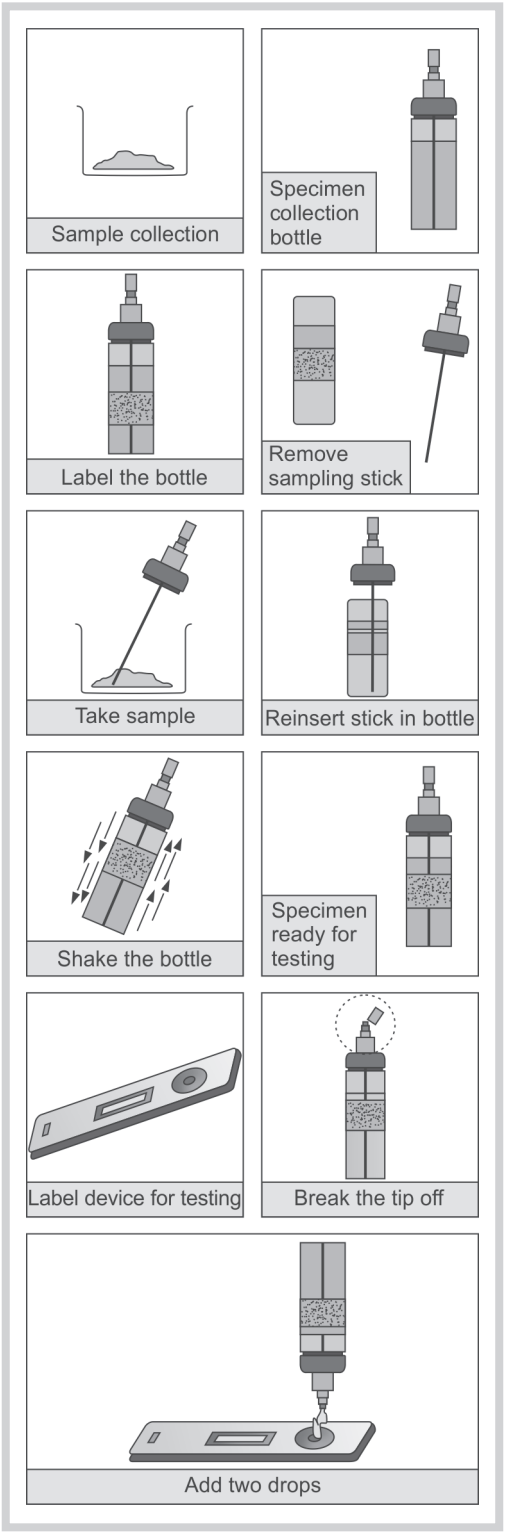


FIG. 7.1: Using Cancheck FOBT

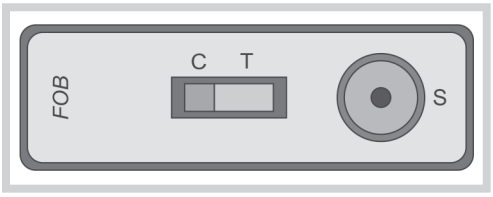


FIG. 7.2: Cancheck FOBT negative result

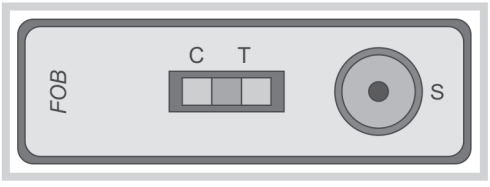


FIG. 7.3: Cancheck FOBT positive result

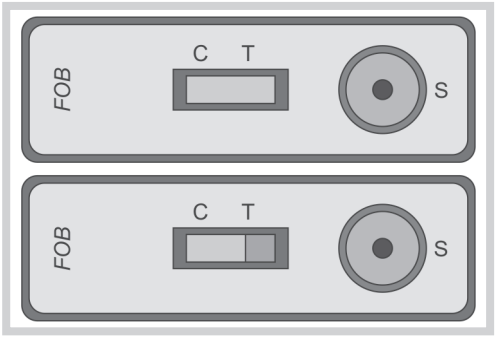


FIG. 7.4: Canceck FOBT invalid results

Remarks

1. Stool samples collected during menstrual bleeding, constipation induced bleeding, bleeding hemorrhoids or when rectal medication is used may cause positive results.
2. Hands, collection containers and test area should be kept free of blood as they may cause false positive results.
3. Certain medications may induce gastrointestinal bleeding and cause false positive reactions, hence should be avoided during and prior to the testing period.
4. Diet containing exogenous peroxidases may induce false positive results.
5. Dosages of vitamin C more than 250 mg per day will cause a false negative result.
6. If the test is developed before the sample smear dries completely on the test card, the results obtained may not be accurate.
7. Only use the sample applicators provided in the kit for applying the samples.

Rapid Immunochromatographic Device: Testing of FOBT (Human)

Cancheck®-FOBT is a rapid, qualitative, two-site sandwich immunoassay for the detection of fecal occult blood concentration in human feces.

Summary

Colorectal cancer (CRC) is a major cause of death from cancer. The risk of CRC increases with age, with an approximate doubling of the incidence in each decade from 40 to 80 years of age. It has been estimated that the lifetime risk of developing CRC is 1:50. Fecal Occult Blood Test (FOBT) provides the most cost-effective way to screen for CRC. It has been reported that screening for CRC by FOBT decreases CRC mortality by 15–33%. FOBT is the test to detect the presence of occult blood in the feces. Small amounts of blood is present in the feces of normal healthy individuals due to bleeding from the gastrointestinal tract like bleeding gums and bleeding from minor abrasions. The presence of small amounts of blood in feces may not alter the color or appearance of the stool. The detection of fecal occult blood can be useful in detecting bleeding resulting from gastrointestinal disorders such as colitis, polyps, colorectal carcinomas and diverticulitis. Benzidine and guaiac tests for fecal occult blood detect the peroxidase activity of heme, either as intact hemoglobin or as free heme. Hence, to avoid false positives, for the week before the test, patients need to follow a diet that excludes red meat, turnips, horseradish, broccoli, radishes, cauliflower, cantaloupes and other melons and supplemental vitamin C. Unlike Guaiac tests, Cancheck - FOBT is a third generation immunochromatographic test that is not affected by peroxidase activity.

Principle

Cancheck®-FOBT utilizes the principle of immunochromatography, a unique two-site immunoassay on a nitrocellulose membrane. The conjugate pad contains two components—monoclonal anti-human hemoglobin antibody conjugated to colloidal gold and rabbit IgG conjugated to colloidal gold. As the test specimen flows through the membrane assembly of the device, the highly specific monoclonal anti-human hemoglobin antibody-colloidal gold conjugate complexes with the human hemoglobin in the specimen and travels on the membrane due to capillary action along with the rabbit IgG colloidal gold conjugate. This complex moves further on the membrane to the test region (T) where it is immobilized by another specific monoclonal anti-human hemoglobin antibody coated on the membrane leading

to formation of a colored band. If occult blood level is equal to or higher than the 200 μg/L of feces suspension, the test is positive. The absence of this colored band in the test region indicates a negative test result. The rabbit IgG-colloidal gold conjugate and unbound complex, if any, move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies coated on the membrane at the control region (C), forming a colored band. The control band formation is based on the 'Rabbit/anti-Rabbit globulin' system. Since it is completely independent of the analyte detection system, it facilitates formation of consistent control band signal independent of the analyte concentration. This control band acts as a procedural control and serves to validate the test results.

Reagents and Materials Supplied

Cancheck®- FOBT kit contains:

A. Individual pouches, each containing :
    1. Device membrane assembly pre-dispensed with monoclonal anti-human hemoglobin colloidal gold conjugates, rabbit IgG colloidal gold conjugate, monoclonal anti-human hemoglobin antibody and anti-rabbit antiserum coated at the respective regions.
    2. Desiccant pouch.
B. BUF: 0.1 M Tris, 1% sodium chloride, 0.5% Brij 35, 0.1% sodium azide.
C. Package insert.

Storage and Stability

The sealed pouches in the test kit and the kit components may be stored between 4–30°C for the duration of shelf life as indicated on the pouch/carton. **Do not freeze**.

Notes

1. Read the instructions carefully before performing the test.
2. For in vitro diagnostic use only. Not for medicinal use. For professional use only.
3. Do not use the kit beyond expiry date and do not re-use the test device.
4. Do not intermix reagents from different lots.
5. Handle all specimens as if potentially infectious. Follow standard biosafety guidelines for handling and disposal of potentially infectious material.
6. If desiccant color at the point of opening the pouch has turned from blue to pink or colorless, another test device must be run.
7. Specimen extraction buffer contains sodium azide (0.1%), avoid skin contact with this reagent. Azide may react with lead and copper in the plumbing and form highly explosive metal oxide. Flush with large volumes of water to prevent azide build-up in the plumbing.

Specimen Collection and Preparation

1. Cancheck®-FOBT uses human feces as specimen.
2. Collect feces in a clean dry container.
3. Though fresh specimen is preferable, in case of delay in testing, it may be stored at 2–8°C for maximum up to 24 hours.
4. Refrigerated specimens must be brought to room temperature prior to testing.
5. Label the specimen collection bottle with specimen identity.
6. Unscrew and remove the cap (with attached sampling stick) of the specimen collection bottle ensuring that the extraction buffer is not spilt.
7. Take representative amounts of feces specimen from different portions of the sample by introducing the sampling stick at 3–4 different places in the feces specimen.
8. Wipe the sampling stick with an absorbent or tissue paper. The sample taken up by the grooves is sufficient for the test.
9. Reinsert the sampling stick into the bottle and screw the cap tightly.
10. Shake the specimen collection bottle so that there is proper homogenization of feces in buffer solution.

Testing Procedure and Interpretation of Results

1. Bring the kit components of device to room temperature prior to testing.
2. Open a foil pouch by tearing along the "notch".
3. Remove the testing device. Once opened, the device must be used immediately.
4. Label the device with specimen certification.
5. Place the testing device on a flat horizontal surface.
6. Hold the specimen collection bottle in an upward position and break the tip off.
7. Invert the bottle and holding the dropper vertically, carefully dispense exactly two drops of specimen-buffer mixture into the specimen port.
8. Observe the development of visible colored band at test region (T).
9. Positive results may be observed within 5 minutes, depending on the concentration of occult blood in the tested specimen.
10. Do not read and interpret after 5 minutes.
11. In negative specimens only the control band (C) would develop.

Negative Result

Presence of one colored band at Control (C) region indicates absence of Occult blood or the concentration of Occult blood in the specimen is below the detection limit of 200 μg/L of feces suspension.

Positive Result

If concentration of Occult blood in specimen is above 200 µg/L of feces suspension, two colored bands appear at Test (T) and Control (C) regions. The intensity of the test band may be more or less than the Control band, depending upon the concentration of Occult blood in specimen.

Invalid Result Cancheck-FOBT: The test is invalid if no band is visible at 5 minutes. The test should also be considered invalid if only the test band appears and no control band appears. Verify the test procedure and repeat the test with a new device.

Performance Characteristics

The detection limit of Cancheck® -FOBT is up to 200 µg/L of feces suspension, i.e. equivalent to 100–200 µg/g of feces.

Sensitivity

The detection limit of Cancheck®-FOBT device is up to 200 µg/L feces suspension, (calibrated against Sigma Human Hemoglobin Cat No. H-7309). This corresponds to a concentration of 100 to 200 µg/g hemoglobin/g of feces. No Prozone Effect up to a hemoglobin concentration of 1000 mg/L has been observed.

Specificity

Cancheck® -FOBT is highly specific to human hemoglobin and does not cross-react with the following:

Chicken hemoglobin	500 µg/mL
Pork hemoglobin	500 µg/mL
Beef hemoglobin	500 µg/mL
Goat hemoglobin	500 µg/mL
Rabbit hemoglobin	500 µg/mL
Horseradish peroxidase	2000 µg/mL

Limitations of the Test

1. Cancheck® -FOBT is a highly sensitive and specific test for human hemoglobin in feces. Nonetheless, as with any in vitro diagnostic test, occasional false positive and negatives may occur.
2. False negatives may occur due to improper feces suspension preparation or the lesion did not bleed or bleed sufficiently to produce a positive result.
3. Blood secondary to aspirin use or use of other non-steroidal anti-inflammatory agents may cause GI bleeding and show false positive results.
4. Stool samples collected during menstrual bleeding, constipation induced bleeding, bleeding hemorrhoids and rectal medication may also cause false positive results.
5. Gloves, collection container and test area should be kept free of blood to avoid false positive results.
6. Since benzidine and guaiac-based tests suffer from nonspecific interference of peroxidase activity, exact one-to-one correlation of the results of such tests with a 3rd generation immunochromatographic test like Cancheck-FOBT may not be observed.
7. Cancheck-FOBT should only be used as a screening test. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the result of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.

Fecal Fat

Normal Value

In a normal diet, fat in the stool will be up to 20% of total solids.

Lipids measured as fatty acids: 2–5 g/24 h

It is raised in malabsorption syndromes, the commonest example being steatorrhea. Fecal fat quantitation can be done by:

- Gravimetric method
- Isotopic techniques (radioisotopes)
- Electrical capacitance method
- Titrimetric method of Van de Kamer.

Titrimetric Method

Fats and fatty acids are converted to soap by boiling with alcoholic potassium hydroxide. After cooling, excess hydrochloric acid is added to convert soaps to fatty acids. These are extracted with petroleum ether. An aliquot is evaporated, taken up in neutral alcohol, and titrated with sodium hydroxide. Fats are calculated as fatty acids.

Electrical Capacitance Method

This has replaced the titrimetric method now. An aliquot of fecal suspension is extracted with solvent consisting chiefly of chlorinated benzenes. The extract is filtered and its electrical capacitance is measured and compared with standards of triolein similarly treated.

Interpretation

To quantitate fat excretion, there must be known dietary intake and timed stool collection. The usual technique involves a diet containing 100 g of fat daily, with a 3-day stool collection to measure total fat excretion. Excretion of more than 6 g per day is abnormal and values may range from 50 g or more.

Fecal fat increase occurs in:
- Enteritis and pancreatic diseases when there is lack of lipase
- Surgical removal of a section of intestine
- Malabsorption syndromes
- In chronic pancreatic disease, fat is more than 10 g/24 h
- A stool specimen high in fat content will have a pasty appearance and can be detected by gross examination.

Interfering Factors
1. Increased neutral fat may occur under the following conditions:
   - With use of rectal suppositories
   - With ingestion of castor oil or mineral oil
   - With ingestion of dietetic low calories mayonnaise.
2. Barium interferes with test results.

Other Methods of Assessing Malabsorption

Glucose Tolerance Test

The GTT curve becomes flat since adequate amounts of orally given glucose are not absorbed, whereas in the same patient if glucose is given parenterally, a normal curve is obtained.

Protein Loss

Protein loss estimation is not necessary for diagnosing protein-losing enteropathy.

Proteins within the intestine are reduced enzymatically to their component amino acids, which are then reabsorbed. If mucosal abnormalities prevent reabsorption or if protein leakage exceeds reabsorptive capacity, hypoproteinemia may result. The fecal protein excretion can be documented by administering isotopically labeled albumin or povidone (PVP) rather than by chemical analysis of feces.

Microscopic Examination of Stool Specimens

Stool specimens should be fresh and must not be contaminated with detergents or disinfectants, etc.

Having described the gross appearance, proceed on for microscopic examination for cells and parasites as follows:
1. Place a small piece of stool on a slide and mix with saline until smooth. Cover with a coverslip. If the specimen contains mucus, examine preferably without saline. The mucus is put on the slide and covered with a coverslip.
2. Examine under 10X and 40X objectives, with a reduced condenser aperture.
3. Report the presence of:
   - Large numbers of pus cells or muscle fibers
   - Red blood cells
   - Free living amebae, flagellates or ciliates.
   - Eggs and larvae
   - Cysts
   - Yeast cells.

Parasitic amebae, flagellates, ciliates, eggs, larvae and cysts are usually reported as the number seen in the entire preparation as follows:
- Scanty (rare): 1 to 3
- Few (1+): 4 to 10
- Moderate number: (2+) 10 to 20
- Many (3+): 20 to 40
- Very many (4+): Over 40.

Cells are usually reported as the number seen per high power field as in urine deposit.

Use of Saline

Normal (0.85%) saline is used for routine examination of stool specimens, as it is isotonic with living organisms. Use fresh uninfected saline.

Use of Iodine

Iodine is used to examine the nuclear structure of cysts, the preparation is made in the same way as for saline.

Using an iodine solution, the chromatin granules and karyosome of nuclei stain brown. The glycogen vacuole stains brown and the chromatid bars remain unstained. The solution used is called Dobells's iodine.
- Iodine: 1.0 g
- Potassium iodide: 2.0 g
- Distilled water: 50 mL

Iodine should not replace saline for routine use, as it kills living material, and would therefore, make it impossible to detect motility of amebae, flagellates, ciliates and larvae. In addition, iodine makes the chromatid bars of *E. histolytica* difficult to see.

Use of 1% Eosin

This provides a pink background against which the cysts and amebae stand out as clear unstained objects.

Use of Sargeaunt's Stain

This is used to stain the chromatid bars of cysts, and is of value especially for *E. histolytica*.

The nuclear structure stains pale green, the chromatid bars stain deeper green.

The stain consists of:
- Malachite green: 0.2 gL
- 95% Ethanol: 3 mL
- Distilled water: 100 mL

This stain can only be used after a formol ether stool concentration method, because the use of ether appears to be necessary for the staining reaction.

Stool Concentration Methods

Where heavy infestation is present, this method is not needed.

Concentration Methods may be Used

1. To see whether treatment of the parasites has been successful.
2. To find ova of *S. mansoni* or *Taenia* if few or for other ova and cysts if they have not been seen on routine examination (being very few) and are suspected to be present.
3. To examine stool specimens from patients who do not come from an area where a particular parasite is found.

Flotation Concentration Methods

In flotation methods, the stool is mixed with a solution, e.g. zinc or magnesium sulfate, which has a high specific gravity so that the parasitic contents float to the surface.

Zinc Sulfate Concentration Method

This method can be used to concentrate cysts, larvae and most helminth eggs, except those of *P. westermani*, *F. buski*, *C. sinensis* and *D. latum* and other operculated eggs and also Schistosoma eggs which do not float.

Reagents

Zinc sulfate solution of specific gravity 1.180 is needed. Prepared by dissolving 33 g of chemical in 100 mL of distilled water.

Method

1. Mix a small piece of stool with about 10 mL of water or saline, in a bottle or tube.
2. Sieve the suspension into a beaker, through a strainer with small holes.
3. Pour the contents of the beaker into a centrifuge tube.
4. Centrifuge at 2000–3000 rpm/min for 1 minute.
5. Pour off the supernatant fluid.
6. Resuspend the deposit in clean water and add enough water to fill the tube.
7. Mix well and recentrifuge.
8. Pour off the supernatant fluid.
9. Resuspend in zinc sulfate solution, fill the tube with the solution.
10. Centrifuge at high speed for 1 minute.
11. Transfer the contents from the surface of the tube to a slide, using a bacteriological wire loop. This surface film must be removed immediately.
12. Add small drops of saline and mix.
13. Cover with a coverslip.
14. Examine under 10X and 40X objectives.

Sedimentation Concentration Methods

In sedimentation methods, the parasites are not floated but deposited, usually by centrifuging.

Simple Sedimentation Method

A small piece of stool is mixed with saline in a tube or bottle and sieved through a strainer. The sieved contents are centrifuged and the supernatant fluid poured off. The deposit is resuspended in more saline, mixed, and centrifuged. This is repeated until the supernatant fluid is clear. The deposit is examined directly on a slide.

By this simple method, parasitic cysts, eggs, and free living parasites can be concentrated.

Formol-saline Ether Sedimentation Method

This method gives a good concentration of parasitic contents and is recommended for routine work. This method, however, cannot be used to concentrate free living forms as formalin kills the parasites.

Reagents

10% formol saline
Saline 450 mL
Concentrate formaldehyde solution 50 mL (40% w/v)
Add the formaldehyde solution to the saline and mix well.

Method

1. Mix a small piece of stool in about 10 mL of 10% formol saline, in a tube or bottle.
2. Sieve the suspension into a beaker through a strainer with small holes.
3. Pour about 6 mL of the sieved suspension into a centrifuge tube.
4. Add about 3 mL of ether.
5. Mix well and immediately centrifuge at 3,000 rpm/min for 1 minute.

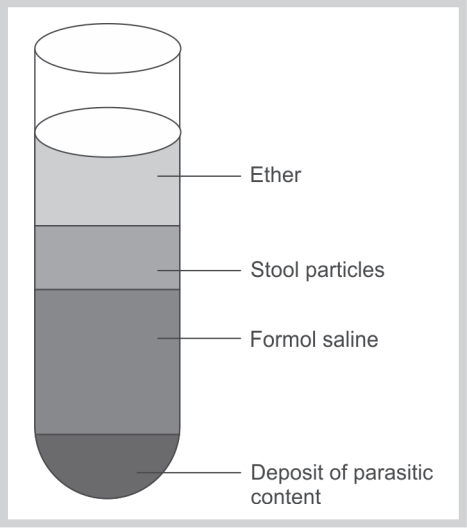


FIG. 7.5: Various layers as seen after centrifugation

6. Four layers are seen (Fig. 7.5)
   a. An upper layer of ether
   b. Middle layer of stool particles
   c. A lower layer of formol saline
   d. The deposit in which parasitic contents will be found.
7. Using an applicator stick, separate the middle layer from the sides of the tube and pour this away together with the ether and formol saline.
8. Resuspend the deposit by tapping the bottom of the tube with the finger.
9. Transfer the deposit to a slide using a pasteur pipette.
10. Cover with a coverslip and examine under 10X and 40X objectives with a reduced condenser aperture. For the identification of cysts; iodine, eosin, or Sargeaunt's stain can be used.

CHAPTER 8

Medical Parasitology

MEDICAL PARASITES

Parasitism is a category of association of living things in which one partner (the parasite) maintains itself at the expense of the energy of the other (the host). By definition, medical parasitology, then, would include even viruses and bacteria, but it is restricted to those animal parasites, chiefly protozoa and helminths, that produce a state of disease in man or are closely related to others that do. Microbial human parasites are included, for convenience, in the field of microbiology (discussed elsewhere), and with the specialized subspecialties of virology, bacteriology, and mycology.

Importance of Morphologic Identification

Recognition and differentiation of the animal parasites of man, often involving separation of pathogenic from very similar nonpathogenic (harmless) forms, need precise knowledge of their morphology. Proficiency in this part of laboratory work is gained by extensive practical experience with properly collected and processed clinical specimens. The diagnosis and differentiation of protozoal diseases, whether intestinal or systemic, often present unusual problems. Differentiation of intestinal amebae demands particular care, especially to distinguish the nonpathogenic small race (*Entamoeba hartmanni*) from the very similar larger form (*E. histolytica*) that is responsible for amebiasis.

The following pages present medical parasitology in tabular and pictorial forms.

INTESTINAL PROTOZOA OF MAN

Infections worldwide prevalence depends on sanitation level and degree of natural or acquired resistance. *Entamoeba histolytica* is found often in Indian rural and urban population. The intestinal protozoans present a serious threat in tropical rather than temperate climates, but usually much less common than asymptomatic infection. Incidence of *Giardia lamblia* varies with age, most common in children, relatively rare in adults. *Balantidium coli* is comparatively rare but may be common where sanitary conditions are very poor. Only these three commonly accepted forms are considered in Tables 8.1A and 8.1B. Pathogenicity of other species is rare or questionable.

Nonpathogenic protozoa are commonly found in the feces of man and should be differentiated from the recognized pathogenic forms. The commoner organisms: amebae—*Entamoeba hartmanni, E. coli, Endolimax nana, Dientamoeba fragilis, Iodamoeba butschlii;* flagellates—*Chilomastix mesnili, Trichomonas hominis*. Their main importance is that they are a sign of environmental pollution with fresh feces and may elicit unnecessary treatment or inaccurate diagnosis.

Trichomonas vaginalis

Trichomonas vaginalis, known only in the trophozoite stage, inhabits the human vagina and urethra of male and female. Produces vaginitis with severe itching and mucopurulent discharge in small proportion of cases. *T. vaginalis* is worldwide in distribution, occurring in

TABLE 8.1A: Tabulated life cycle of common human intestinal pathogenic protozoans

Parasite	*Enters man*	*Infective form*		*Life cycle in man*	*Exit*	*Reservoir host*
Entamoeba histolytica	Orally by contamination	Cyst	Becomes trophozoite in intestine	May invade mucosa or body organs, chiefly liver	Cyst or trophozoites in feces (latter not infective)	Man
Giardia lamblia	-do-	-do-	-do-	Duodeuum and bile passages		Man
Balantidium coli	-do-	-do-	-do-	Many invade mucosa of large intestine	-do-	Man, pig, monkey

TABLE 8.1B: Intestinal protozoal diseases of man

Disease and etiology	*Possible clinical features*	*Laboratory diagnosis*
Amebiasis	Flask-shaped ulcers in mucosa of large intestine which appear as pinpoint dots on surface with mild inflammation but may produce extensive undermining below surface. Localized in cecum and whole large intestine, especially on flexures and sigmoid colon *Blood:* Leukocytosis, anemia. Eosinophilia rare in uncomplicated protozoal infections	Examine feces by smear, concentration, culture Abscess and ulcer material (as for feces) amebic serology (ELISA method)
E. histolytica	*Symptoms:* Dysentery, bloody diarrhea, sometimes followed by constipation; abdominal pain, gas distension; poor appetite, weight loss, headache, nervous manifestations, local tenderness *Complications:* Liver abscess (single or multiple) with liver enlargement and congestion. Pain, swelling, leukocytosis, anemia, fever. Lung abscess primary or, more commonly secondary to liver abscess. Peritonitis—bacterial with usual manifestations. Ulcerations and abscesses of other organs or tissues, manifestations depending upon site infected. (Incubation period: acute, 8–10 days, chronic, 2 months to years.)	
Giardiasis *G. lamblia*	Duodenitis, perhaps choledochitis. Mucosal inflammation possible mechanical and toxic interference with absorption of vitamin A and fats, resulting in diarrhea and steatorrhea	Examine feces by smear, concentration
Balantidiasis *B. coli*	Limited to large intestine where parasites localize, with pathology and symptoms that may resemble amebic dysentery. Most cases asymptomatic with high natural resistance; acute or chronic disease. Epidemic outbreaks may occur with cases of extensive ulceration	Examine feces by smear, concentration, culture

10–40% of women examined. It is mainly transmitted by coitus but may also be transferred by recently contaminated toilet articles. The male is the chief agent of spread, although he seldom suffers symptoms. Treatment of the female, however, should always include treatment of her sexual partner.

The Basis of Serum Biochemical Tests for Leishmaniasis

These tests assess alterations in serum proteins particularly serum gamma globulin. The positive results are obtained after 2 to 3 months or more.

Napier's aldehyde test: When serum of the patient is treated with formaldehyde, it causes flocculation and opacity. To 1 mL of patient's serum, add 2 drops of formaldehyde—if flocculation and opacity occur within 10 minutes, the test is strongly positive. If this change occurs in about 2 hours time, it is labeled as weakly or doubtful positive.

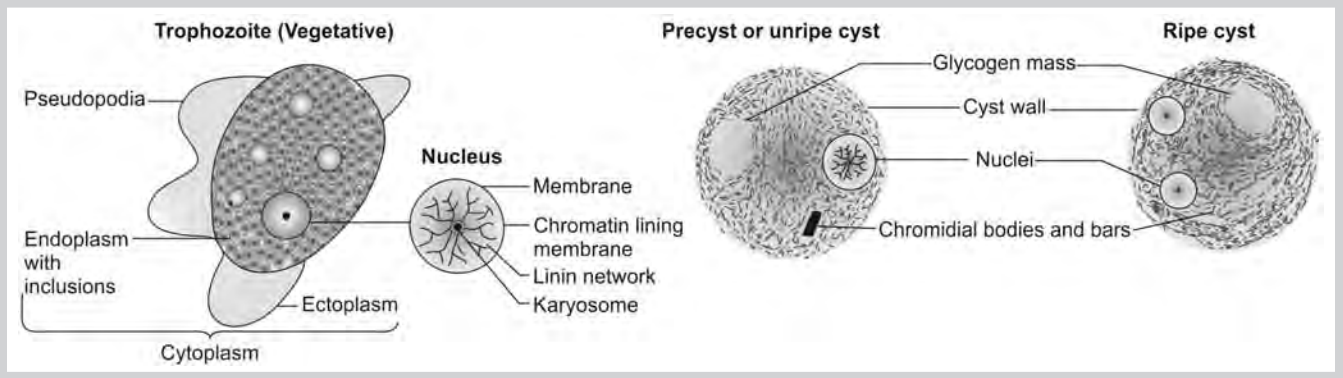
Chopra's antimony test: Dilute patient's serum 10 times with normal saline. To 1 mL of diluted serum, add 2 drops of 4% urea stibamine solution. Immediate appearance of flocculum and turbidity indicates a positive test.

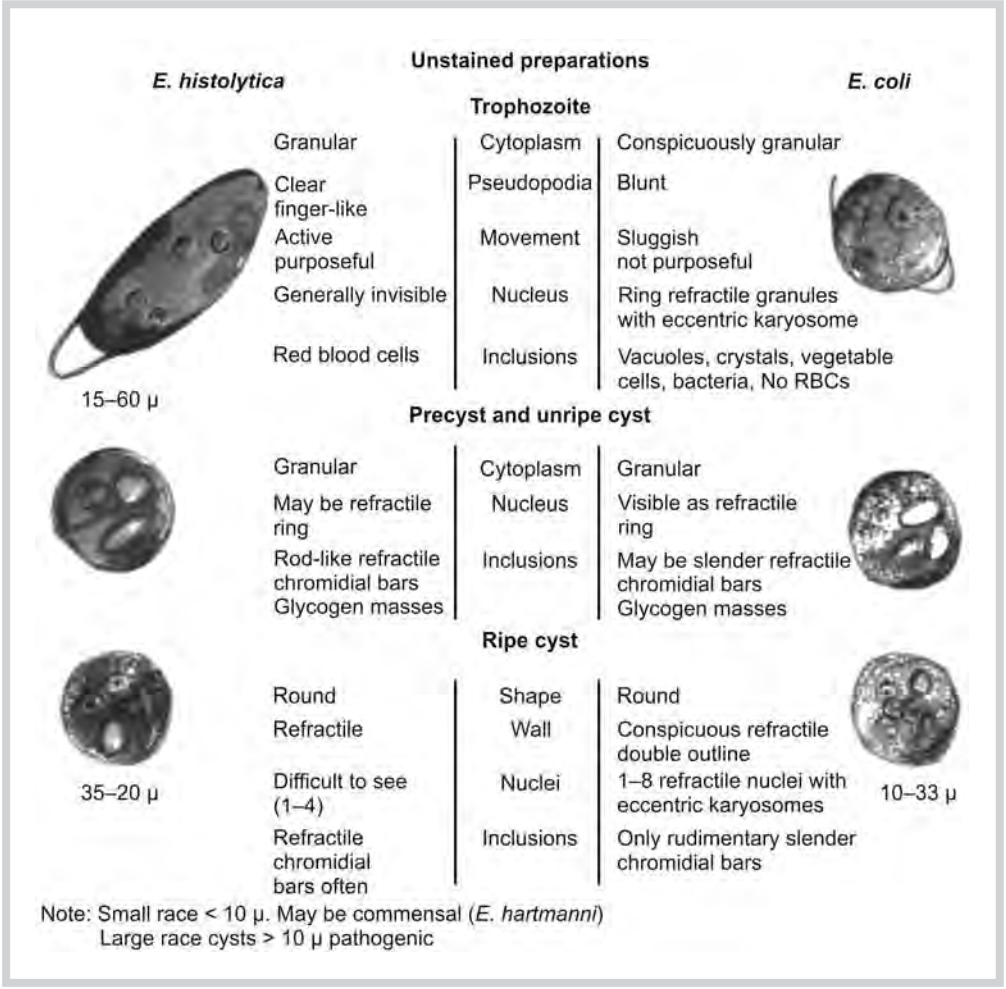
For exact determination of individual classes of gamma globulins, immunoturbidometric or nephelometric techniques may be used. However, for screening purposes and normal routine clinical testing, the given above tests are quite satisfactory.

ELISA techniques are ideal.

Entamoeba histolytica

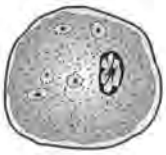
Morphology



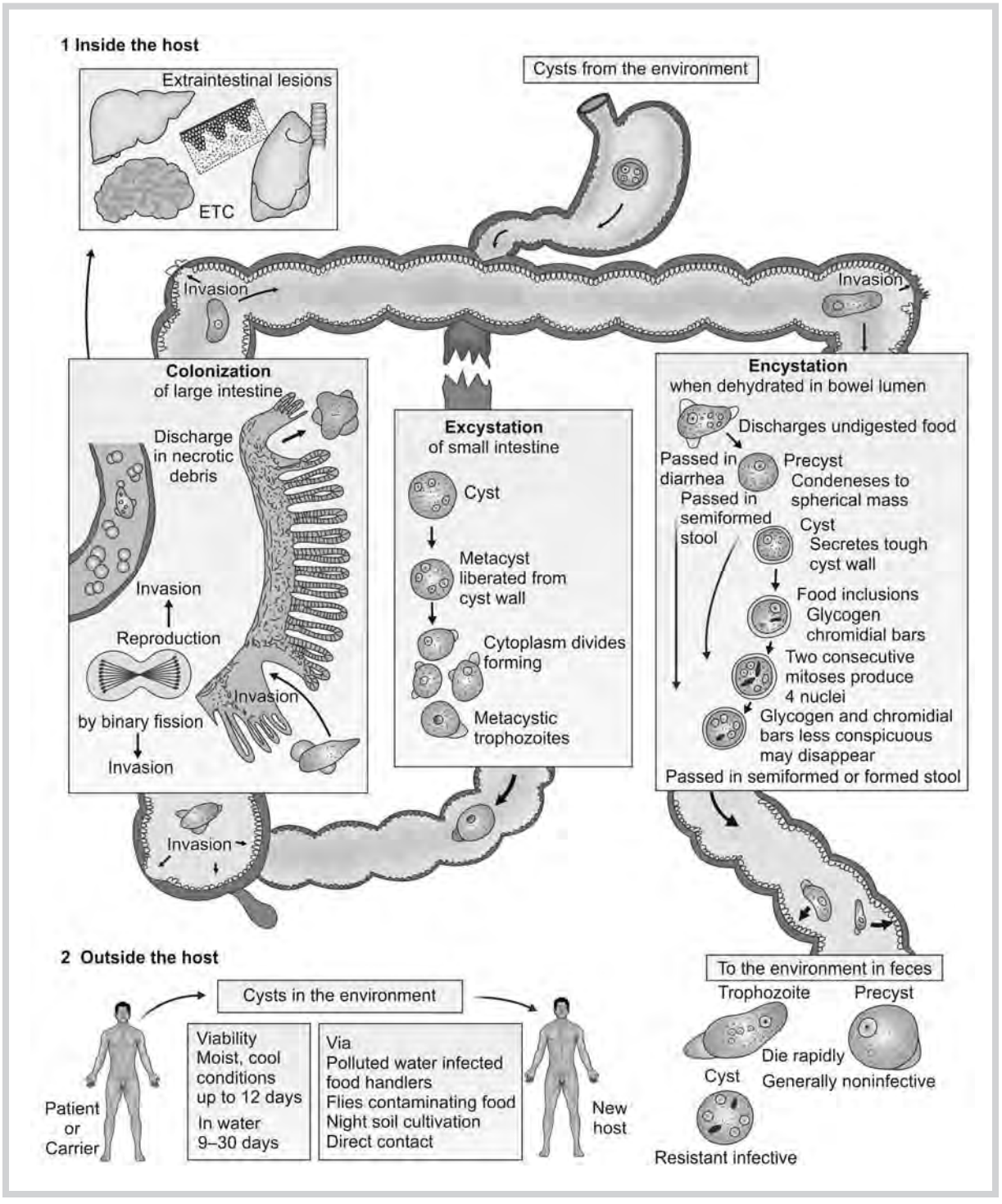


Contd...

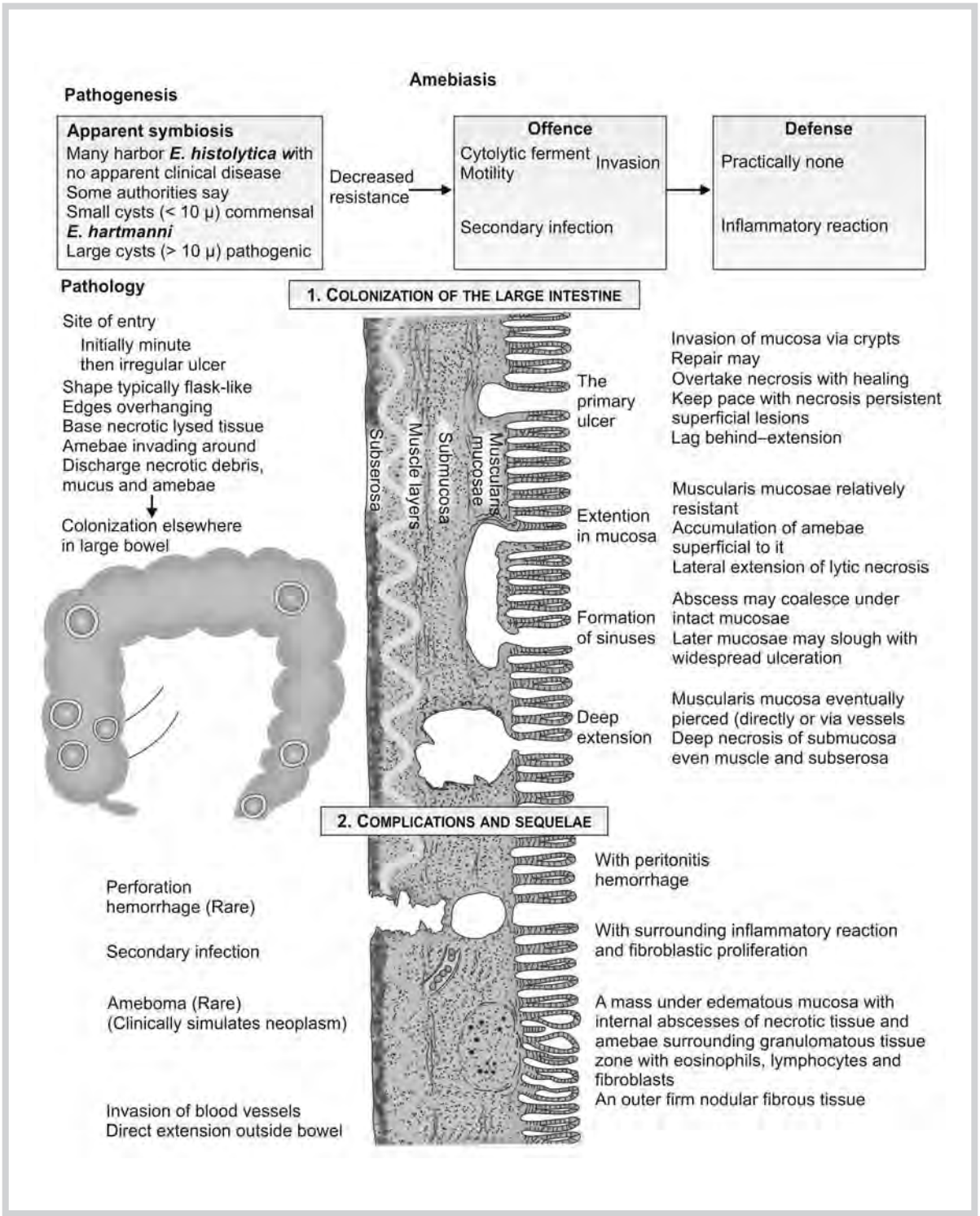
Contd...

E. histolytica		**Iodine preparations**		*E. coli*
		Trophozoite		
	Finely granular yellow green	Cytoplasm	Conspicuous granularity	
	Red cells (yellow)	Inclusions	Bacteria, etc. No RBC	
	Yellow ring with center yellow dot (karyosome)	Nucleus	Nuclear membrane with eccentric karyosome easily recognized	
		Precyst		
	Brown, diffuse	Glycogen	Brown, compact	
	As above	Cytoplasm Nucleus	As above	
		Stained by iron hematoxylin		
		Trophozoite		
	Purplish brown Faintly granular	Cytoplasm	Grayish blue coarsely granular	
	RBC black	Inclusions	Vacuoles black, as are bacteria, etc.	
	Lined with minute granules	Nucleus membrane	Thick plaques of black chromatin	
	Small black central dot	Karyosome	Eccentric black dot or plaque	
	Trace only seen	Linin network	More conspicuous may have chromatin plaques	
		Precyst		
	Round	Shape	Round	
	As trophozoite	Cytoplasm nucleus	As trophozoite	
	Black chromidial bodies or bars Glycogen (dissolved) replaced by vacuoles	Inclusions	May have slender black chromidial bars Glycogen (dissolved) replaced by vacuoles	
		Cyst		
	Gray-blue	Cytoplasm	Grayish blue, granular	
	As precyst, less conspicuous or absent	Inclusions	As precyst, less conspicuous or absent In 2 nuclei stage glycogen vacuoles may be dumb bell shaped	
	Unstained, hyaline	Wall	Unstained, hyaline	
	As trophozoite 1–4	Nuclei	As trophozoite 1–8	

Entamoeba histolytica *(causing amebiosis) life cycle*



Pathogenesis



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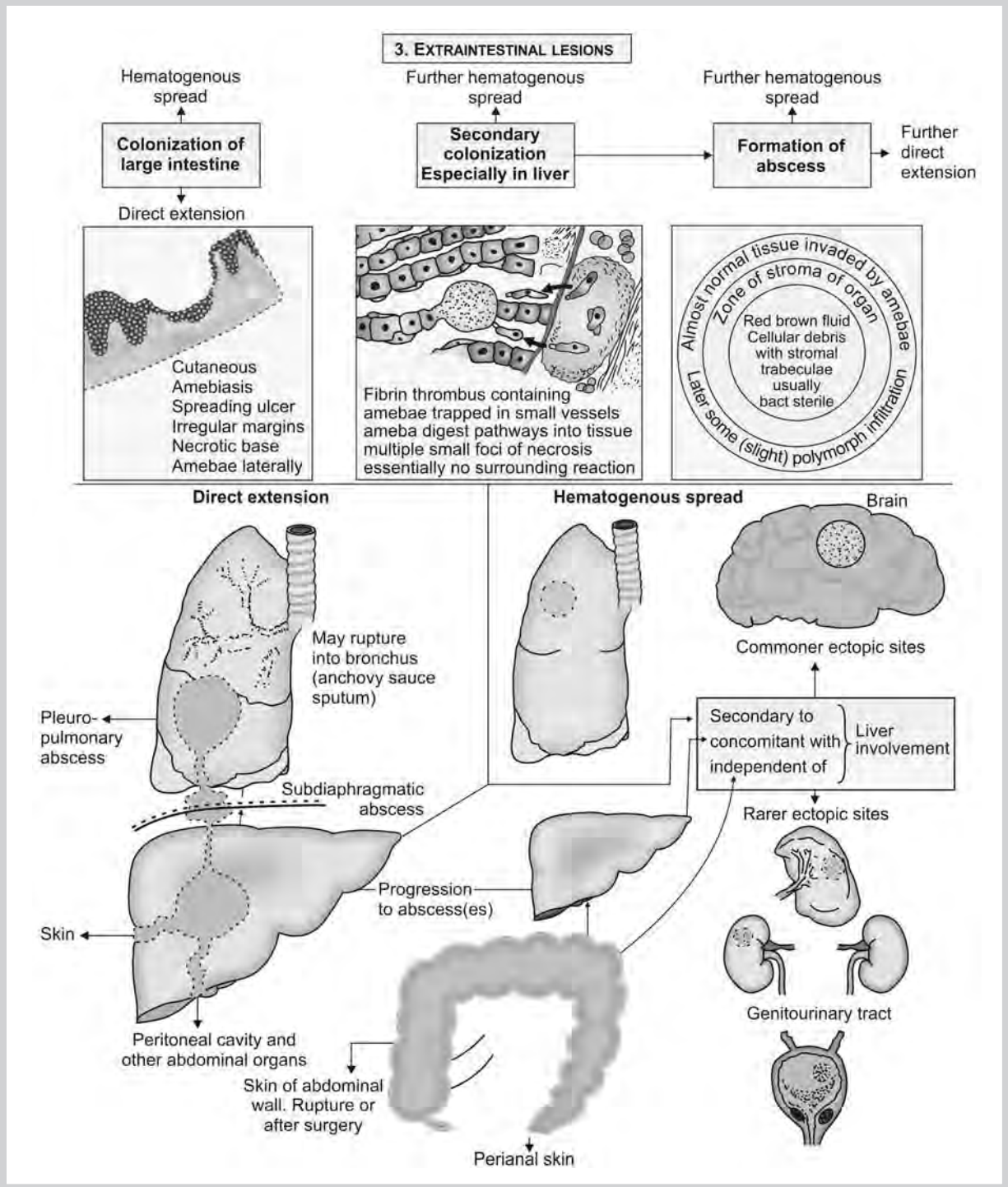
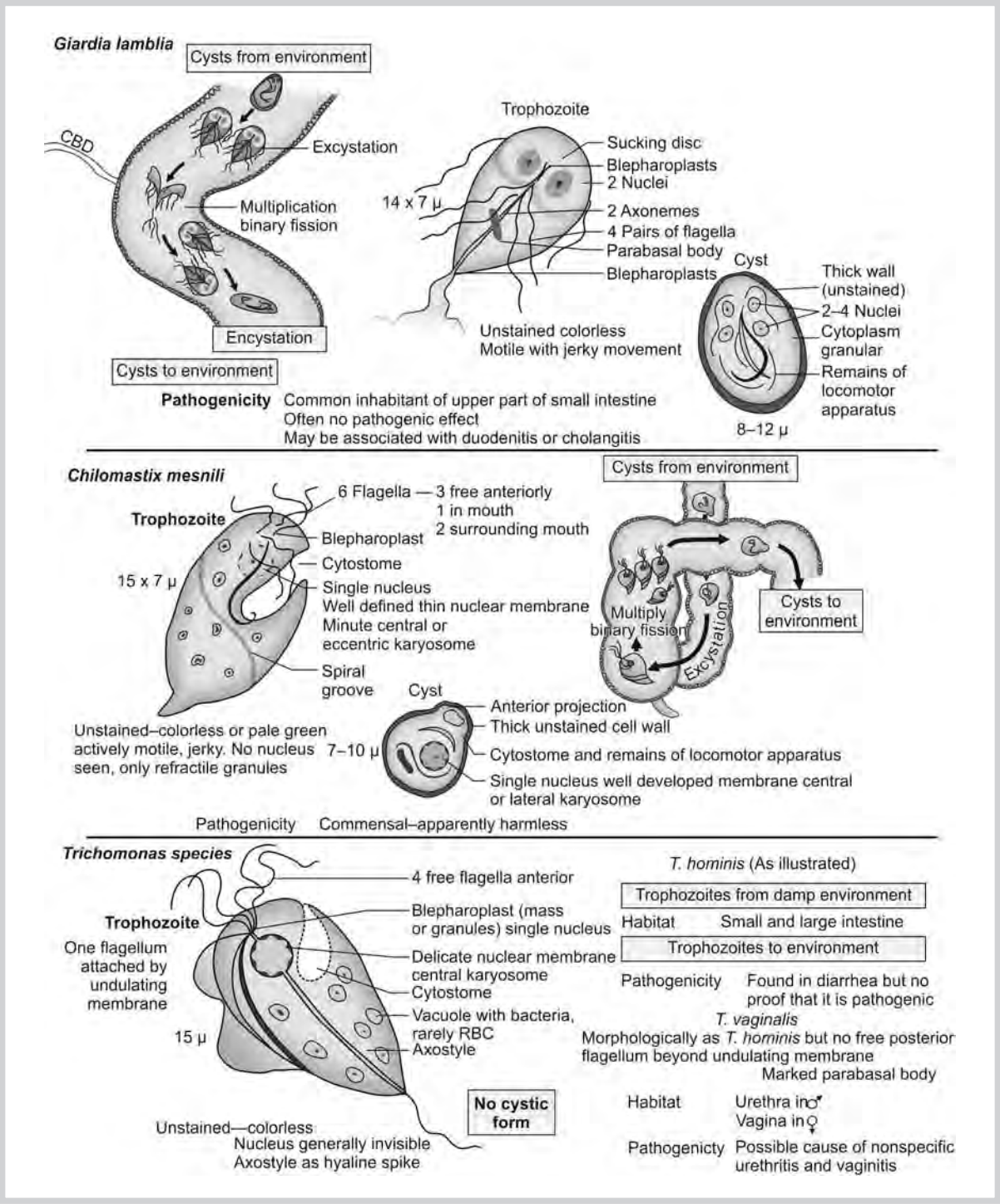
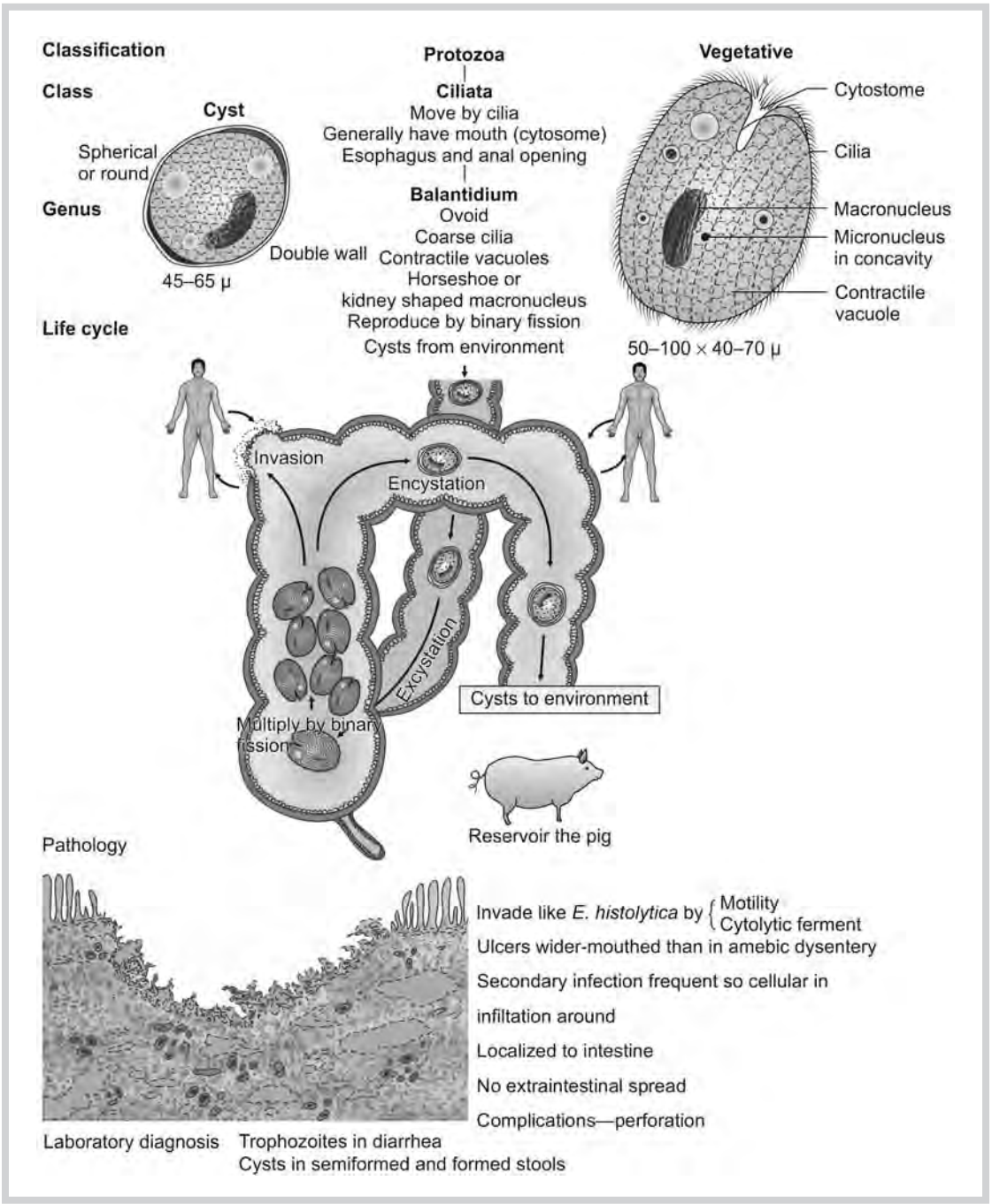

3. EXTRAINTESTINAL LESIONS
Hematogenous spread
Colonization of large intestine
Direct extension
Further hematogenous spread
Secondary colonization Especially in liver
Further hematogenous spread
Formation of abscess
Further direct extension
Cutaneous Amebiasis
Spreading ulcer
Irregular margins
Necrotic base
Amebae laterally
Fibrin thrombus containing amebae trapped in small vessels
ameba digest pathways into tissue
multiple small foci of necrosis
essentially no surrounding reaction
Almost normal tissue invaded by amebae
Zone of stroma of organ
Red brown fluid
Cellular debris
with stromal
trabeculae
usually
bact sterile
Later some (slight) polymorph infiltration
Direct extension
Hematogenous spread
Brain
Commoner ectopic sites
May rupture into bronchus (anchovy sauce sputum)
Pleuro-pulmonary abscess
Subdiaphragmatic abscess
Secondary to
concomitant with
independent of
Liver involvement
Rarer ectopic sites
Progression to abscess(es)
Skin
Genitourinary tract
Peritoneal cavity and other abdominal organs
Skin of abdominal wall. Rupture or after surgery
Perianal skin

The Intestinal Flagellates

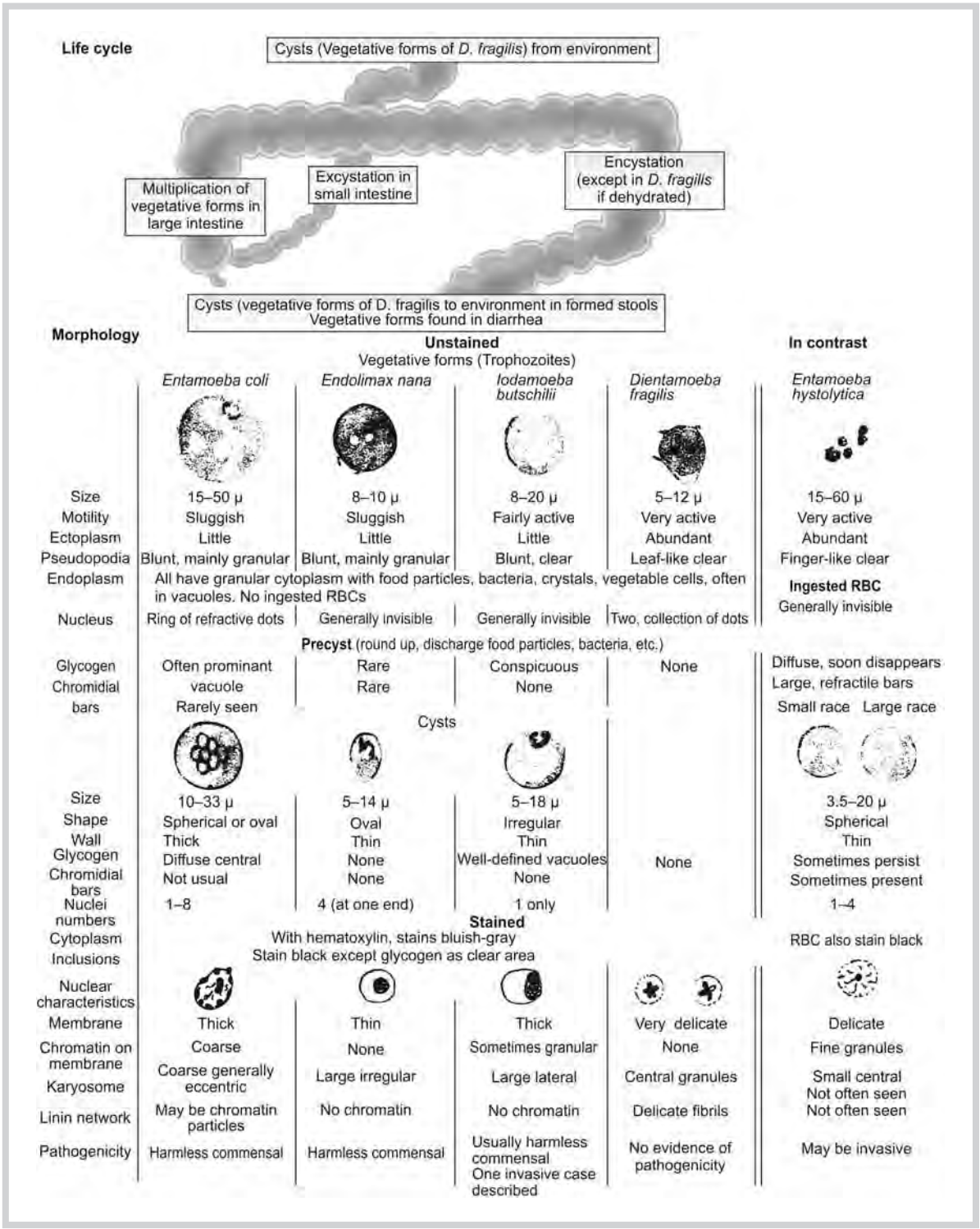


Intestinal Cilliate

Balantidium coli (Causing Balantidiasis)



The Nonpathogenic Intestinal Amebae



MALARIAL PARASITES OF MAN (TABLE 8.2)

TABLE 8.2: Morphology of different erythrocytic forms of plasmodia of man

	P. vivax	*P. falciparum*	*P. malariae*	*P. ovale*
Trophozoite				
Size	Relatively large, 2.5 μ	Small; < 1.5 μ	Relatively large, 2.5 μ	Relatively large, 2.5 μ
Shape	Round or oval, delicate ring	Round or oval, very delicate ring	Round or oval, compact ring	Round or oval, dense ring
Chromatin	Prominent dot in thin part of cytoplasm or in vacuole; at times two dots	Fine dot; frequently two or bar shaped	A prominent mass often inside the vacuole of the ring. 'Bird' eye form is common	A dense well-defined mass at the thin segment of the cytoplasm
Accole form	At times thickened	Frequent	None	None
Cytoplasm	Opposite to chromatin	No thickening opposite to chromatin	Thicekned all through, more opposite to chromatin	Thickened opposite to chromatin
Pigment	Nil	Nil	May be present	Nil
Number in an RBC	One	May be more	One	One
Growing form rarely seen in peripheral blood				
Size	Large	Small	Small	Small
Shape	Irregular; ameboid with fine streaming cytoplasmic pseudopodia	Compact	Compact; cytoplasm more collected together; egg form, equatorial band form, Ribbon, comet form	Compact; may be slightly ameboid
Vacuole	Prominent	Inconspicuous	Disappears early	Inconspicuous
Chromatin	Dots or threads	Dots or threads; chromatin is relatively more compared to cytoplasm	Dots or threads	Large irregular clumps
Pigment color	Yellowish brown	Black or pepper-like	Dark brown	Dark yellowish brown
Microgametocyte (male)				
Size	Large (10 to 12 μ); fills enlarged RBC	Large (8 to 10 μ × 2 to 3 μ); larger than RBC	Smaller than size of a normal RBC	About the size of normal RBC
Shape	Round or oval, compact	Kidney or bean shaped; ends bluntly rounded	Round; compact	Round; compact
Cytoplasm	Light blue	Pinkish blue	Reddish blue (stains badly)	Pale blue
Chromatin	Fibril in skin; large, diffuse; stains poorly; lies across equator, surrounding area unstained	Fine granules; scattered through 1/3 of the body of the parasite lie amongst pigment granules, stains lightly in the central part	Fibril in skin; medium, diffuse; arranged in zone like bands, surrounding area unstained	As in *P. vivax*
Pigment	(i) Fine granular (ii) Light brown to yellow brown (iii) Scattered throughout cytoplasm	(i) Fine granular (ii) Blackish (ii) Scattered throughout.	(i) Coarse granular (ii) Dark brown (iii) Scattered and also aggregated in chunks and masses	
Macrogametocyte (female)				
Size	Large (12 to 14 μ; larger than male); fills enlarged RBC	Larger (10 to 12 μ × 2 to 3 μ) larger than male; larger than RBC	Smaller than size of RBC	Size of RBC
Shape	Round or oval	Crescent shaped; ends sharply rounded or pointed	Round; compact	Round; compact

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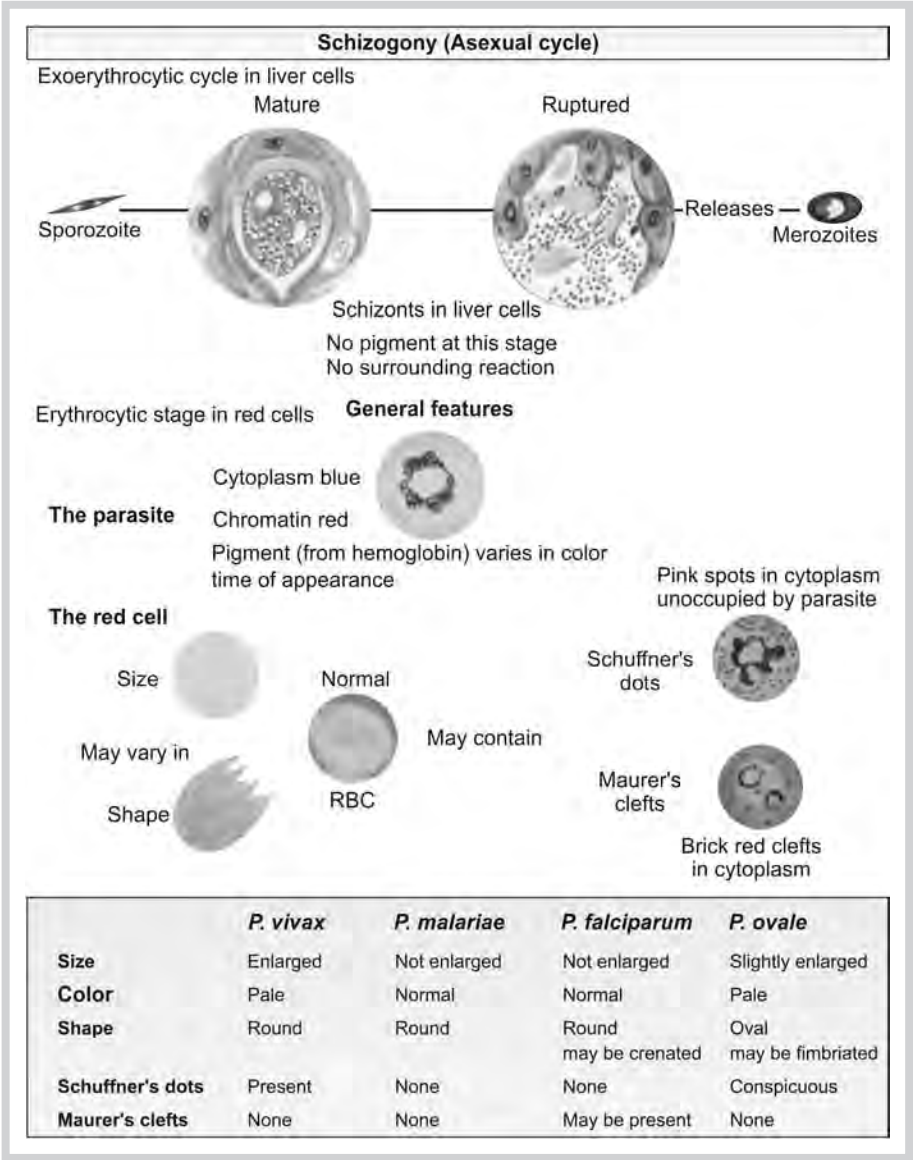
	P. vivax	*P. falciparum*	*P. malariae*	*P. ovale*
Cytoplasm	Deep blue	Deep blue	Deep blue	Deep blue
Chromatin	Condensed into compact mass; eccentric in position often surrounded by a halo	Condensed into a small deep staining compact mass; in position lies in the midst of pigment (no halo)	As in *P. vivax*	As in *P. vivax*
Pigment	(i) Yellow brown (ii) Aggregated in small masses (iii) Arranged at periphery or wreath-like	(i) Black (ii) Granular or small clumps (iii) Arranged round the chromatin masses	(i) Dark brown (ii) and as in *P. vivax*; abundant pigment—pigmented parasite	(i) Dark yellow brown; (ii) and as in *P. vivax*

Morphology of Malarial Parasites Stained by Leishman of Giemsa



	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
Size	Enlarged	Not enlarged	Not enlarged	Slightly enlarged
Color	Pale	Normal	Normal	Pale
Shape	Round	Round	Round may be crenated	Oval may be fimbriated
Schuffner's dots	Present	None	None	Conspicuous
Maurer's clefts	None	None	May be present	None

Morphology of Malarial Parasites Stained in Thin Films

Morphology of Malarial Parasites Stage in Thin Films

	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
RING FORMS (EARLY TROPHOZOITES)				
				
Size	Up to 1/3 red cell	Up to 1/3	Up to 1/5	Up to 1/3 red cell
Shape	Delicate ring	Compact ring	Very delicate ring	Delicate ring
				
Chromatin	Fine dot sometimes two	One mass often inside ring	Fine dots frequently two	Dense, well defined mass
Accole forms	Sometimes	None	Frequent	None
Pigment	None at this stage	May be present	None at this stage	None at this stage
DEVELOPING TROPHOZOITES				
				
			Rarely seen in (peripheral blood)	
Size	Large	Small	Small	Small
Shape	Very irregular	Compact, often band forms	Compact	Compact
Vacuole	Prominent	Inconspicuous	Inconspicuous	Inconspicuous
Chromatin	Dots or threads	Dots or threads	Dots or threads	Large irregular clumps
Pigment texture	Fine	Coarse	Coarse	Coarse
Color	Yellow brown	Dark brown	Black	Dark yellow brown
Quantity	Medium	Abundant	Medium	Medium
Distribution	Scattered fine particles	Scattered clumps and rods	Aggregated in two clumps	Scattered coarse particles

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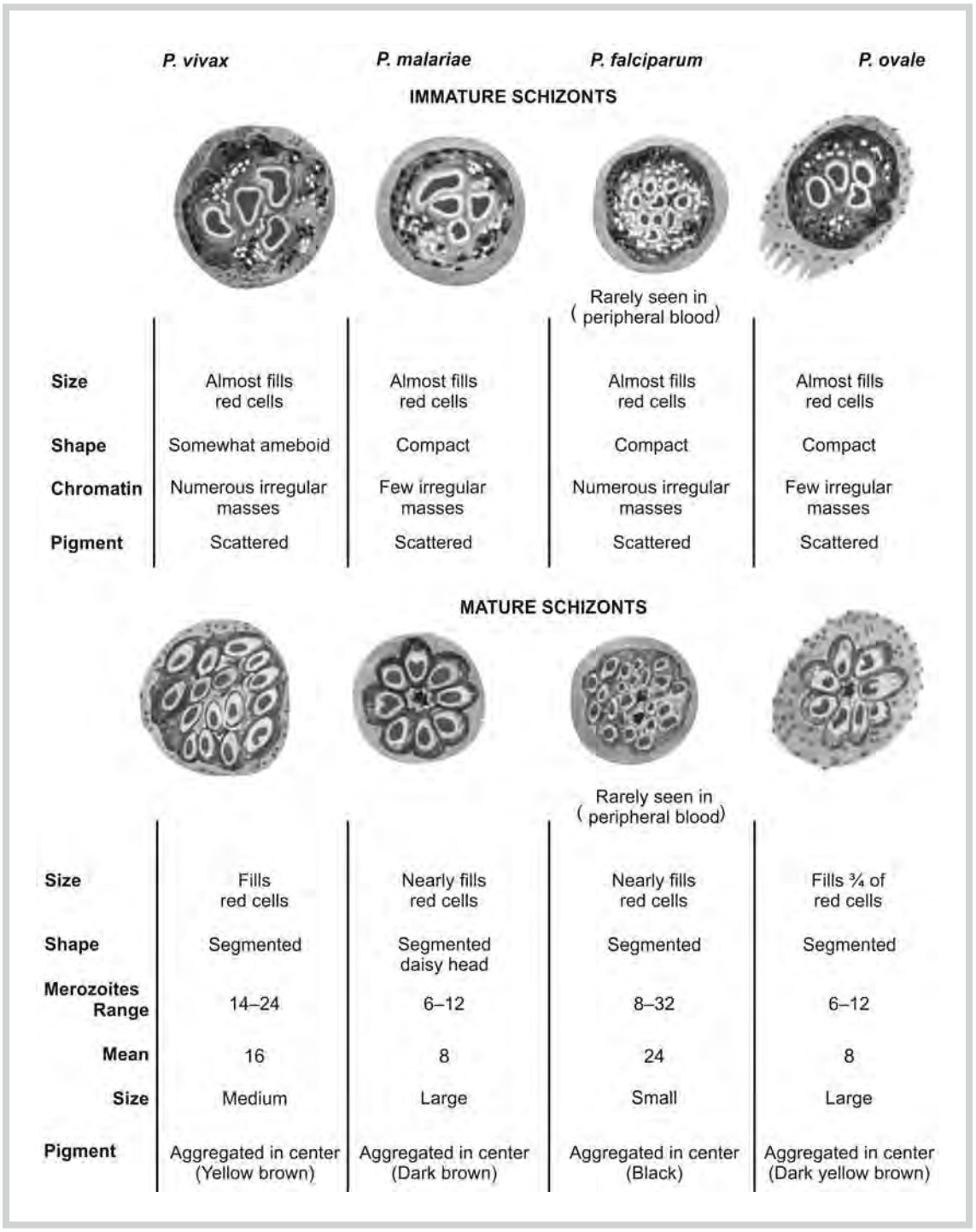

	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
IMMATURE SCHIZONTS				
			Rarely seen in (peripheral blood)	
Size	Almost fills red cells	Almost fills red cells	Almost fills red cells	Almost fills red cells
Shape	Somewhat ameboid	Compact	Compact	Compact
Chromatin	Numerous irregular masses	Few irregular masses	Numerous irregular masses	Few irregular masses
Pigment	Scattered	Scattered	Scattered	Scattered
MATURE SCHIZONTS				
			Rarely seen in (peripheral blood)	
Size	Fills red cells	Nearly fills red cells	Nearly fills red cells	Fills ¾ of red cells
Shape	Segmented	Segmented daisy head	Segmented	Segmented
Merozoites Range	14–24	6–12	8–32	6–12
Mean	16	8	24	8
Size	Medium	Large	Small	Large
Pigment	Aggregated in center (Yellow brown)	Aggregated in center (Dark brown)	Aggregated in center (Black)	Aggregated in center (Dark yellow brown)

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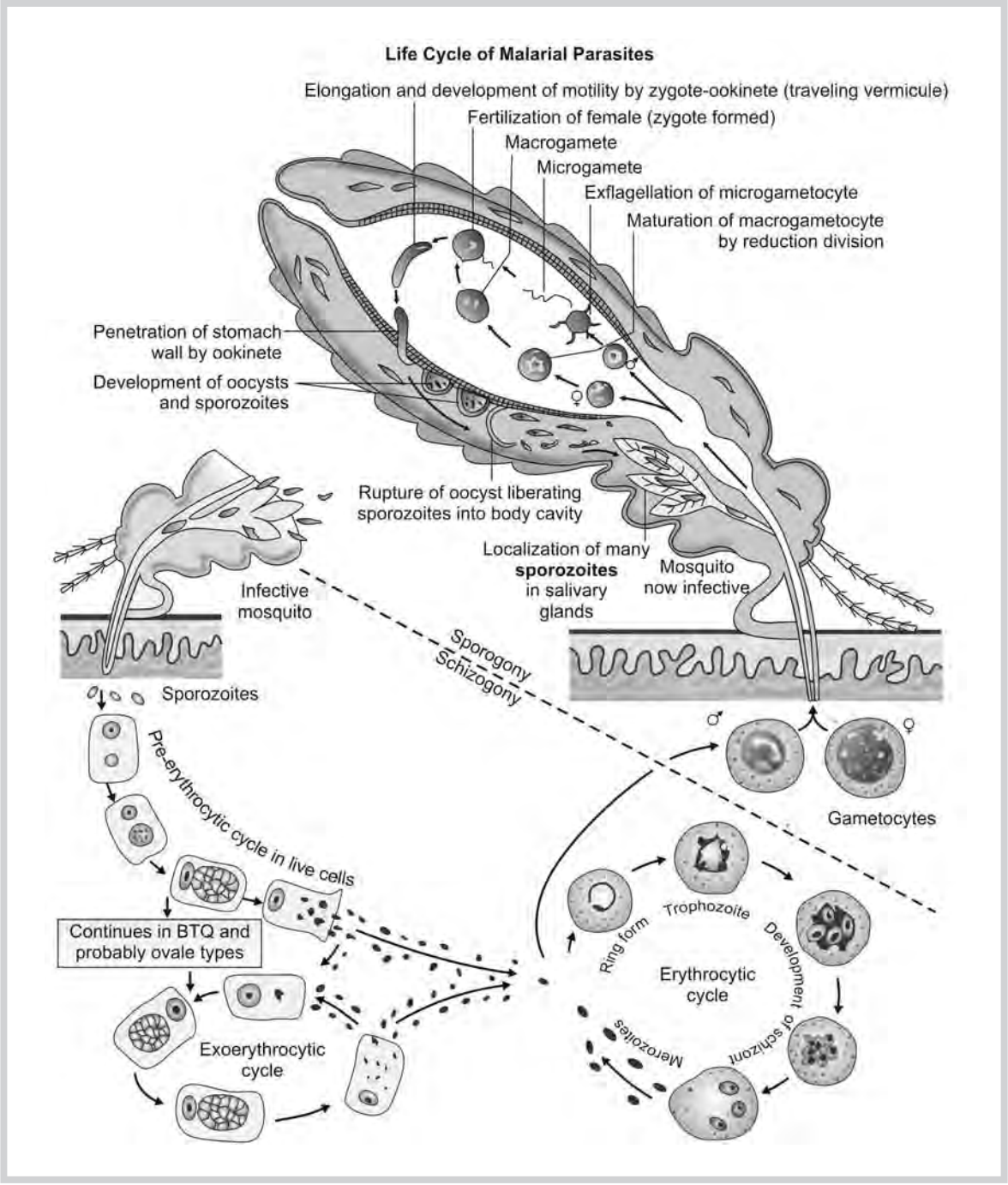
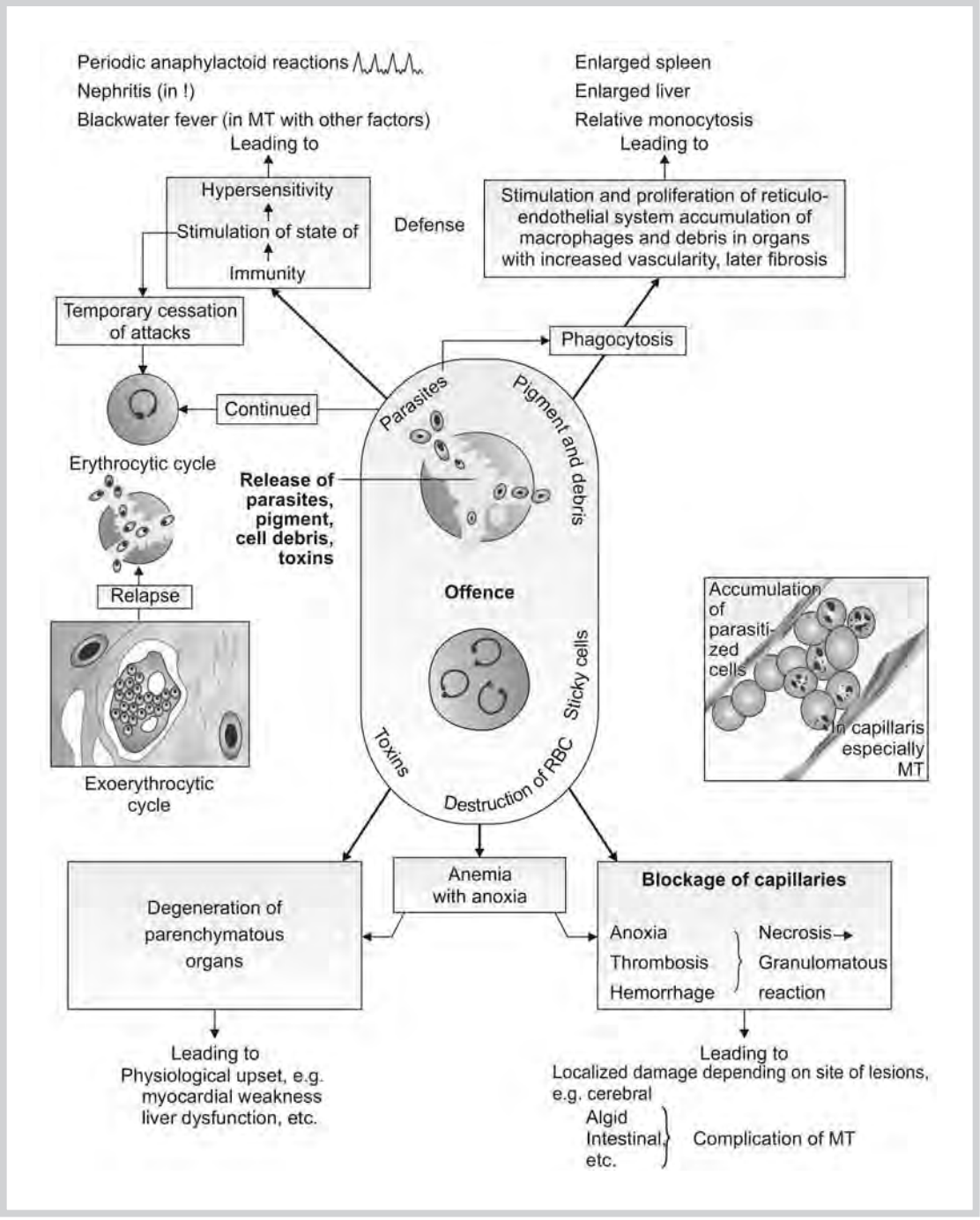
	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
MICROGAMETOCYTES				
				
Time of appearance	3–5 days	7–14 days	7–12 days	12–14 days
Number in bloodstream	Many	Scanty	Many	Scanty
Size	Fills enlarged red cell	Smaller than red cell	Larger than red cell	Size of red cell
Shape	Round or oval compact	Round compact	Kidney shaped bluntly round ends	Round compact
Cytoplasm	Pale blue	Pale blue	Reddish blue	Pale blue
Chromatin	Fibrils in skin with surrounding unstained area	As for ***P. vivax***	Fine granules scattered throughout	As for ***P. vivax***
Pigment	Abundant brown granules throughout	As for ***P. vivax***	Dark granules throughout	As for ***P. vivax***
MACROGAMETOCYTES				
				
Time of appearance	3–5 days	7–14 days	7–14 days	12–14 days
Number in bloodstream	Many	Scanty	Many	Scanty
Size	Fills enlarged red cell	Smaller than red cell	Larger than red cell	Size of red cell
Shape	Round or oval compact	Round compact	Crescentic–sharply rounded or pointed ends	Round compact
Cytoplasm	Dark blue	Dark blue	Dark blue	Dark blue
Chromatin	Compact peripheral mass	As for ***P. vivax***	Compact masses near center	As for ***P. vivax***
Pigment	Small masses round periphery	As for ***P. vivax***	Black granules round–nucleus	As for ***P. vivax***

Life Cycle of Malarial Parasites

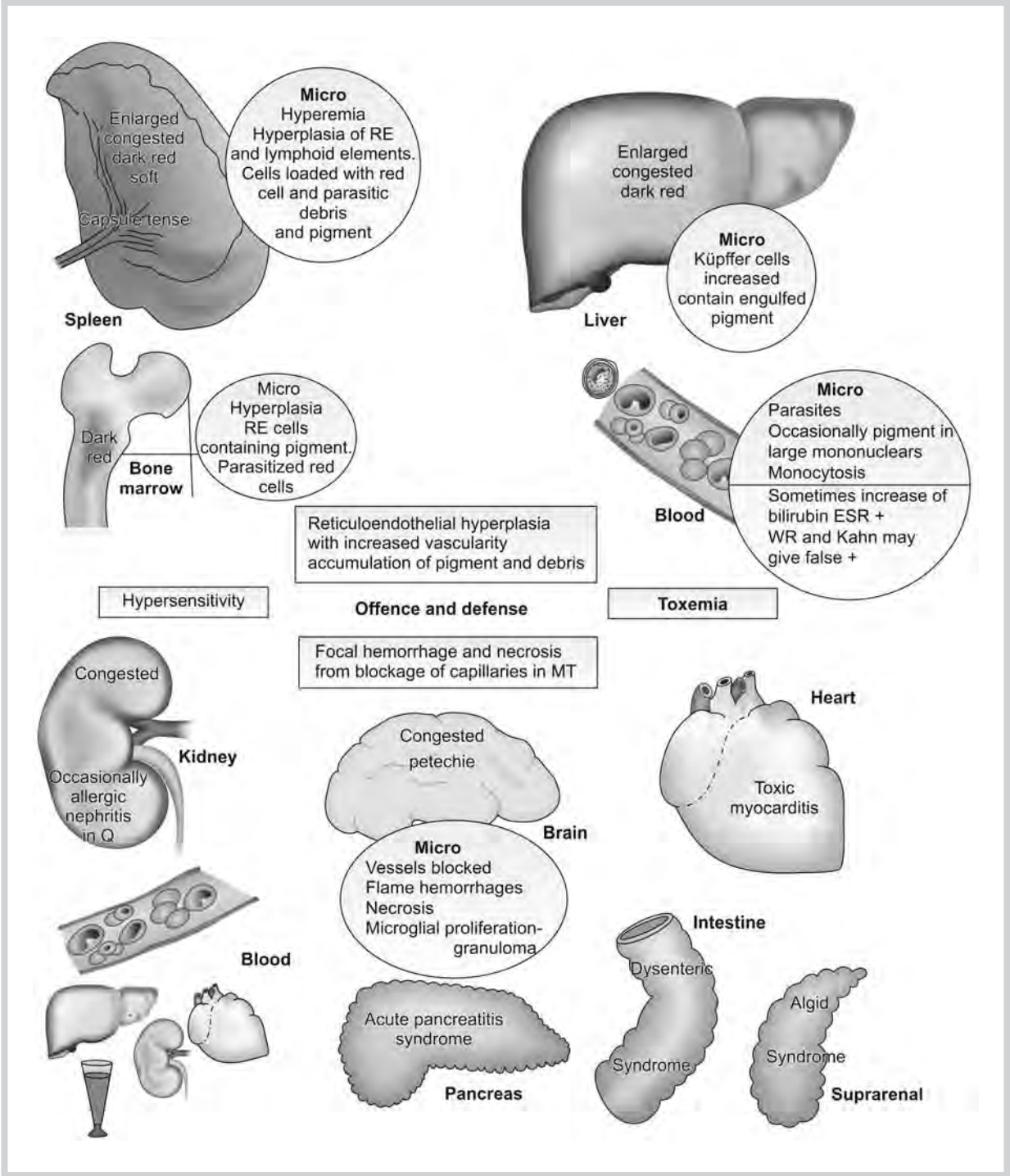


The Pathogenesis of Malaria



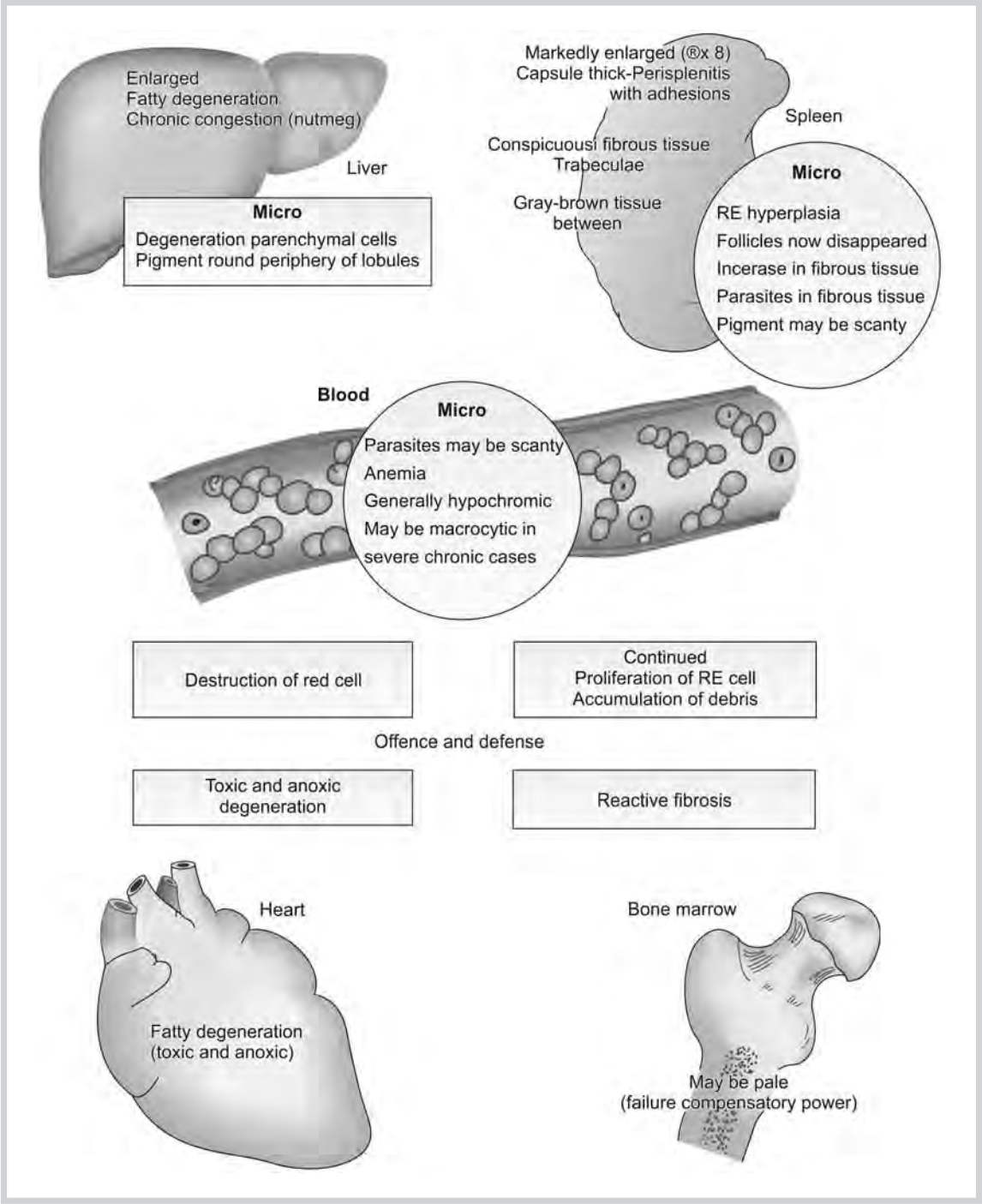
Pathogenesis of Malaria

1. Acute Phase



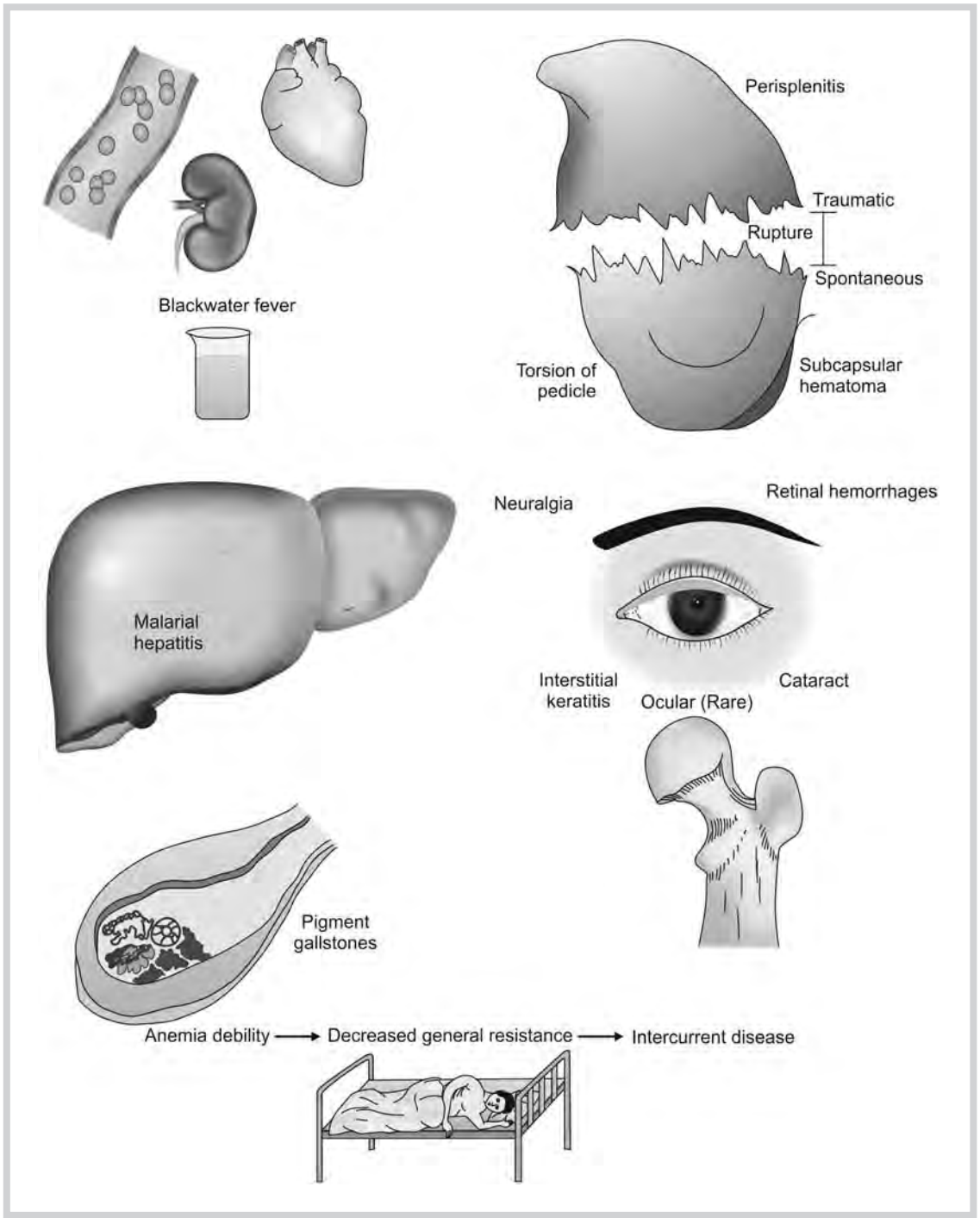
Pathogenesis of Malaria

2. Chronic Phase



Pathogenesis of Malaria

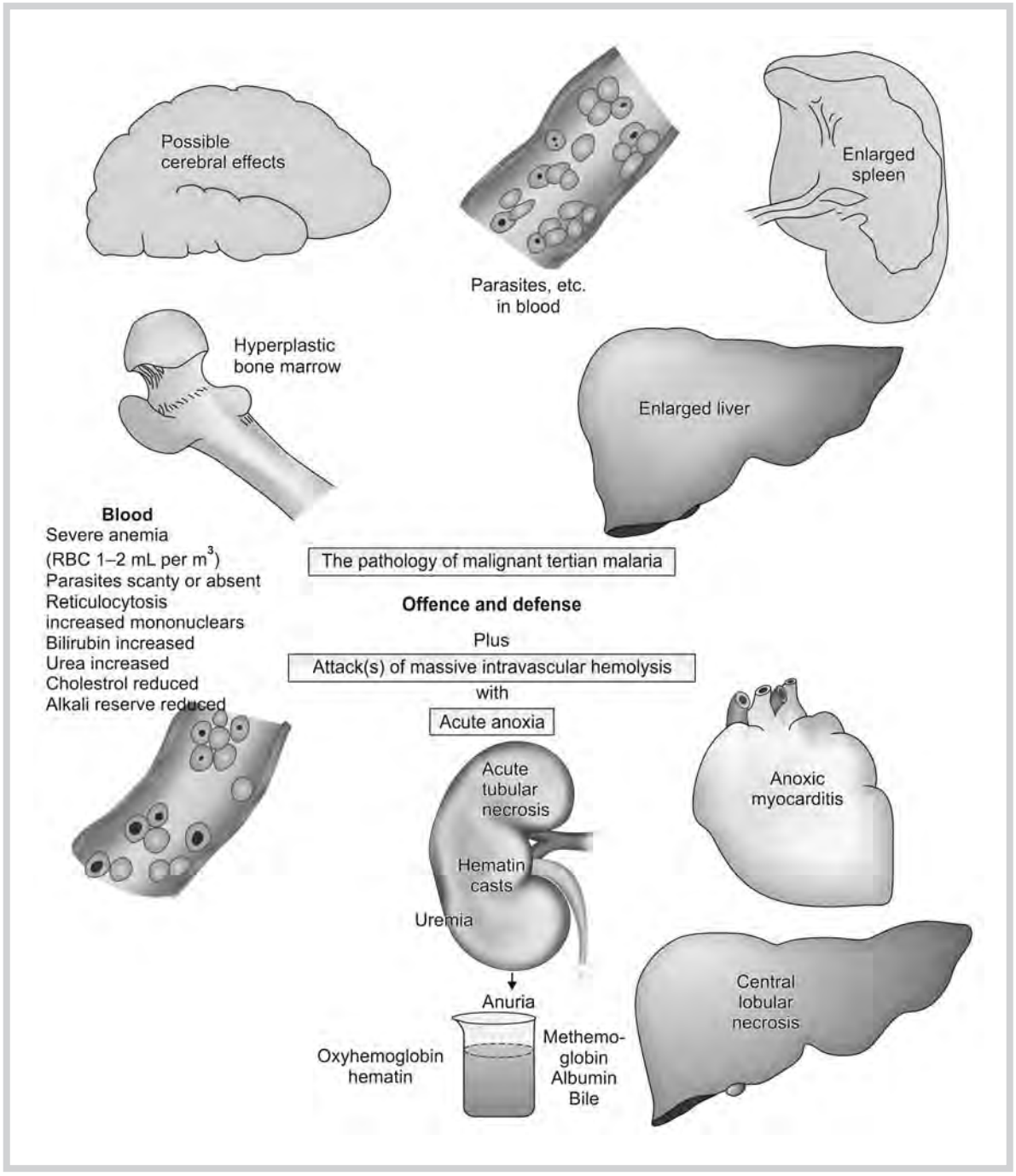
3. Complications and Sequelae



Pathogenesis of Malaria

4. Blackwater Fever

Acuter hemolytic attacks in MT malarias; associated with taking of quinine; numerous theories as to mechanism



BLOOD FLAGELLATES OF MAN (TABLES 8.3 AND 8.4)

TABLE 8.3: Blood flagellates of man

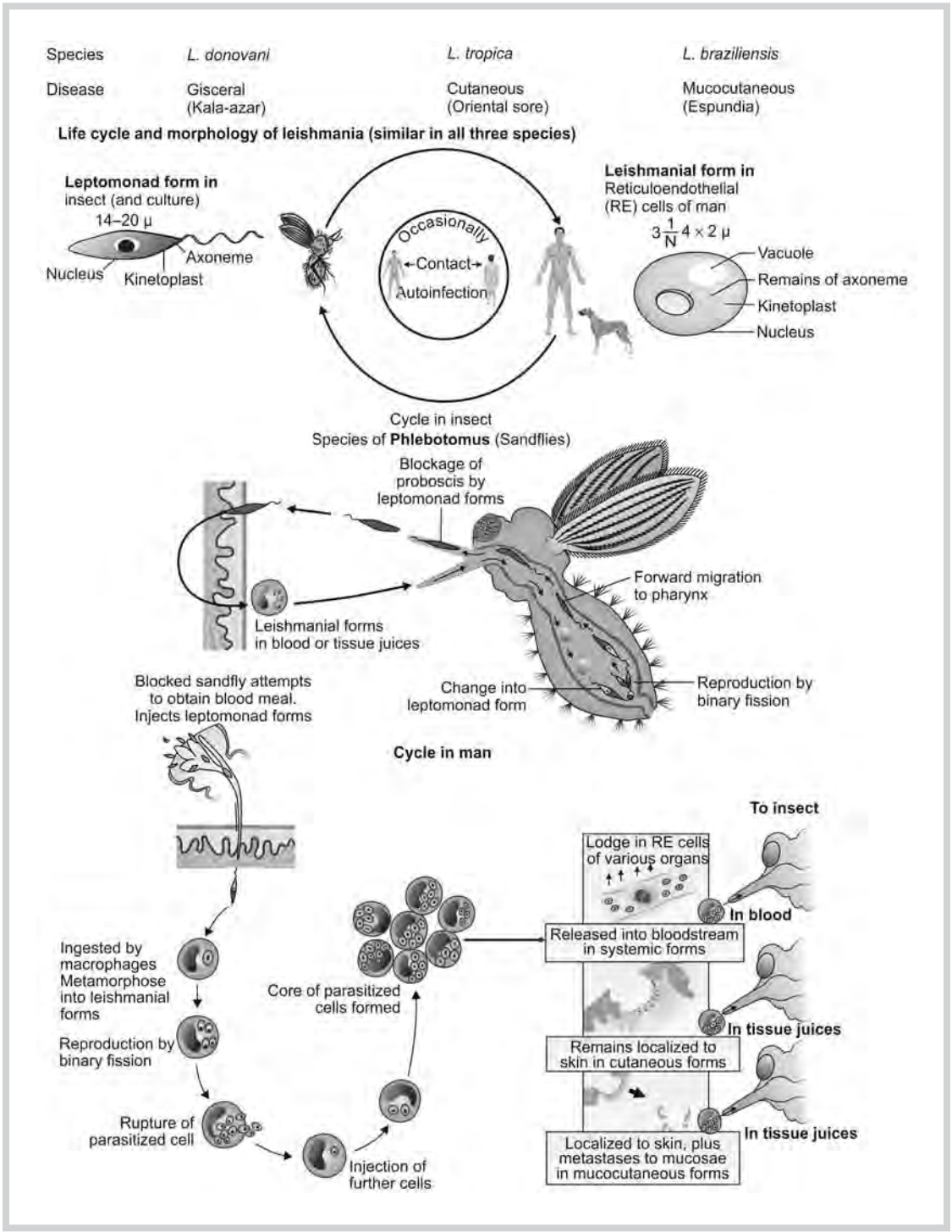
Prevalence depends upon presence of vertebrate reservoirs and proper insect hosts and varies with human habitation, habits, and agricultural practices						
Parasite and distribution	*Fly host*	*Enters man*	*Life cycle in man*	*Exit*	*Cycle in fly*	*Reservoir host**
Leishmania donovani Asia, Africa, tropical South America, Middle East, Mediterranean basin	*Phlebotomus* or *Lutzomyia* (Sandfly)	Plug of promastigotes (leptomonad) injected with bite of fly	Become amastigotes (leishmania or LD bodies) in RES macrophages	Sucked into fly with blood of host	Become promastigotes (leptomonads) in the intestine of fly	Man, dogs, foxes or other carnivores, wild rodents
Leishmania tropica Asia, South and Central America, Middle East, Europe	*Phlebotomus* or *Lutzomyia* (Sandfly)	-do-	Become amastigotes in endothelial cells of skin	-do-	-do-	Man, various wild rodents, possibly dogs
Leishmania braziliensis Central and South America	*Lutzomyia* (Sandfly)	-do-	Become amastigotes in endothelial cells of skin and secondarily in mucous membranes of nasopharynx	-do-	-do-	Man, various wild rodents, possibly dogs
Trypanosoma gambiense Central and West Africa	*Glossina palpalis* (Tsetse fly)	Fly bites; metacyclic trypomastigotes (trypanosomes) injected with saliva	Trypomastigotes in lymph and blood; later in spinal fluid	-do-	Become epimastigotes (crithidia) and then metacyclic trypomastigotes in the intestine and salivary glands of fly	Man, domestic animals
Trypanosoma rhodesiense Central and East Africa	*Glossina morsitans* (Tsetse fly)	-do-	-do-	-do-	-do-	Man, wild game animals (antelopes)
Trypanosoma cruzi Central and South America	*Panstrongylus megistus* (kissing bug) and other reduvid bugs	Metacyclic trypomastigotes in feces scratched into skin or rubbed into mucous membrane of eye	Become amastigotes in tissue cell (particularly cardiac muscle) and trypomastigotes in bloodstream	Sucked into bug with blood of host	Become epimastigotes and then metacyclic trypomastigotes in the hind intestine of bug	Man, dogs, cats, foxes, armadillo, opossum, rodents

*Varies in each area, in India and Africa, the dog apparently is not involved in *L. donovani* transmission. RES = Reticuloendothelial system.

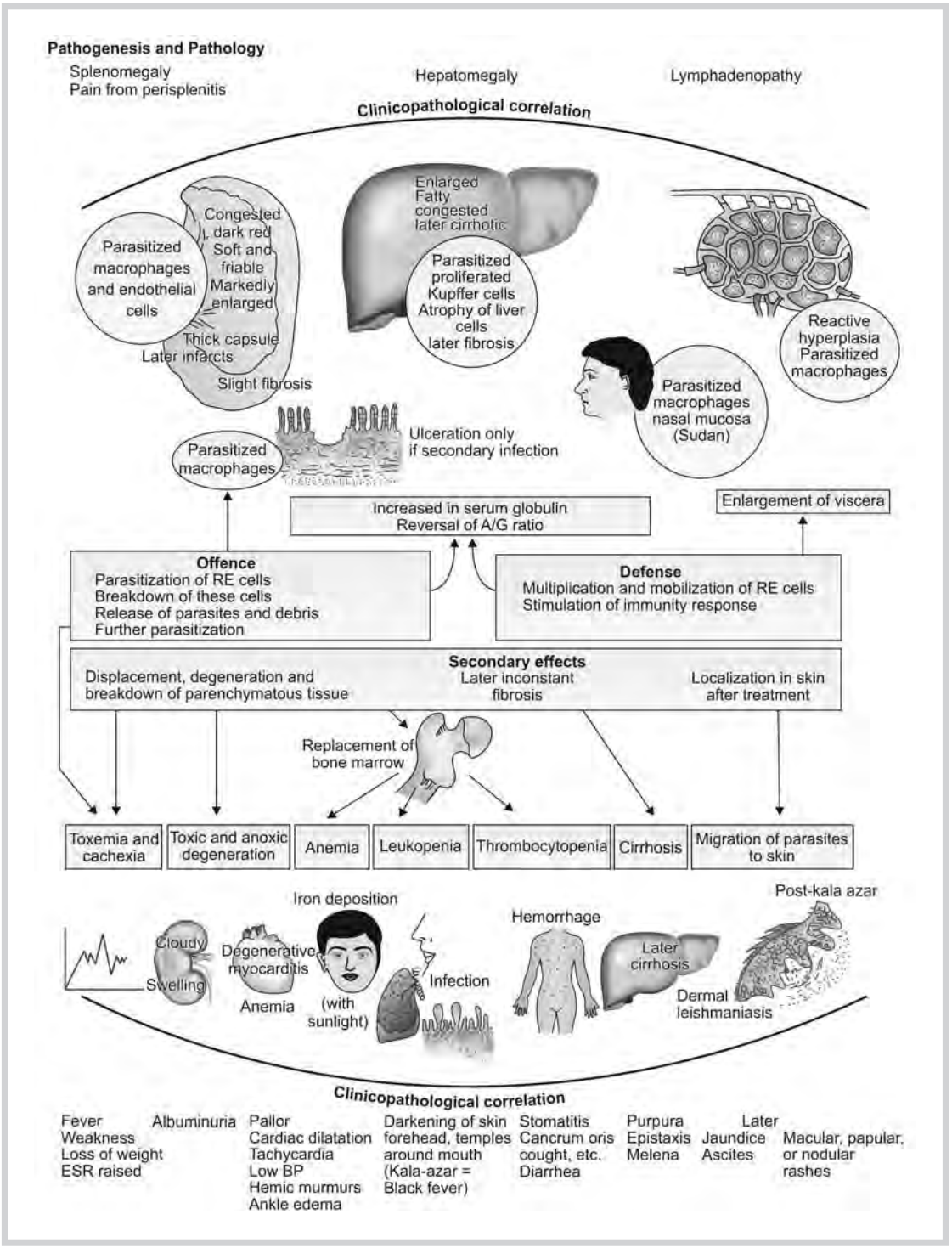
TABLE 8.4: Blood flagellate diseases of man

Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Kala-azar (visceral leishmaniasis) *L. donovani*	Chronic febrile disease, causing hyperplasia and then blockage of the reticuloendothelial system, particularly spleen and liver. Irregular fever (may spike twice a day) with chills, sweating, diarrhea, edema, cachexia, leukopenia, anemia. Splenomegaly and leukopenia characteristic. (Incubation period: 2 to 9 months.) Untreated cases usually end fatally; with proper treatment, fatality is under 5%	Blood: Thick and thin smears, culture in NNN or Tobie's diphasic blood agar. CF test diagnostic; nonspecific tests such as Napier and Chopra serum tests for screening; skin tests for past infection. Nasal scrappings, lymph node biopsy, sternal marrow and splenic or hepatic aspirate. Stained smears, culture, inoculation of hamsters
Oriental sore (cutaneous leishmaniasis) *L. tropica*	Endothelial cells and lymphoid tissue of skin parasitised. Itching red papule → scaling → crusted ulcer → ulcer enlargement → healing. May be multiple [Incubation period: several days to months, depending on strain, (1) dry (urban), relatively benign, slowly ulcerating form; or (2) moist (rural) acute, rapidly ulcerating zoonotic form]	Ulcer curettings (from margin, not center of ulcer): Stained smears, culture
American leishmaniasis (espundia, forest yaws, uta; mucocutaneous leishmaniasis) *L. braziliensis*	Initial ulcers similar to oriental sore, but this enlarges, producing weeping 'saucer' ulceration. Destructive and deforming secondary lesions occur at mucocutaneous junctions, particularly of nasopharynx. Produces fever, pain, malaise, and anemia (Incubation period: indeterminate). Initial lesion—few days, complications—months to years. Nutrition probably very important in severity	Ulcer curettings (from margin not center, of smear): Stained smears, culture
Sleeping sickness West African (*Trypanosoma gambiense*) East African (*Trypanosoma rhodesiense*)	Local lesion at fly bite followed by fever, adenitis, rash, transitory edemas. May fulminate (*T. rhodesiense*) or go on to meningoencephalitis and meningomyelitis, with mental and physical wasting leading to coma and death (*T. gambiense*). (Incubation period: *T. gambiense*, 1–3 weeks; *T. rhodesiense*, 1–2 weeks.)	Blood: Thick and thin smears, concentration, culture. Spinal fluid sediment smears. Lymph node fluid: Smears and culture
Chagas' disease *T. cruzi*	Acute, usually in children: Febrile illness with generalized adenopathy lasting a few months; placental infection common. Chronic cardiac involvement, particularly of right ventricle, consisting of degeneration of cardiac muscle. Patient seldom lives beyond age 50. Megacolon or megaesophagus a sequel. Anemia. Romana's sign (palpebral edema) most probably an allergy to insect bite (Incubation period: 1 to 2 weeks)	Blood: Thick and thin smears only in initial phases. Culture. Xenodiagnosis. Complement fixation most reliable serologic test
* Direct blood film stain positive except in heavy infections; buffy coat smear or culture better.*		

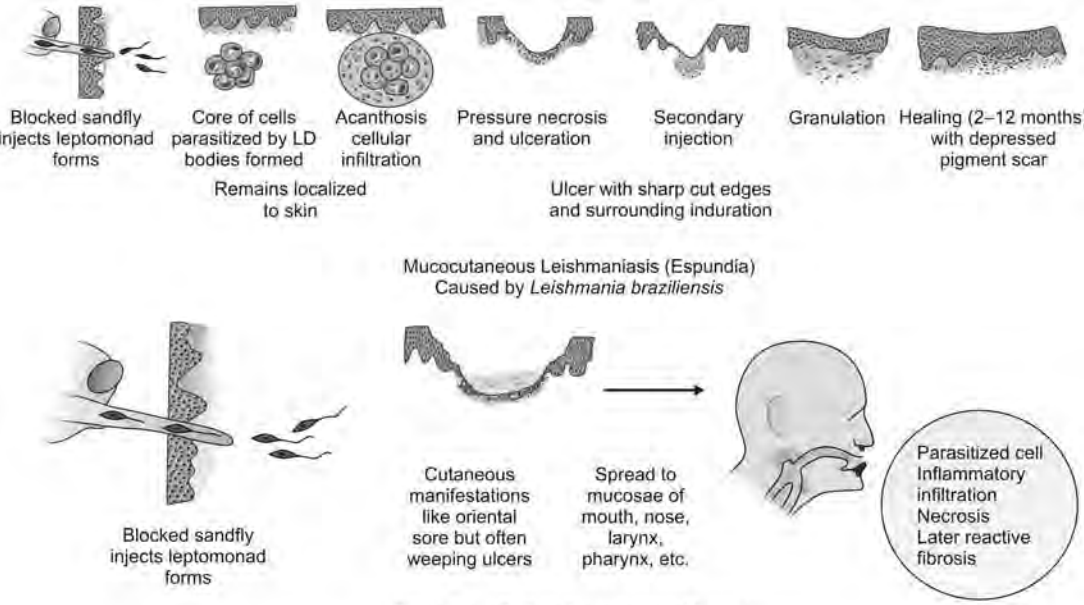
Leishmaniasis



Visceral Leishmaniasis (Kala-Azar) Caused by *Leishmania donovani*



Cutaneous Leishmaniasis (Oriental Sore, Chiclero's Disease, Uta, etc.) *Caused by Leishmania tropica*



Mucocutaneous Leishmaniasis (Espundia)
Caused by *Leishmania braziliensis*

Secondary effects in loose mucosal tissues

Edema and capillary involvement Interference with local blood supply Necrosis–Extensive destruction	Secondary infection Deep erosion locally Spread of infection to lungs or elsewhere	Healing with fibrosis

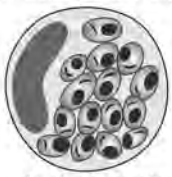
Leading to

Extensive disfiguring lesions | General constitutional upset (fever, pain, anemia) | Bronchopneumonia and septicemia

Diagnosis of leishmaniasis

Visceral

1. Demonstration of parasite by stained film — In — Blood, Bone marrow, Lymph node juice, Nasal scrapings (Sudan), Spleen puncture, Liver puncture (not free from danger)
   Culture (NNN)
   Animal inoculation (Hamsters)

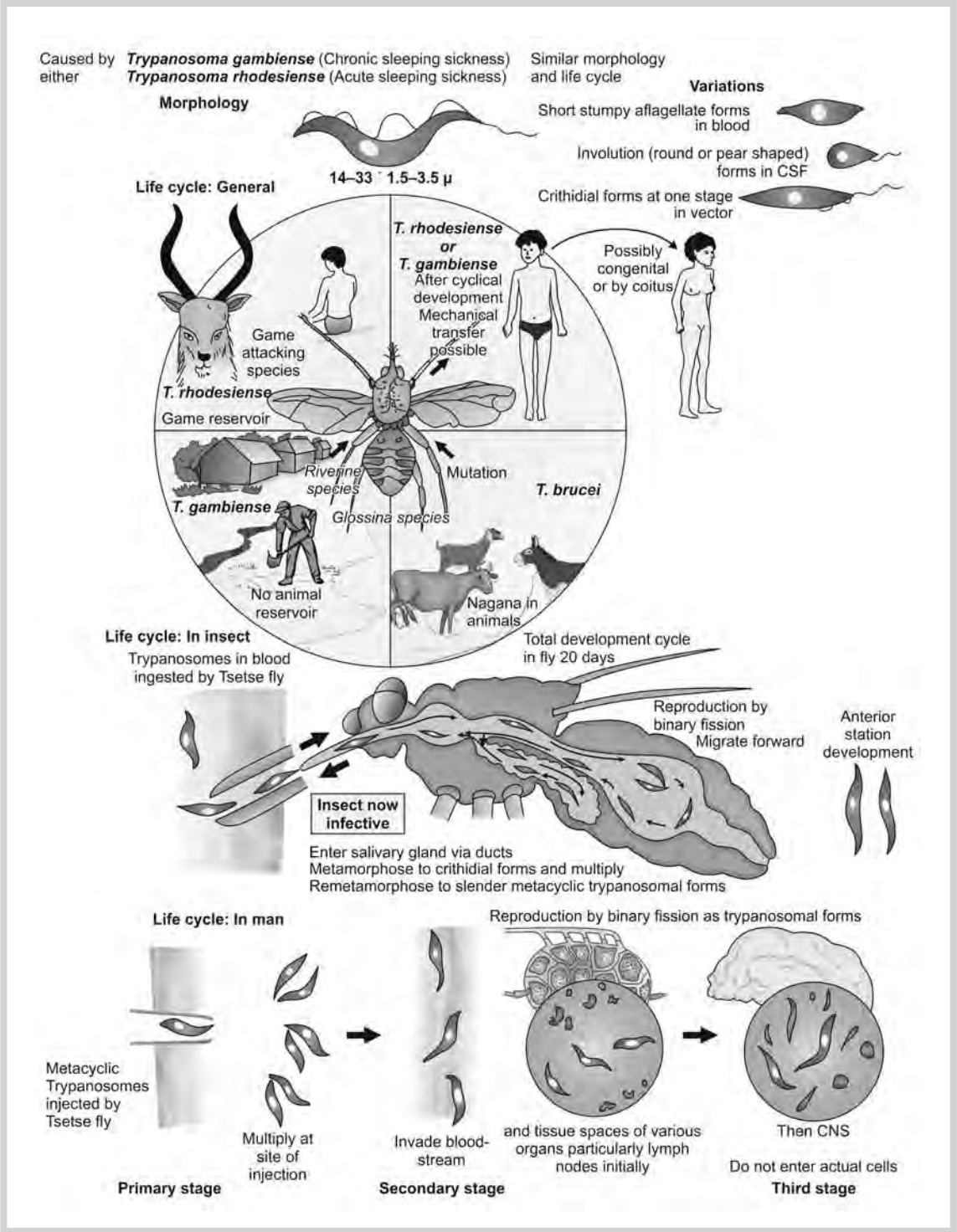


2. Serological tests
   - Specific: CFT (antigen, *Leishmania* cultures)
   - Nonspecific: Depending on reversal AG ratio, Formal gel (Napier), Aldehyde (Chopra), Euglobulin precipitation (Sia), Electrophoresis

Cutaneous and Mucocutaneous

1. Demonstration of parasite by Stained film — In — Serum from indurated edge of ulcer, Biopsy of margin ulcer, Mucosal scraping in mucocutaneous type
   Culture
   Animal inoculation
2. Intradermal test (Montenegro). Antigen culture of appropriate *Leishmania*
3. Serological tests in mucocutaneous type

Trypanosomiases: African Type: Sleeping Sickness



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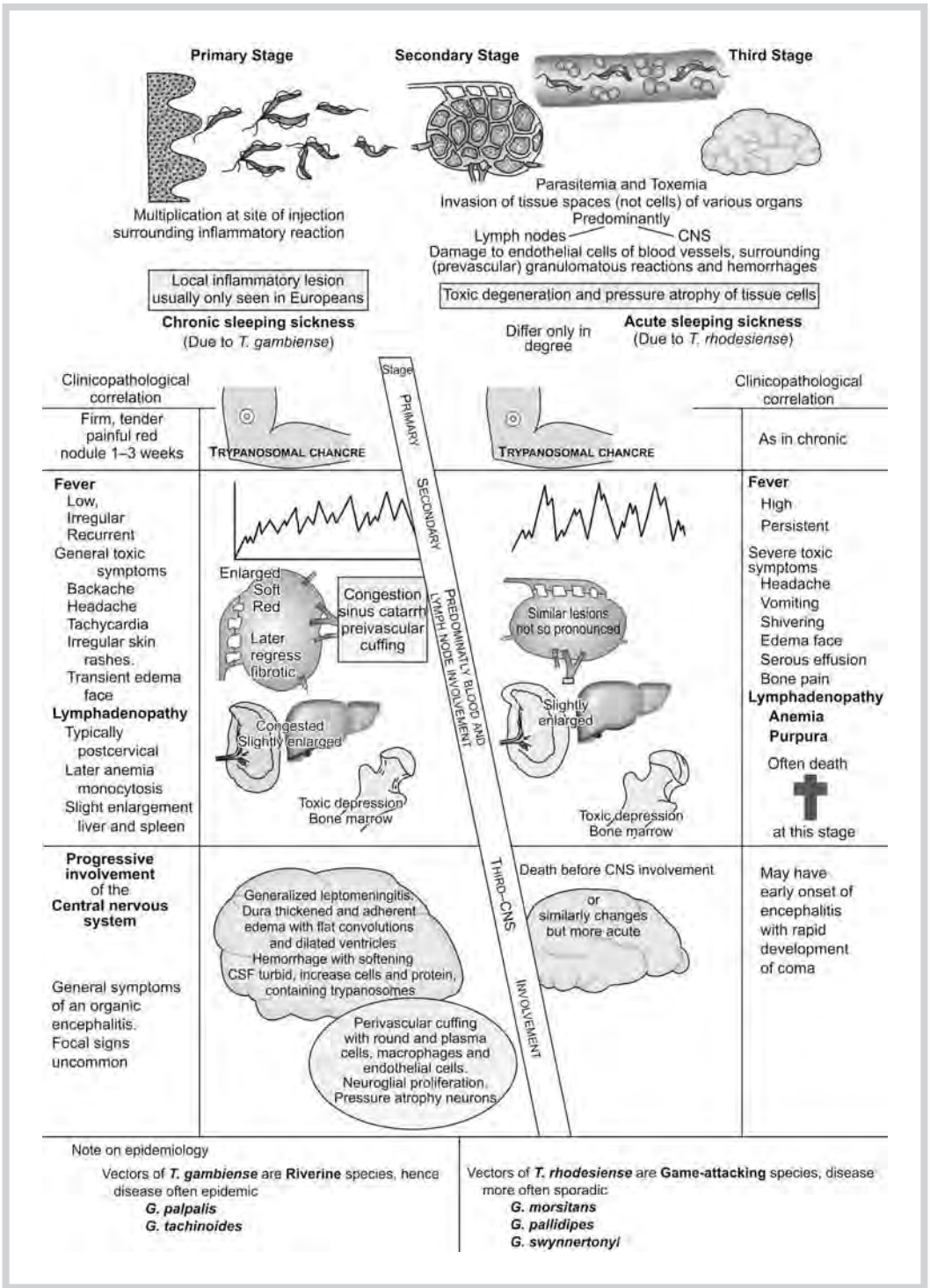
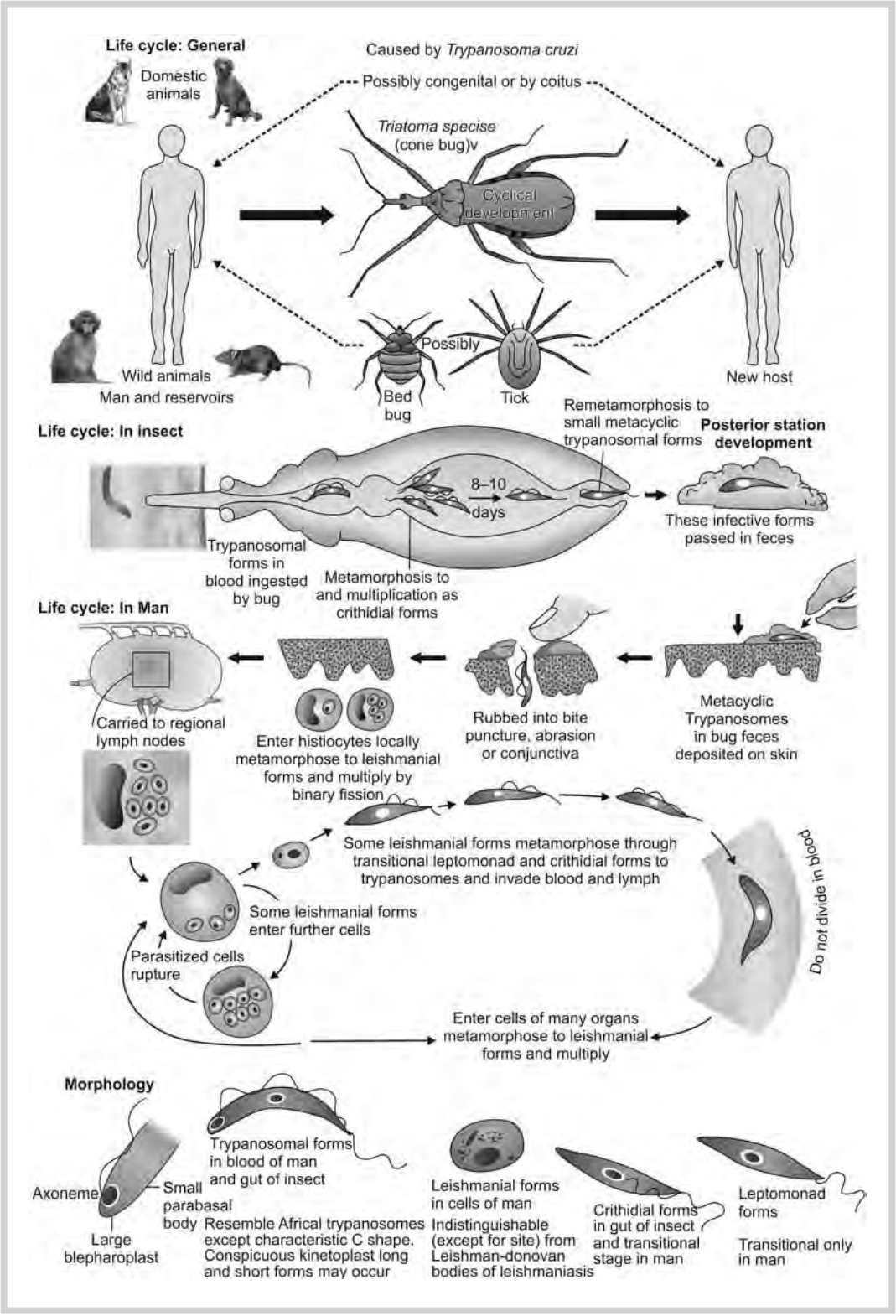

Primary Stage
Secondary Stage
Third Stage
Multiplication at site of injection surrounding inflammatory reaction
Parasitemia and Toxemia
Invasion of tissue spaces (not cells) of various organs
Predominantly
Lymph nodes
CNS
Damage to endothelial cells of blood vessels, surrounding (prevascular) granulomatous reactions and hemorrhages
Local inflammatory lesion usually only seen in Europeans
Toxic degeneration and pressure atrophy of tissue cells
Chronic sleeping sickness
(Due to T. gambiense)
Differ only in degree
Acute sleeping sickness
(Due to T. rhodesiense)
Clinicopathological correlation
Stage
Clinicopathological correlation
Firm, tender painful red nodule 1–3 weeks
TRYPANOSOMAL CHANCRE
PRIMARY
TRYPANOSOMAL CHANCRE
As in chronic
Fever
Low, Irregular Recurrent
General toxic symptoms
Backache
Headache
Tachycardia
Irregular skin rashes.
Transient edema face
Lymphadenopathy
Typically postcervical
Later anemia monocytosis
Slight enlargement liver and spleen
SECONDARY
PREDOMINANTLY BLOOD AND LYMPH NODE INVOLVEMENT
Enlarged
Soft
Red
Later regress fibrotic
Congestion sinus catarrh preivascular cuffing
Congested
Slightly enlarged
Toxic depression Bone marrow
Similar lesions not so pronounced
Slightly enlarged
Toxic depression Bone marrow
Fever
High
Persistent
Severe toxic symptoms
Headache
Vomiting
Shivering
Edema face
Serous effusion
Bone pain
Lymphadenopathy
Anemia
Purpura
Often death
at this stage
Progressive involvement of the Central nervous system
General symptoms of an organic encephalitis. Focal signs uncommon
THIRD-CNS INVOLVEMENT
Death before CNS involvement
or similarly changes but more acute
Generalized leptomeningitis. Dura thickened and adherent edema with flat convolutions and dilated ventricles Hemorrhage with softening CSF turbid, increase cells and protein, containing trypanosomes
Perivascular cuffing with round and plasma cells, macrophages and endothelial cells. Neuroglial proliferation. Pressure atrophy neurons
May have early onset of encephalitis with rapid development of coma
Note on epidemiology
Vectors of T. gambiense are Riverine species, hence disease often epidemic
G. palpalis
G. tachinoides
Vectors of T. rhodesiense are Game-attacking species, disease more often sporadic
G. morsitans
G. pallidipes
G. swynnertonyi

Trypanosomiasis South American Type: Chagas' Disease



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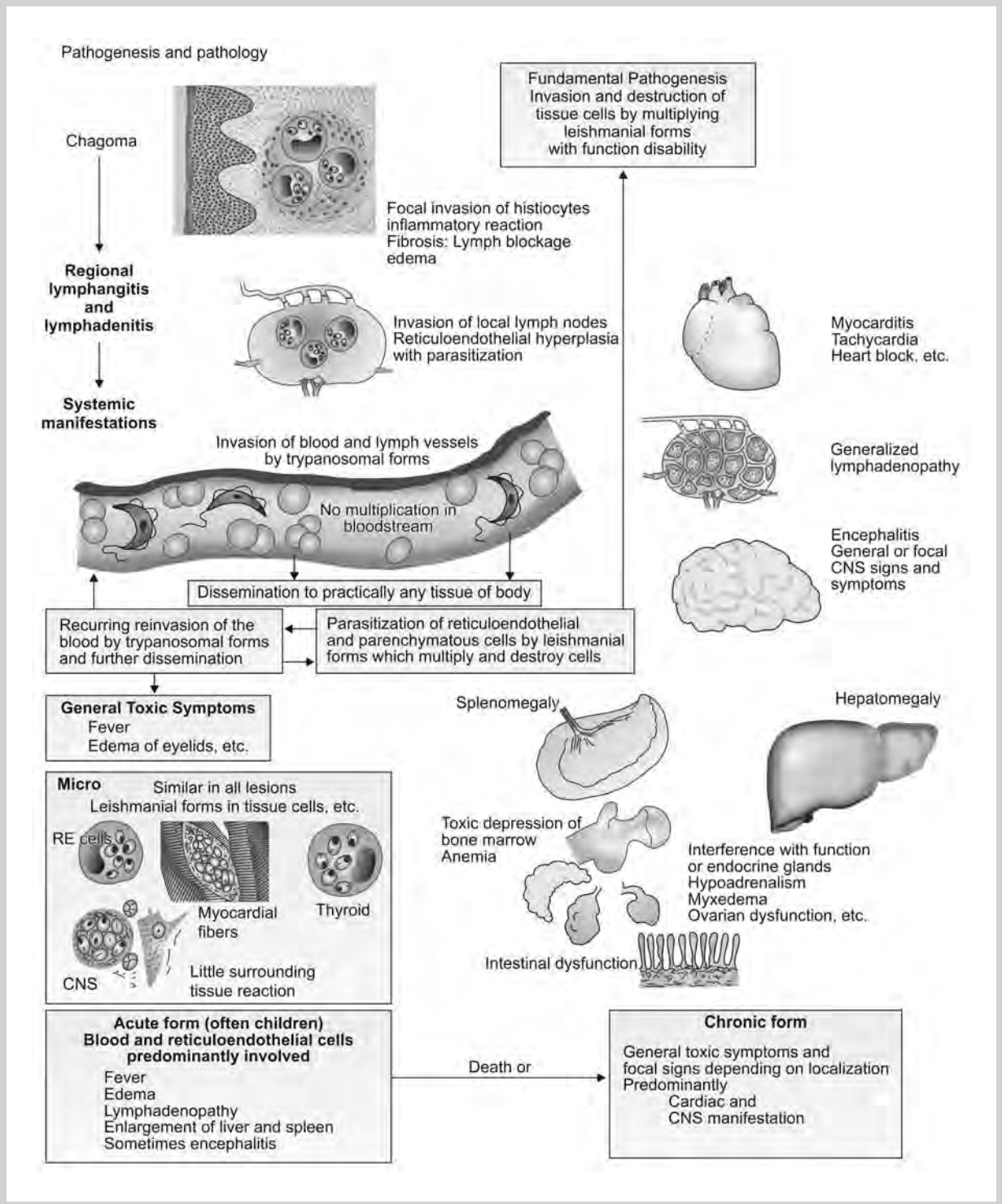

Pathogenesis and pathology
Chagoma
Regional lymphangitis and lymphadenitis
Systemic manifestations
Fundamental Pathogenesis
Invasion and destruction of tissue cells by multiplying leishmanial forms with function disability
Focal invasion of histiocytes
inflammatory reaction
Fibrosis: Lymph blockage
edema
Invasion of local lymph nodes
Reticuloendothelial hyperplasia
with parasitization
Myocarditis
Tachycardia
Heart block, etc.
Generalized lymphadenopathy
Encephalitis
General or focal CNS signs and symptoms
Invasion of blood and lymph vessels by trypanosomal forms
No multiplication in bloodstream
Dissemination to practically any tissue of body
Recurring reinvasion of the blood by trypanosomal forms and further dissemination
Parasitization of reticuloendothelial and parenchymatous cells by leishmanial forms which multiply and destroy cells
General Toxic Symptoms
Fever
Edema of eyelids, etc.
Micro
Similar in all lesions
Leishmanial forms in tissue cells, etc.
RE cells
Myocardial fibers
Thyroid
CNS
Little surrounding tissue reaction
Splenomegaly
Hepatomegaly
Toxic depression of bone marrow
Anemia
Interference with function or endocrine glands
Hypoadrenalism
Myxedema
Ovarian dysfunction, etc.
Intestinal dysfunction
Acute form (often children)
Blood and reticuloendothelial cells predominantly involved
Fever
Edema
Lymphadenopathy
Enlargement of liver and spleen
Sometimes encephalitis
Death or
Chronic form
General toxic symptoms and focal signs depending on localization
Predominantly
Cardiac and
CNS manifestation

Common Intestinal Roundworms of Man—Pathology (Table 8.5)

TABLE 8.5: Common intestinal roundworms of man—pathology

Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Enterobiasis Pinworm infection *Enterobius vermicularis*	Symptomless to perianal itching and irritation. Vaginal itch when female worms leave anus and enter genital passage. Nervousness, insomnia in persistent or heavy infection, especially from biting finger-nails after scratching infected rectal area	Cellulose (Scotch) tape swab
Trichuriasis *Trichuris trichiura*	Depend on worm load. Symptomless to chronic debilitating diarrhea and anemia, with damage to physical and mental development. Lower right quadrant pain is quite a common complaint. Chronic diarrhea, bloody stools, and tenesmus in heavier infections. Weight loss, wasting, rectal prolapse in massive trichuriasis, especially in children. Worms visible attached to mucosa under sigmoidoscopy in heavy infections, mucosa hyperemic, friable, edematous, stools are mucoid and sticky with sreaks of blood, numerous Charcot-Leyden crystals, eosinophils, trichuris eggs	Feces: Direct smear, concentration
Ascariasis *Ascaris lumbricoides*	Larvae (migratory phase): Rarely pneumonitis with cough, hemoptysis, hemorrhages, lung consolidation, focal eosinophilic inflammation. Eosinophilia (usually under 30%) during larval migration, falls rapidly afterwards. Adults (intestinal phase): Symptomless to serious intestinal mechanical complications (pancreatitis, appendicitis, diverticulitis) especially after disturbance of worms causing obstruction or perforation; metabolic complication (malabsorption, nutritive drain). Nausea, vomiting, aggravation of malnutrition	Feces: Direct smear, concentration
Hookworm infection *Ancylostoma duodenale* *Necator americanus*	Larvae (migratory phase): Intense skin invasion may produce "ground itch", pruritic vesicular lesions followed by lung reactions (less intense than in ascariasis); cough, tracheal irritation, eosinophilia. Nausea, vomiting, dyspnea may result from larvae of Ancylostoma (Wakana disease in Japan)	Feces: Direct standardized smear to count eggs or examine after concentration, cultivation of feces on filter paper strips in test tubes (Harada-Mori technique)
	Adults (intestinal phase): Hypochromic, microcytic anemia is the chief clinical feature, varies with intensity and duration of infection, iron intake nutritive state, age and condition of patient. Hypoproteinemia, edema, trophic skin disorders, growth reduction, and mental retardation may follow. Allergic urticaria, diarrhea, abdominal pain in heavy infections. Intestinal malfunction through malabsorption and possible metabolic disturbance probably of significant importance, particularly in children and undernourished populations	
Strongyloidiasis *Strongyloides stercoralis* with larvae in focal lesions	Larvae: Invasion of skin may cause "ground itch," similar to hookworm. Malaise and cough, pulmonary infiltration may occur; high eosinophilia in colon, abdominal lymph nodes, liver, lungs following autoreinfection	Feces: Larvae (not eggs) in direct smear, concentration. Cultivation of feces on filter paper strips in test tubes technique)
(Harada-Mori)	Adults: Alternate diarrhea and constipation; inflammation of intestinal mucosa; may be hemorrhage and microulceration with watery, mucoid diarrhea, colicky abdominal pain, tenderness, flatulence. Heavier. infections produce atrophy of mucosa, ulcerous enteritis, edema, and fibrosis of intestinal wall. Extreme cases (usually after autoreinfection) with rapid deterioration, asthenia, anorexia, and death or chronic invalidism. Intestinal malfunction with impaired protein digestion and fat absorption may produce a condition similar to sprue	

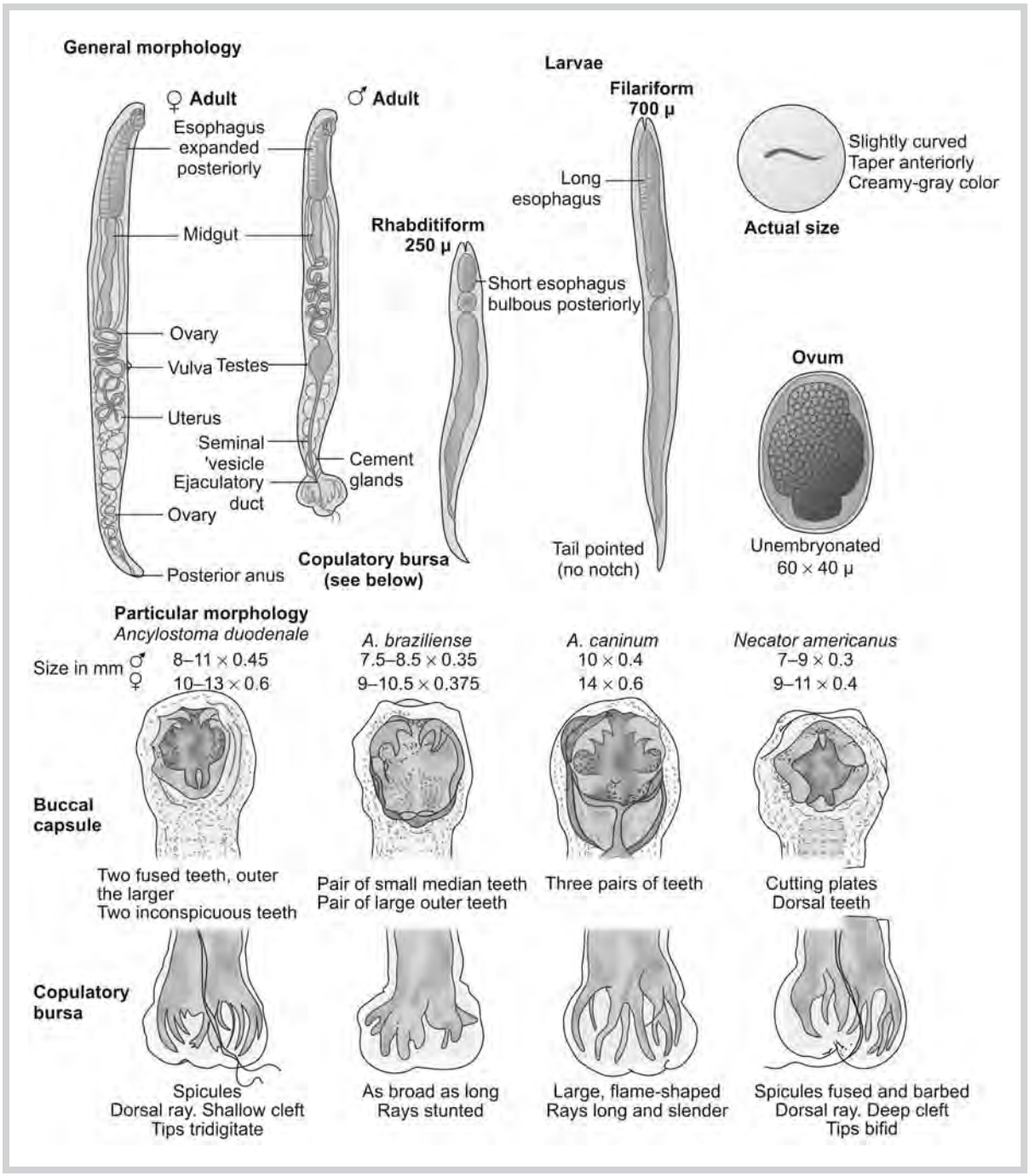
Extraintestinal Roundworm Infection of Man—Larval Worm Pathology (Table 8.6)

TABLE 8.6: Extraintestinal roundworm infections of man—larval worm pathology

Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Trichinosis *Trichinella spiralis*	Adults (intestinal phase): Irritate and inflame the intestinal mucosa, vomiting, diarrhea, pain Larval migration and penetration (1–6 weeks after infection): Muscular rheumatic pains, edema of the eyes, face and hands. Irregular persistent sweating and fever; difficulty with breathing, swallowing, and speech; rash and high eosinophilia (50–90%) Larval encystment (muscle phase) (after 6 weeks): Cachexia, toxic edema, skin eruptions, anemia, dehydration, and gradual subsidence of muscular pains. Fatalities usually occur 4–6 weeks after ingestion of heavily infected pork by nonimmune individuals. Using a home-butchered hog implicated	Biopsy material: Examination by compression of tissue between glass slides or by digestion. Intradermal test, complement fixation, bentonite flocculation and latex agglutination tests
Cutaneous larva migrans *Ancylostoma braziliense* and other non-human hookworms, species of animal *Strongyloides, Gnathostoma spinigerum,* and possibly other nematodes	Intracutaneous violently itching, serpiginous tunnels, which are caused by wandering of hookworm larvae unable to complete normal penetration, migration, and development. Worms move about in the area of initial penetration, producing irregular papulovesicular lesions. Dry crust may form with local eosinophilia and cellular infiltration. This condition usually is transitory but larvae may also penetrate to deeper tissues; produce pulmonary infiltration and be recovered in sputum. The larvae may last several weeks to months, moving at intermittent periods of 1–3 cm/day	Clinical signs sufficient. No worms identified except in experimental animals
Visceral larva migrans: *Toxocara canis, T. cati* (dog, cat ascarids); also *Ancylostoma caninum*, *A. braziliense, Capillaria hepatica,* possibly *Ascaris lumbricoides* var suum and filariae of the genus *Dirofilaria*, other animal nematodes	Chiefly in children aged 1–4, often benign, asymptomatic; later there may develop 20–90% eosinophilia and hepatomegaly. Fever, cough, joint and muscle pains, anorexia, weight loss, nervousness, abdominal pain, pneumonitis, splenomegaly all reported. Symptoms vary with number and location of larvae and patient's allergic response. Chief result of wandering of larvae is indication of a series of focal eosinophilic inflammations succeeded by granulomatous reactions. Endophthalmitis reported in young adolescents who apparently harbored larvae in their tissues from childhood	Heavy infections show larvae and eosinophilic granulomatous lesions in liver biopsy. Chiefly a clinical diagnosis (persistent eosinophilia, hepatomegaly, hyperglobulinemia and frequent pneumonitis); hemagglutination technique useful for confirmation

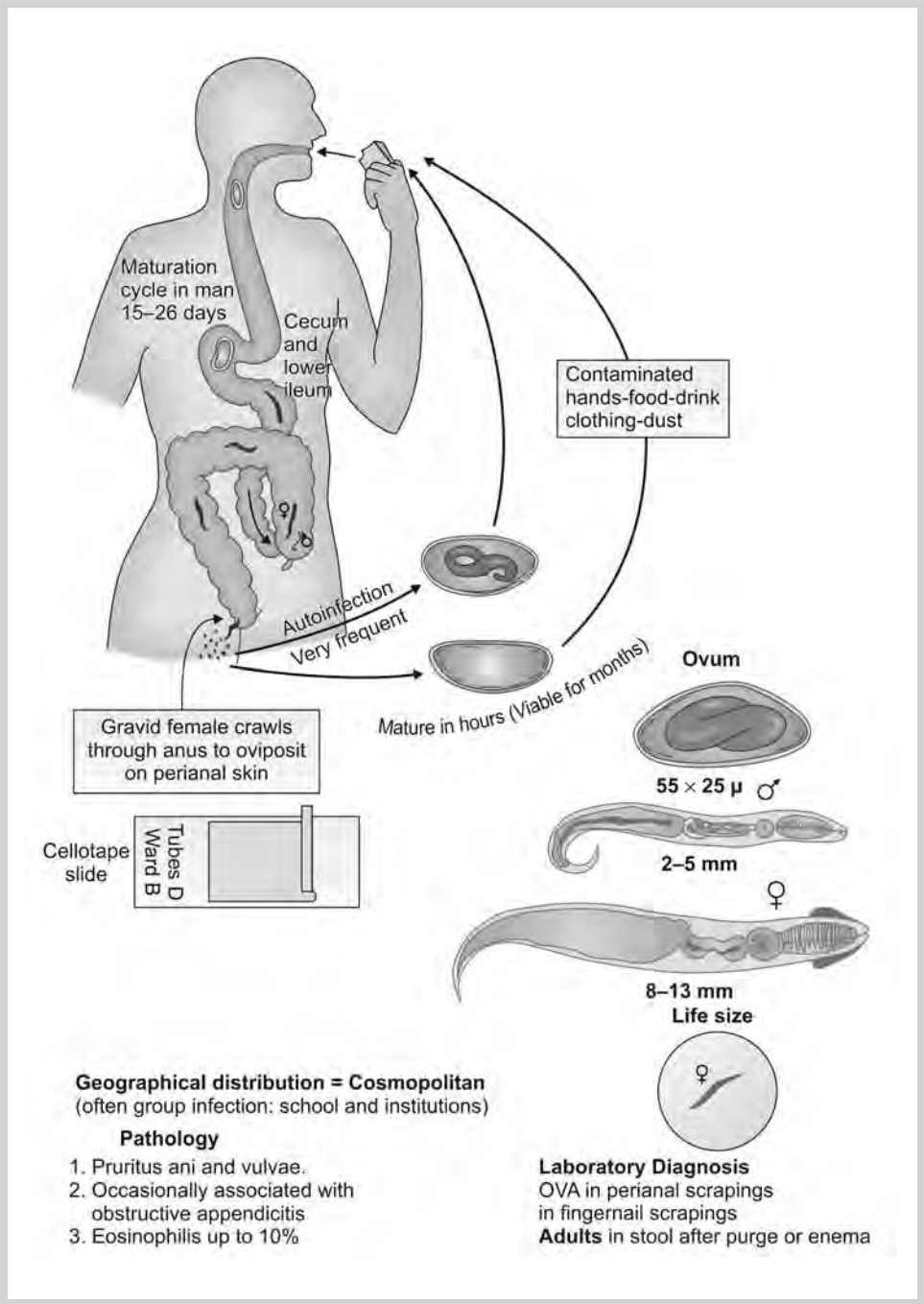
Plasmid Nematodes

Hookworms

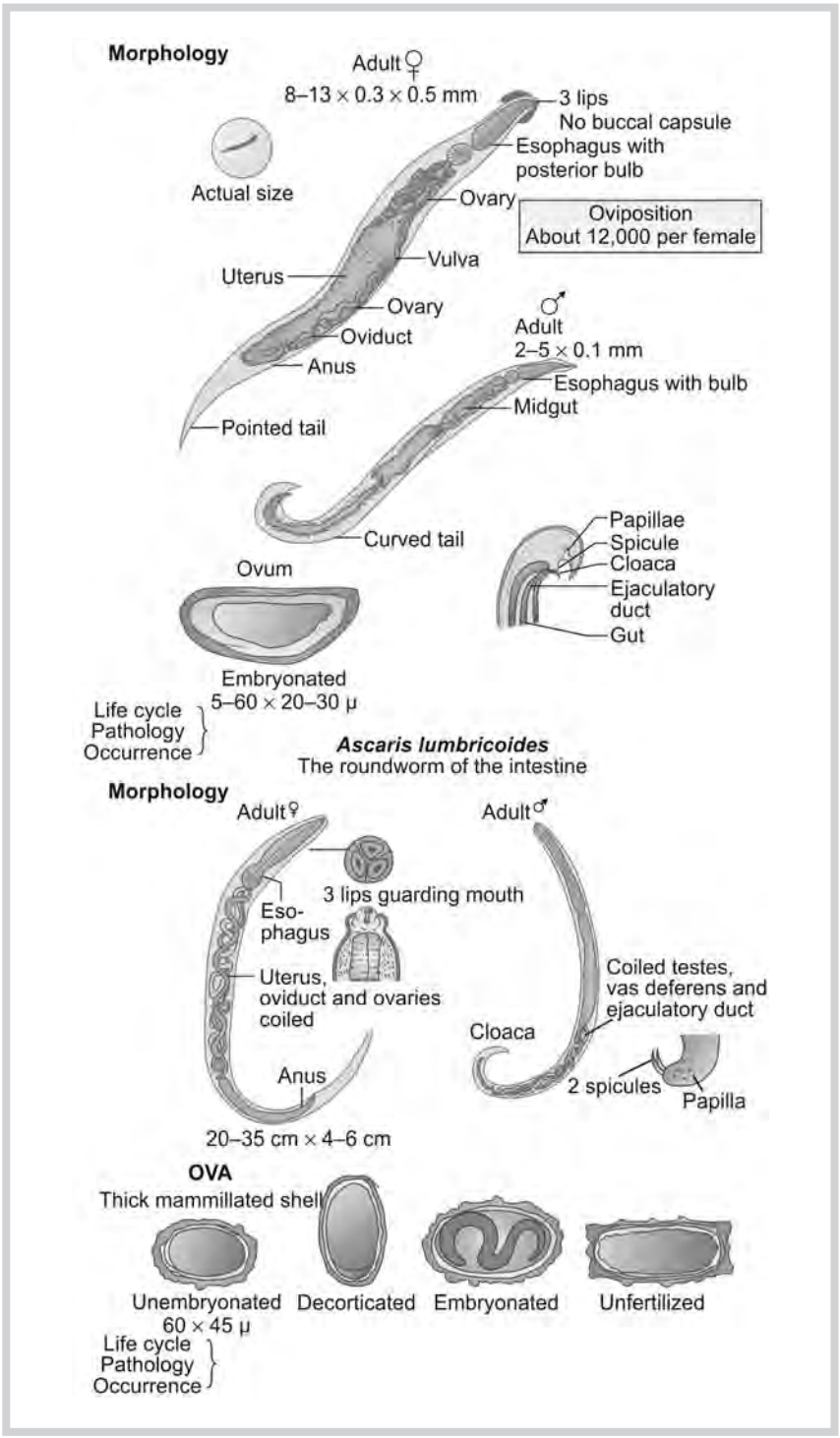


Enterobius vermicularis (Thread, Pin or Seat Worm)

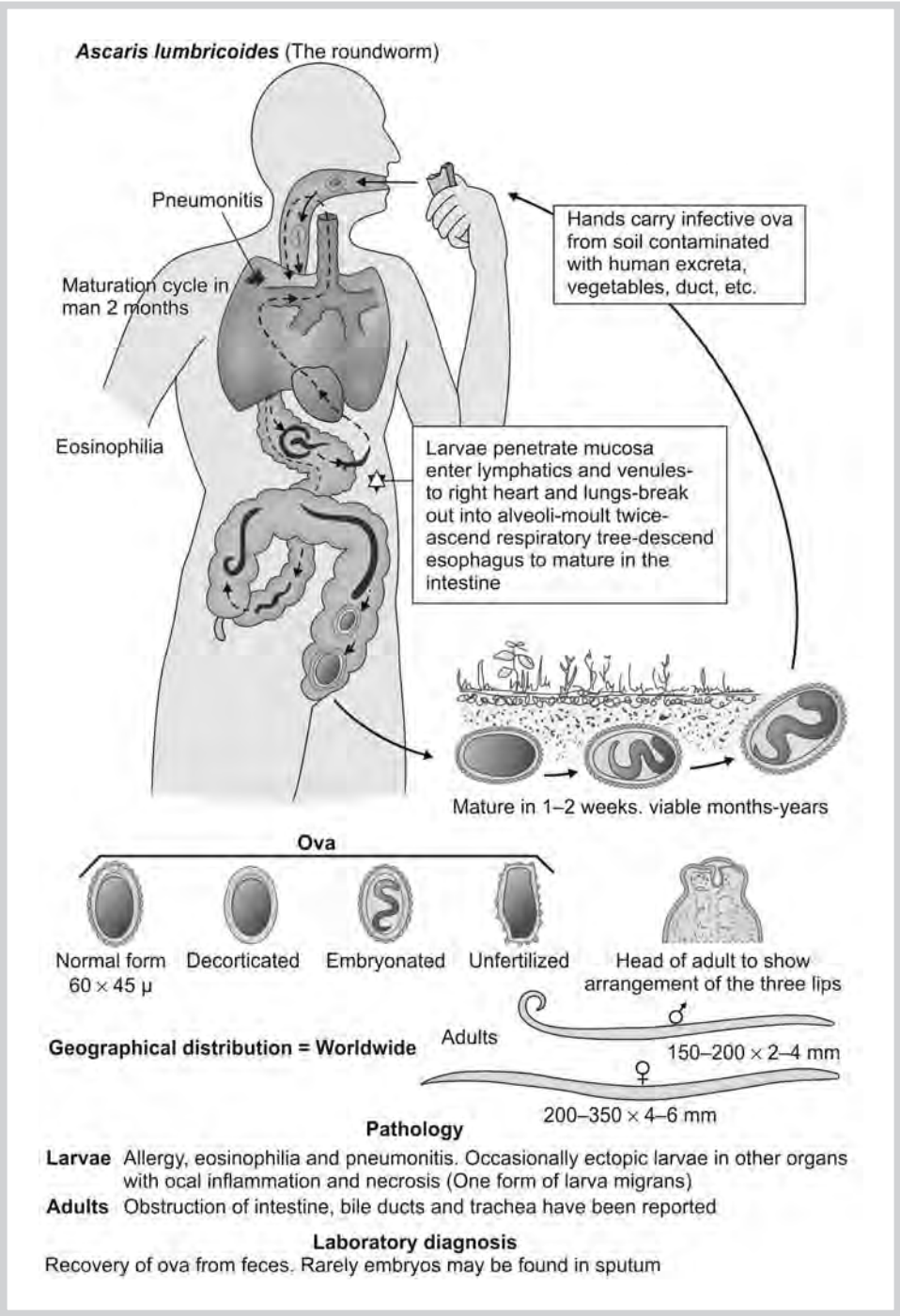
Synonym: Oxyuris vermicularis



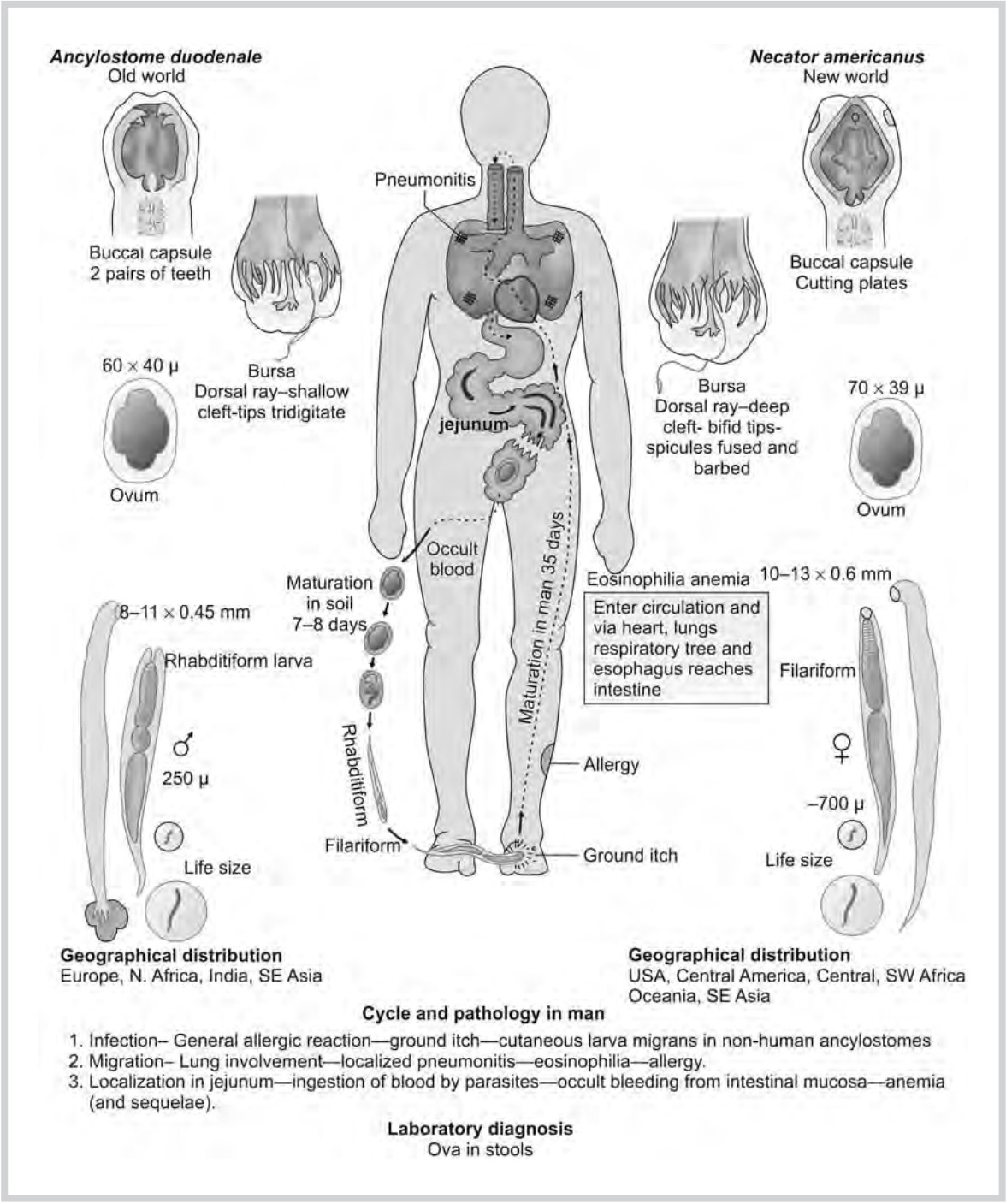
Enterobius vermicularis



Ascaris lumbricoides (The Roundworm)



The Hookworms



Cycle and pathology in man

1. Infection– General allergic reaction—ground itch—cutaneous larva migrans in non-human ancylostomes
2. Migration– Lung involvement—localized pneumonitis—eosinophilia—allergy.
3. Localization in jejunum—ingestion of blood by parasites—occult bleeding from intestinal mucosa—anemia (and sequelae).

Laboratory diagnosis
Ova in stools

Tissue Roundworms of Man, Chiefly Filariae (Tables 8.7 and 8.8)

TABLE 8.7: Tissue roundworms of man—chiefly filariae, life cycle

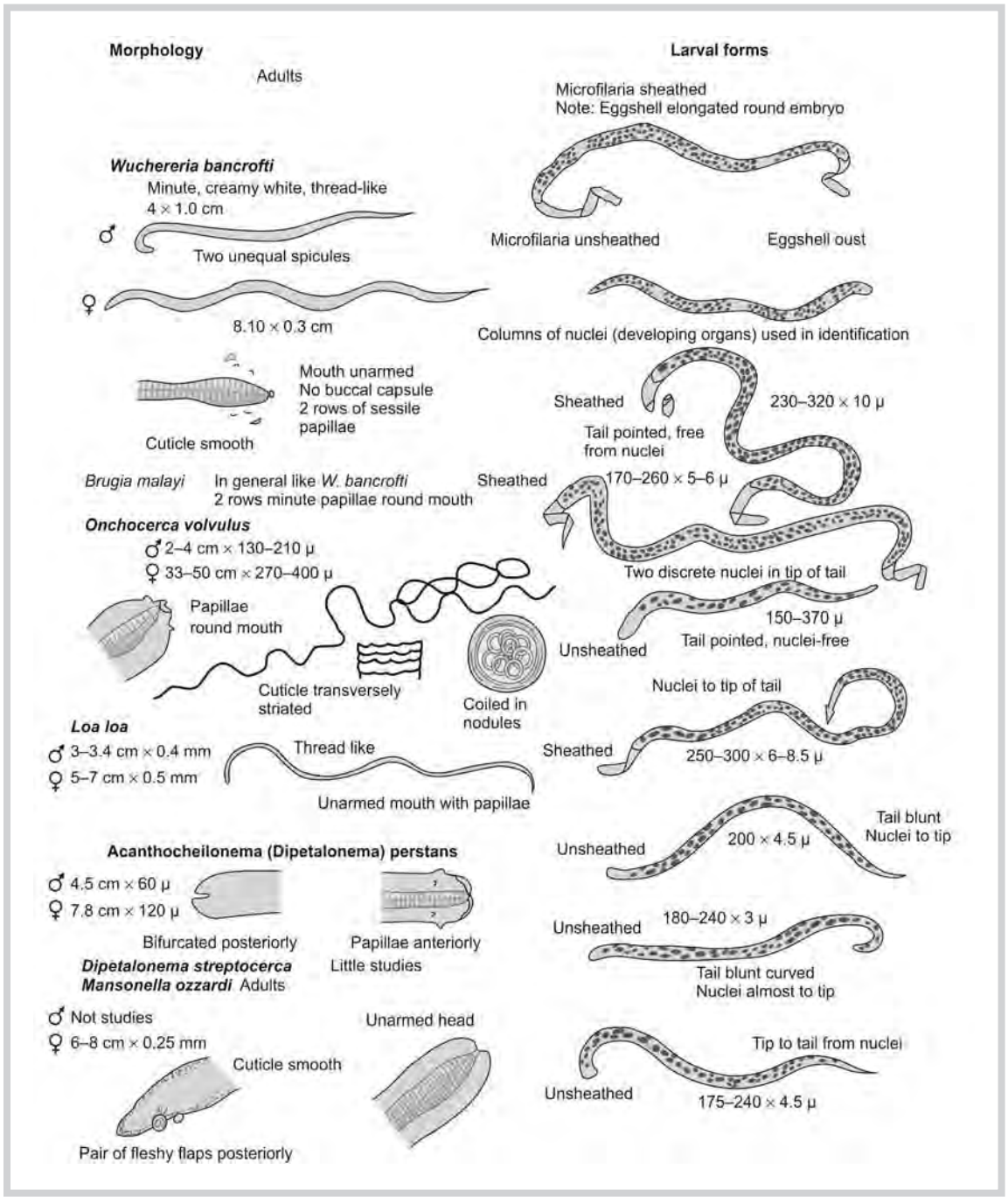
Parasite and distribution	*Intermediate host*	*Mode of human infection*	*Site of worm maturation*	*Larvae found in*	*Exit*	*Larval development in*	*Reservoir host*
Wuchereria bancrofti *Brugia malayi* Tropics and subtropics	Culex, aedes, anopheles, Mansonia, and other mosquitoes	Filariform larvae actively leave mosquito at time of biting, usually enter skin via puncture	In lymph vessels and nodes	Bloodstream (nocturnal periodicity)* certain strains sub- or non-periodic	Microfilariae taken up by insect when biting	Thoracic muscles; larvae migrate to proboscis (8–15 days)	For *B. malayi*—cats, dogs monkeys
Loa loa Tropical Africa	Chrysops (deer or mangrove flies)	Probably same	In subcutaneous tissues	Bloodstream (diurnal periodicity)	Same	Abdomen, larvae migrate to proboscis	Primates?
Onchocerca volvulus Tropical Africa Venezuela, and mountainous portions of Central Africa	Simulium (buffalo gnat)	Probably same	Same, host reaction, produces nodule around cluster of adults	Subcutaneous tissue; eye (no periodicity)	Same	Thoracic muscles; larvae do not concentrate in proboscis	Man
Dracunculus medinensis Africa, India, Far and Middle East, Indonesia	Cyclops (water flea)	Infected cyclops accidentally ingested in water	Digested out of cyclops, then worm migrates into tissues; mature female passes to skin (may be 1 m long)	Adult female in skin causes host to form blister near head of worm. Blister then filled with larvae, bursts when skin immersed in water, discharging larvae. New blister later forms as female moves to new site	Cyclops feeds on larva, which penetrates gut, develops in hemocoel (18–21 days)	Man	

*Periodicity of microfilarial appearance in peripheral blood. May be nocturnal in circulating blood from 8 pm, after which microfilariae confined to pulmonary capillaries

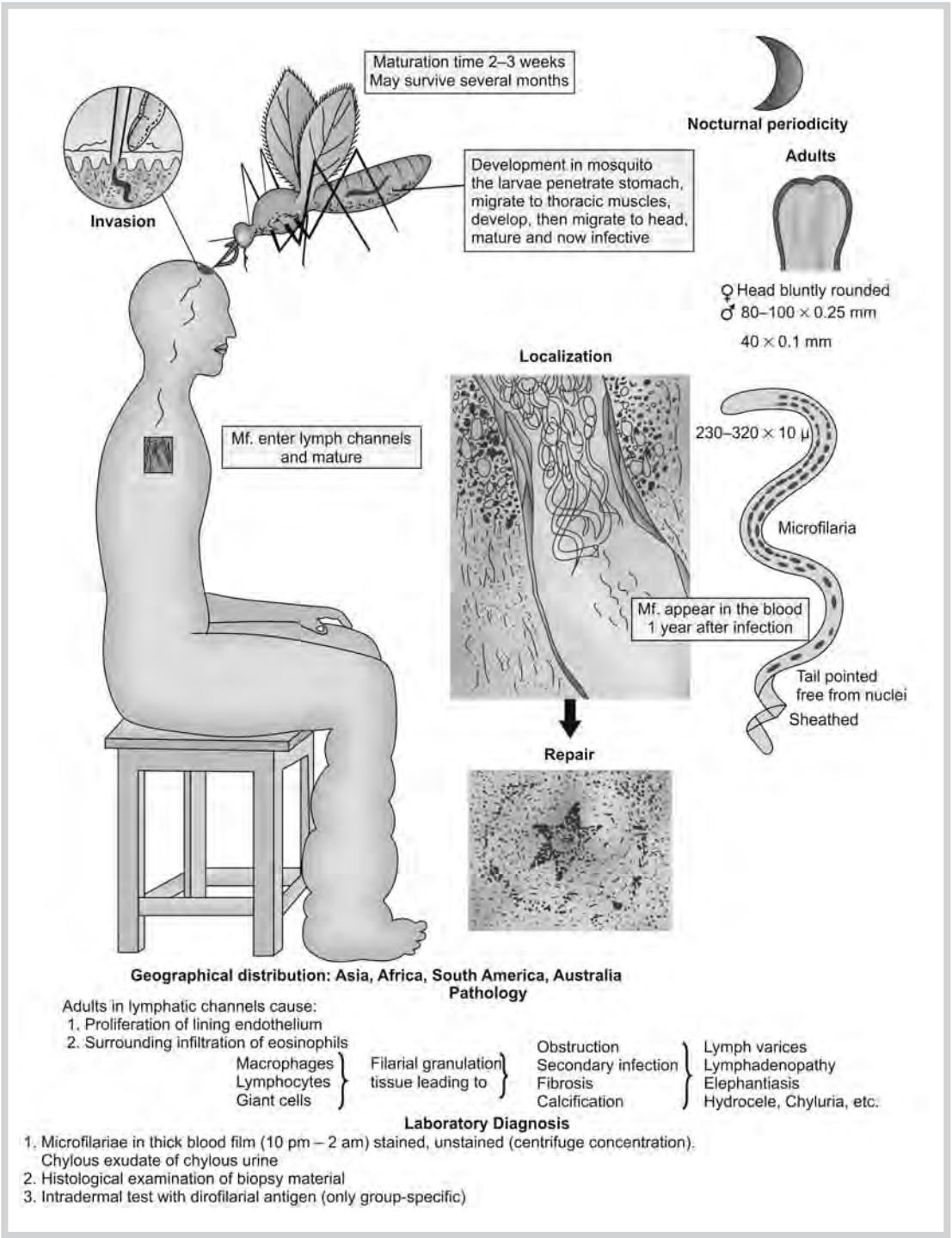
TABLE 8.8: Tissue roundworms of man—chiefly filariae, pathology

Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Bancroft's filariasis *W. bancrofti* **Malayan filariasis** *B. malayi*	Filariae in afferent lymph nodes (of lower extremities, male genitalia, vulva, mammary gland) may cause inflammation followed by intensely fibrotic reaction involving whole area in a mass of scar tissue. Pain, fever, chills, toxemia, eosinophilia. Chronic stage (after disappearance of microfilariae) varies from microscopic lesion to lymph varicosity to marked elephantiasis. High eosinophilia and superficial lymphadenopathy in Malayan filariasis. Pathology probably a general and focal sensitization response	Blood: Thick and thin smears, concentration. Intradermal test gives useful group filaria reaction, indirect hemagglutination, fluorescent antibody tests
Loiasis *Loa loa*	Fugitive swelling ("Calabar swelling") in skin due to local edema and skin reaction against migrating adult worm. The latter commonly moves across surface of eyeball or under skin at bridge of nose (best times for removal with local anesthesia). Eosinophilia with occasional proteinuria produced	Microfilariae in nodule aspirate or skin snip; repeated skin snips may be required; scapular cutaneous region area of choice, manifestations: eosinophilia ocular lesions, pruritus may follow diethylcarbamazine (Hetrazan) therapy
Onchocerciasis *O. volvulus*	Small cutaneous fibrous skin nodules with filariae entwined in center. Microfilariae in nodule, in neighboring tissue, or in normal skin far from nodules, rarely in blood. Skin reactions particulary common in Africa; reduction of elastic fibers, depigmentation, progressive thickening, pruritus, papulovesicular lesions or hyperkeratotic patches with microfilariae in scrapings. Eosinophilia and transient urticaria during incubation. Ocular involvement leading to blindness, a common result of prolonged infection. Conjunctiva and vitreous humor with numerous microfilariae. Pathology due to mechanical action, toxins, hypersensitive response of patient; ocular symptoms after 7–9 years	
Dracontiasis *D. medinensis*	Asymptomatic until reddish papular lesion appears, usually on legs. Lesion forms blister bearing head of female worm and numerous larvae. Blister bursts when immersed in water, releasing larvae. Urticaria, pruritus, allergic symptoms, eosinophilia. Accidental rupture of worm may produce intense inflammatory reaction with secondary infection	Local lesion with head of worm and larvae in blister X-ray reveals calcified worm; reflected light shows worm under skin. Intradermal test
Eosinophilic lung describes a host allergic response to migration of microfilariae that are trapped in lungs, producing an allergic, asthma-like response. Probably, caused by human filariae or closely related species, as completion of worm development and microfilariae production required. Diagnosed by clinical signs, eosinophilia (3000 or more absolute count, usually over 35%), high hemagglutination or complement fixation titer that drops following Heterazan therapy, elevated ESR, mottled lung lesions visible under X-ray		

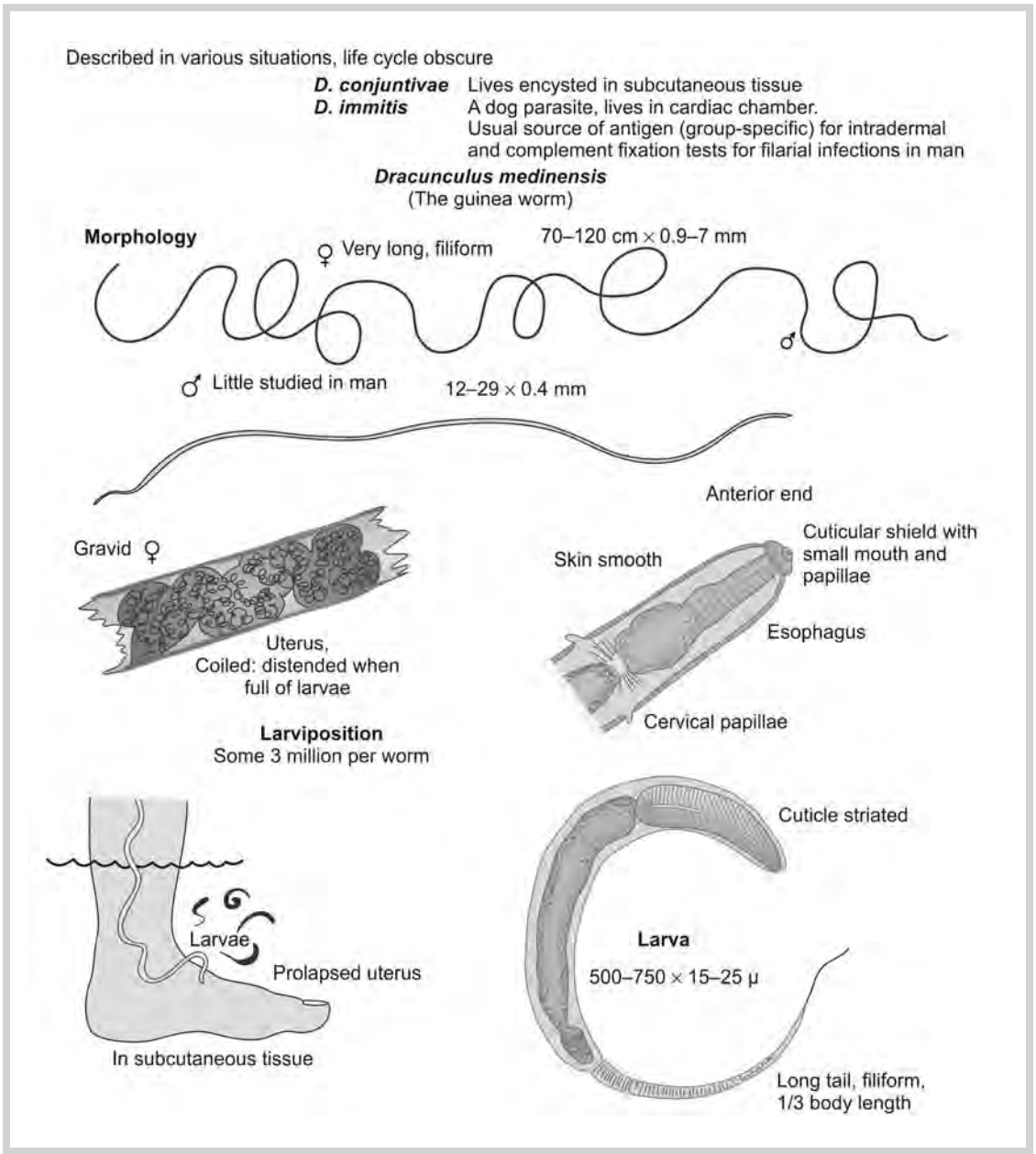
The Filarial Worms



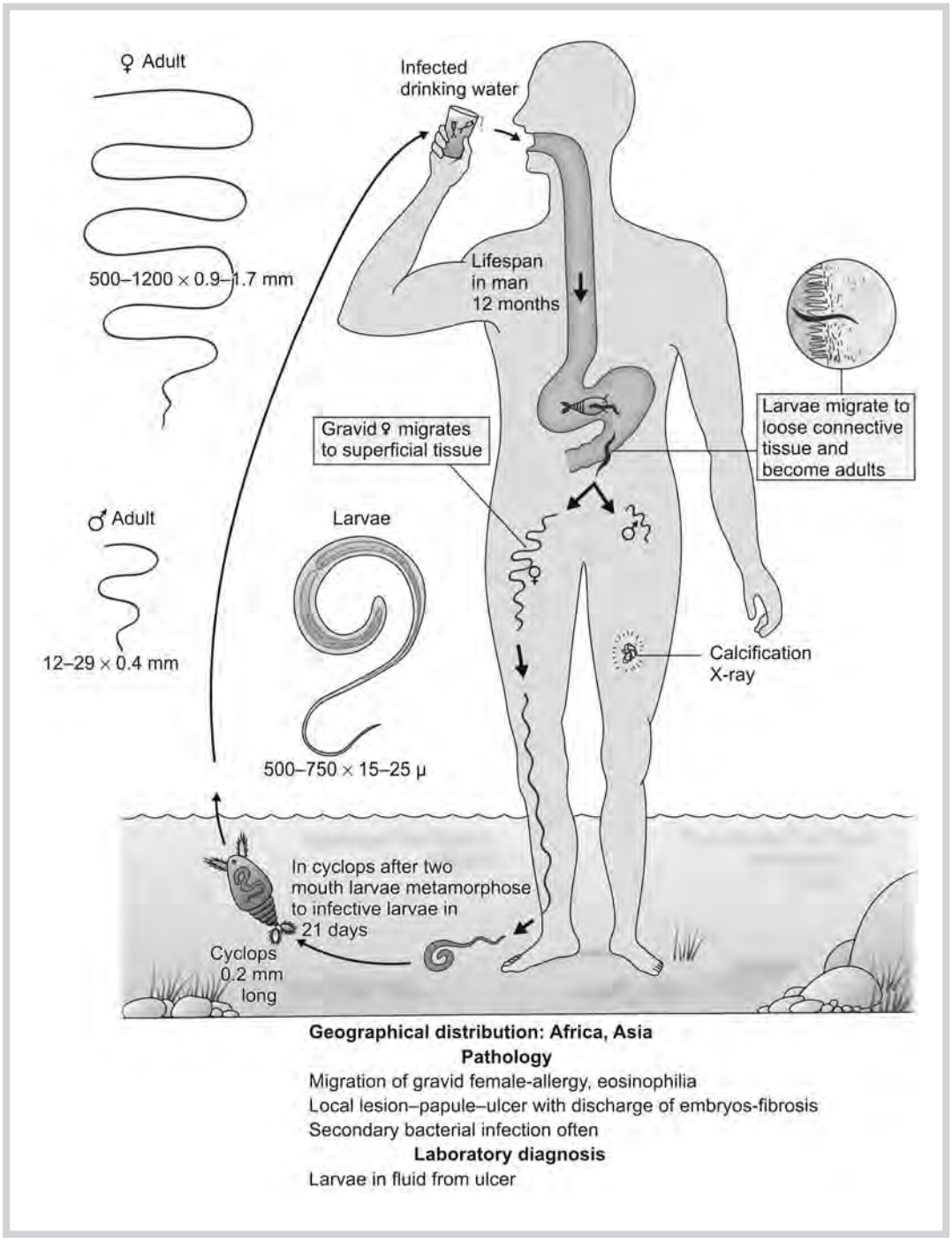
Wuchereria bancrofti



(Filarial Worms)



Dracunculus medinensis (The Guinea Worm)



Geographical distribution: Africa, Asia

Pathology

Migration of gravid female-allergy, eosinophilia
Local lesion–papule–ulcer with discharge of embryos-fibrosis
Secondary bacterial infection often

Laboratory diagnosis

Larvae in fluid from ulcer

Tapeworms of Man (Tables 8.9 and 8.10)

TABLE 8.9: Tapeworms of man

Diphyllobothrium latum, 80–100% in highly endemic areas; *Taenia saginata* commonest of the large tapeworms; *Hymenolepis nana*, probably quite common. *Echinococcus granulosus*, 3–16% in some endemic areas*; Taenia solium,* where pork is eaten raw.

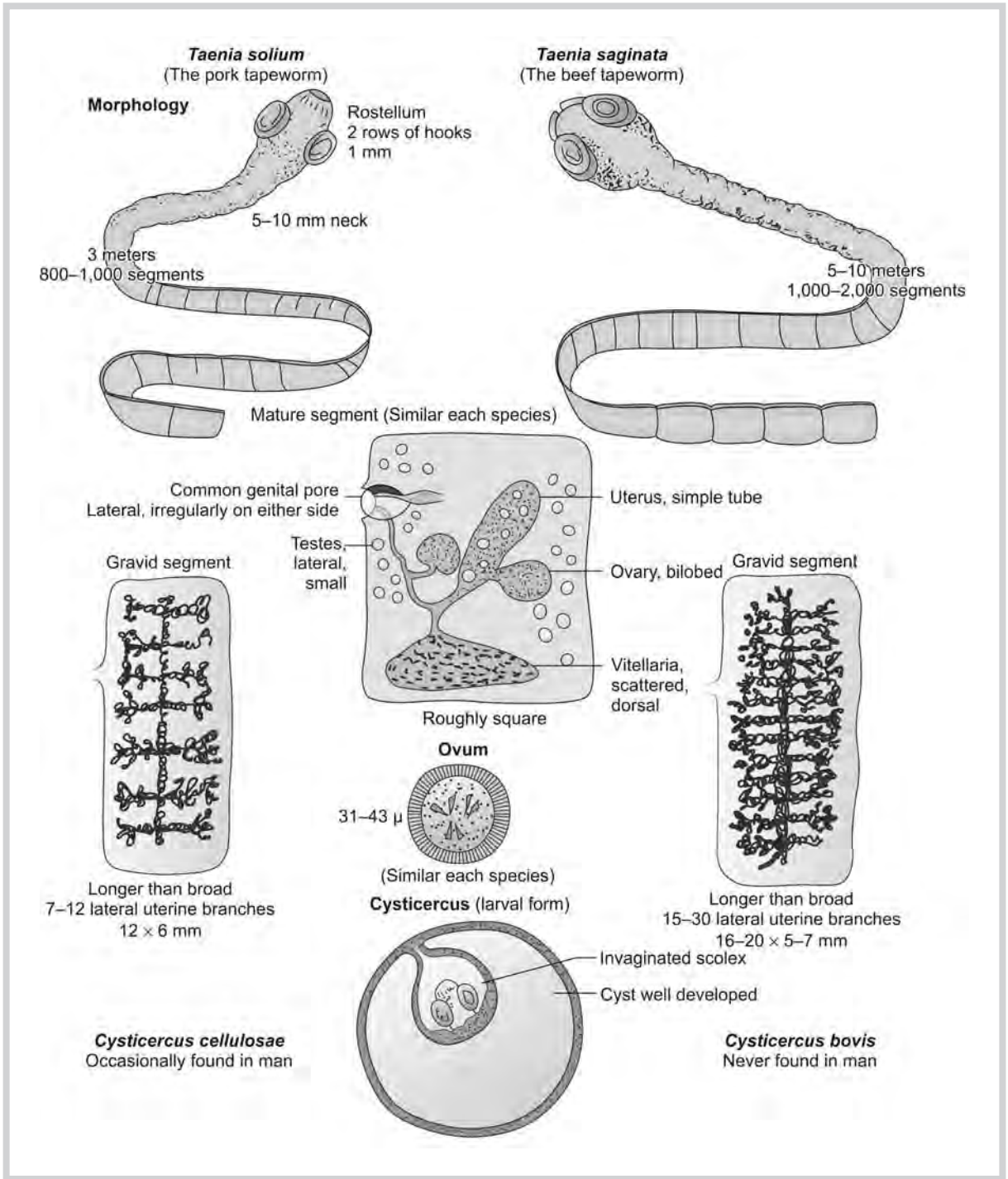
Parasite and distribution	*Infection*	*Cycle in intermediate route*	*Host fate*	*Cycle in definitive maturation of worm*	*Host exit*	*Summary of hosts*
Taenia saginata (beef tapeworm) worldwide	Ingestion of egg (cattle)	Egg hatches in intestine; *Oncosphere* released, penetrates intestine, enters bloodstream of vertebrate	Encysts in muscles or organs, forms a *Cysticercus* larva (bladder worm) in cattle called *Cysticercus bovis*	Man ingests cysticercus in raw beef: scolex attaches to duodenum, becomes adult in 6–12 months	Gravid segments per anus	*Definitive:* Man *Intermediate;* Cattle buffalo, giraffes. llamas, goats
Taenia solium (pork tapeworm) worldwide	Ingestion of egg (hogs, man); autoreinfection	-do-	Similar to *T. saginata,* cysticercus larva (in hogs) called *C. cellulosae*	As for *T. saginata,* but infection source is undercooked pork	-do-	*Definitive:* Man *Intermediate:* Pigs, man (autoreinfection)
Echinococcus granulosus (hydatid worm) Sheep raising areas	Ingestion of egg (sheep, accidental ingestion in man)	-do-	Forms *hydatid cyst* with thousands of infective scoleces in fluid, although cysts may also be sterile. Chiefly in liver, also in lungs rarely in brain	Sheep, dogs ingest hydatid sand (infective scoleces) from hydatid cyst in sheep carcass Worms attach to canine intestinal wall; become adult	Eggs in feces	*Definitive:* Dogs, all canids; rarely cats. *Intermediate:* Sheep, hogs, cattle, man
Hymenolepis nana (dwarf tapeworm) worldwide	Ingestion of egg by man or rodent (direct cycle) or by various insects (indirect cycle)	Egg hatches in intestine; oncosphere released. In man, it invades villus; in insect it penetrates gut and enters hemocoel	*Cysticercoid* larva containing scolex of future adult worm, formed either in villus of human host or hemocoel of insect	In man, larva leaves villus, attaches to small intestine, becomes adult. If infected insect ingested, cysticercoid digested out, hatches, attaches, grows to adult in 10–12 days Cysticercoid derived either from insect or direct egg-to-cercoid cycle in man can produce infection	Eggs in feces	Man, rats and mice, gerbils in Africa. Common tapeworm of man; possibly distinctive strain in man and rodents
Diphyllobothrium latum (fish or broad tapeworm) Orient, Latin America, Great Lakes, Northern Europe	Water flea ingests swimming embryo (coracidium) hatched from egg in water	Hooked embryo penetrates gut wall, develops into *procercoid* larva in hemocoel	Freshwater fish eats water flea; larva digested out in intestine, penetrates to muscles or organs, becomes third stage larva (*plerocercoid* or *sparganum)*	Man ingests fish with sparganum; larva liberated in intestine, attaches to intestinal wall, becomes adult	Eggs in feces into water	*Definitive:* Fish-eating mammals *Intermediate:* Water fleas (Diaptomus), then various freshwater fish

TABLE 8.10: Tapeworm diseases of man

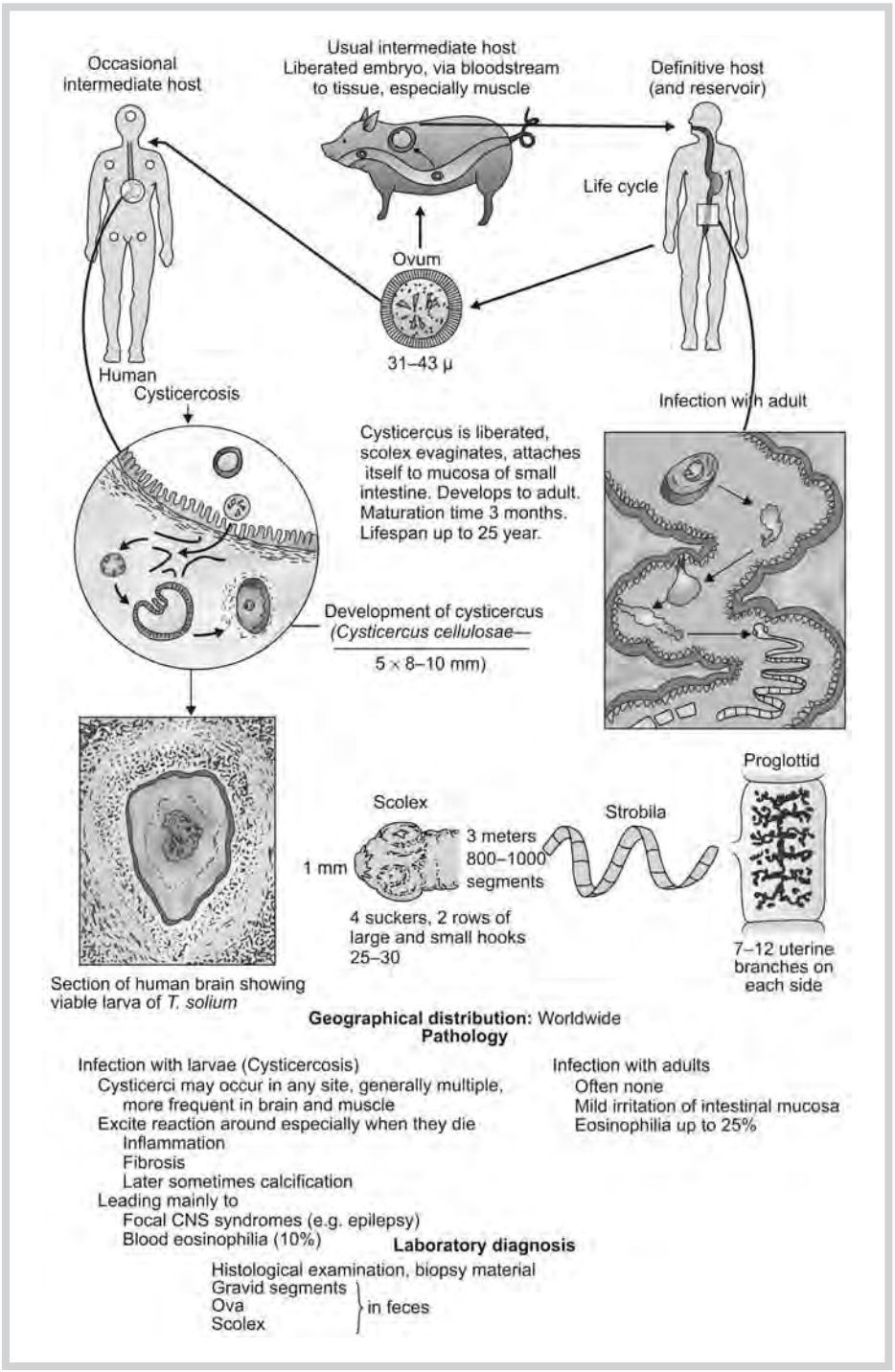
Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Hymenolepiasis *Hymenolepis nana*	Symptomless to systemic toxemia depending on worm load Eosinophilia, nervous manifestations, with or without diarrhea, and pain. Heavy worm load, probably following autoreinfection, may produce convulsions, insomnia, dizziness	Feces: Direct smear or concentration to show eggs
Teniasis saginata *Taenia saginata*	Abdominal and hunger pains, chronic indigestion, weight loss, persistent diarrhea or alternating with constipation; nervous manifestations. Eosinophilia	Feces: Not reliable. Recovery of gravid segments which actively crawl from anus; can be found in underwear or bed linen. Segments or eggs may be rare in feces
Teniasis solium *Taenia solium*	*Intestinal:* Same as *T. saginata. Cysticercosis:* Symptoms may vary with number of larvae and site in tissues. Foreign body response and inflammation, followed by fibrosis and necrosis of parasite, later calcification. Shows affinity for CNS, symptoms resemble brain tumor, epilepsy, and other disorders. Chief sites: Subcutaneous tissues, eye, brain	Feces: Recovery of gravid segments Recovery of larvae by biopsy from infected tissue. Detection of calcified larvae by X-ray
Hydatid disease *Echinococcus granulosus* *E. multilocularis*	*E. granulosus* produces unilocular cysts, 80–90% in liver and lungs. The host becomes sensitized following escape of fluid through fissures Pressure symptoms. Anaphylactic shock may occur upon rupture. Cachexia results from secondary metastases, pulmonary or cerebral emboli may occur. Manifestations resemble cholelithiasis or renal, hepatic or intestinal colic, sometimes of long standing. *E. multilocularis* produces uncontrolled, untreatable metastases in liver with final destruction of most of parenchyma	Cyst contents in urine. Sputum: Direct smear Serology: Complement fixation, bentonite flocculation, hemagglutination, intradermal tests. X-rays for pulmonary cysts or calcified cysts elsewhere Clinical history and picture of great value
Diphyllobothriasis (fish tapeworm disease); *Diphyllobothrium latum*	Symptomless to systemic toxemia. Pain, Weight loss, diarrhea, eosinophilia. Severe macrocytic anemia, similar to pernicious anemia, found in some cases. Worm competes with host for vitamin B_{12}	Feces: Direct smear or concentration to show eggs

Cestoda

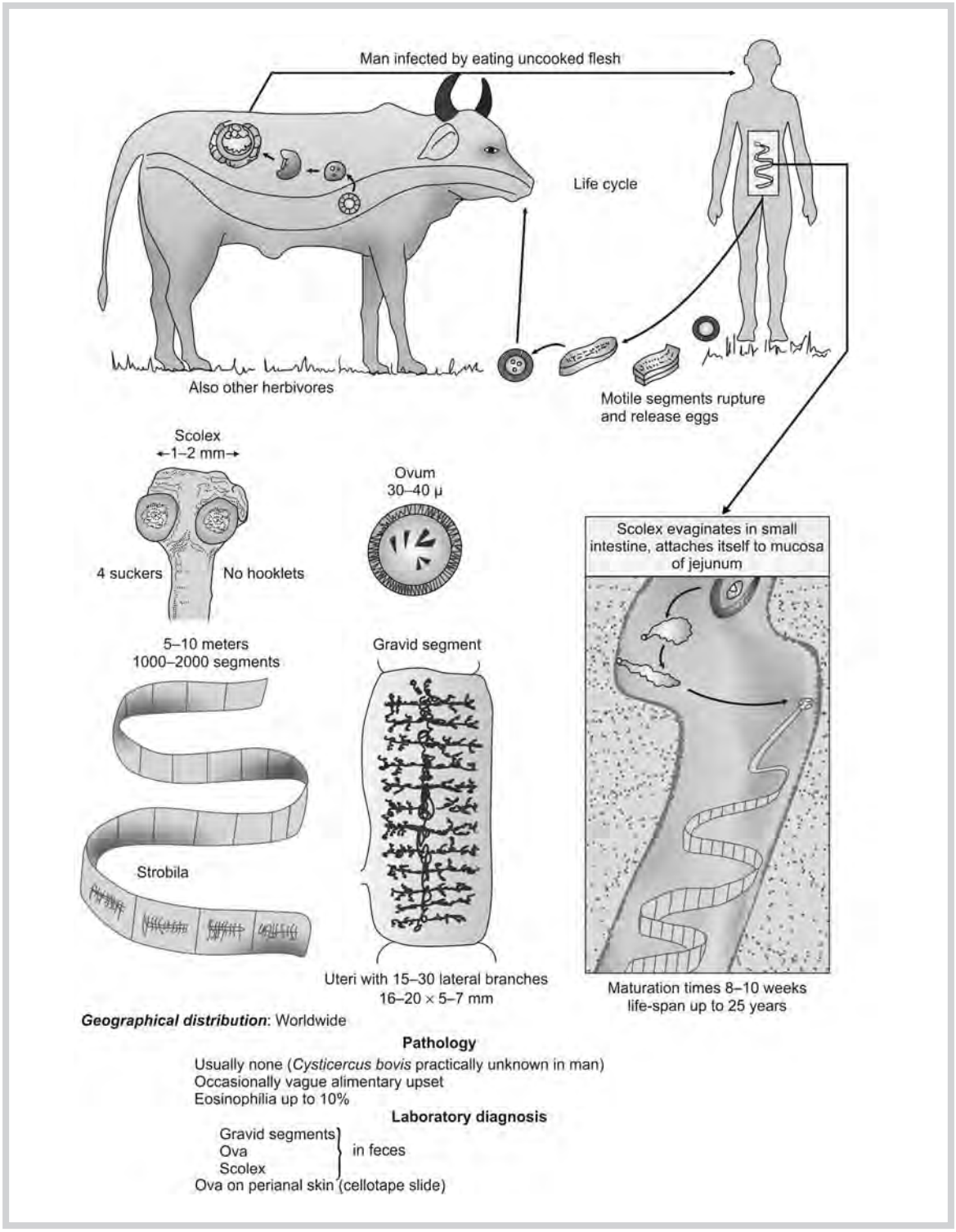
Cyclophyllidean Tapeworms of Man



Taenia solium (The Pork Tapeworm)



Taenia saginata (The Beef Tapeworm)



Geographical distribution: Worldwide

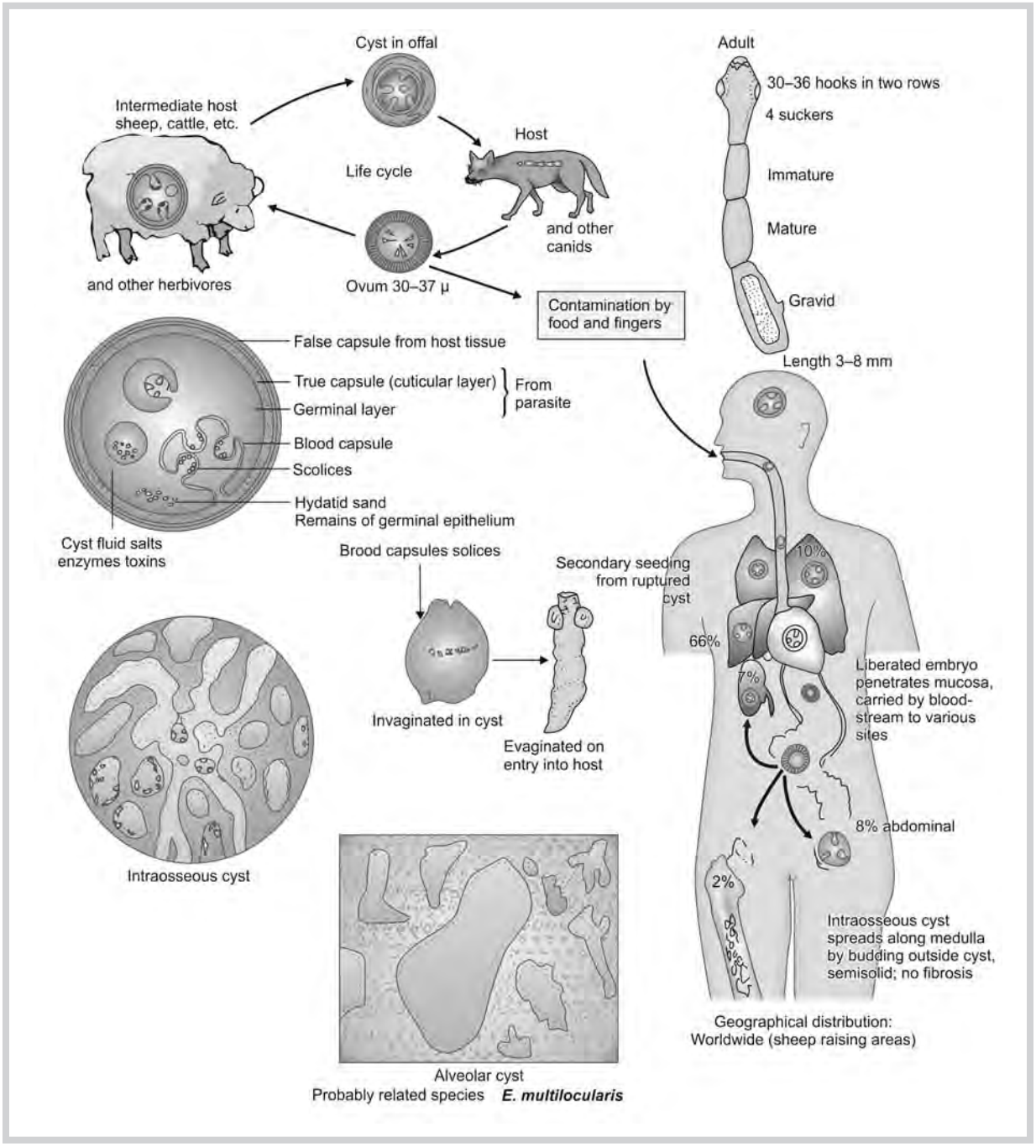
Pathology

Usually none (*Cysticercus bovis* practically unknown in man)
Occasionally vague alimentary upset
Eosinophilia up to 10%

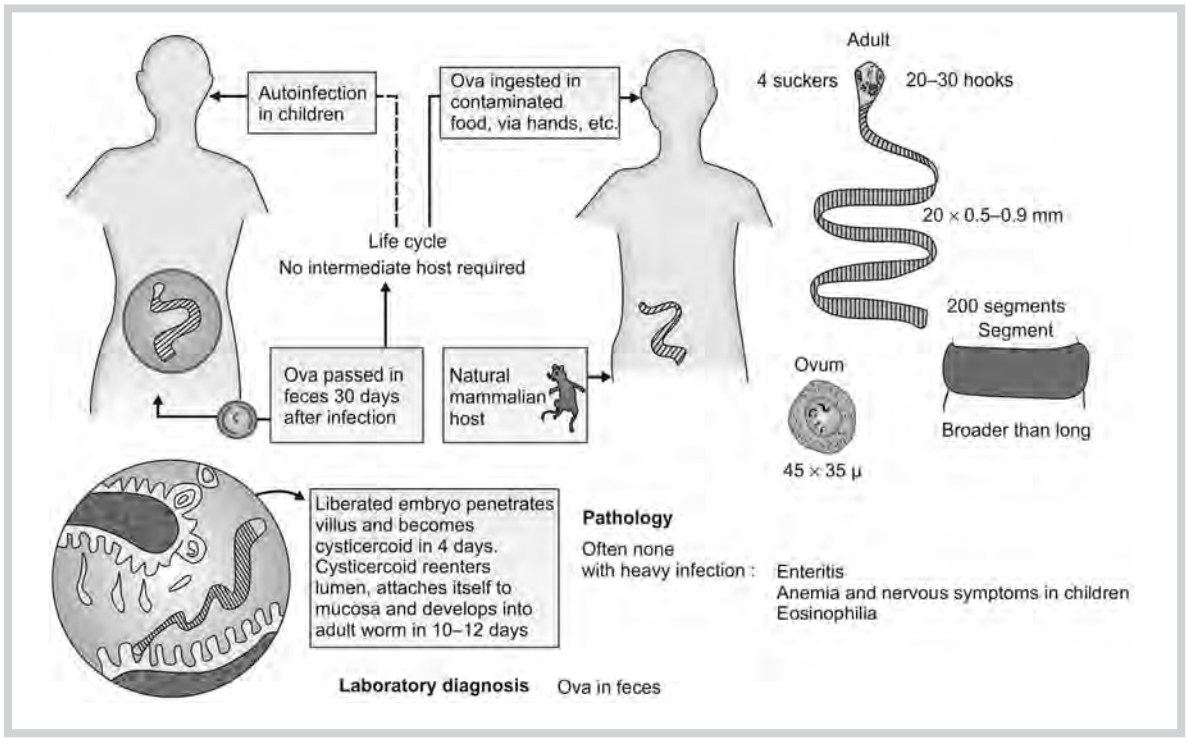
Laboratory diagnosis

Gravid segments, Ova, Scolex } in feces
Ova on perianal skin (cellotape slide)

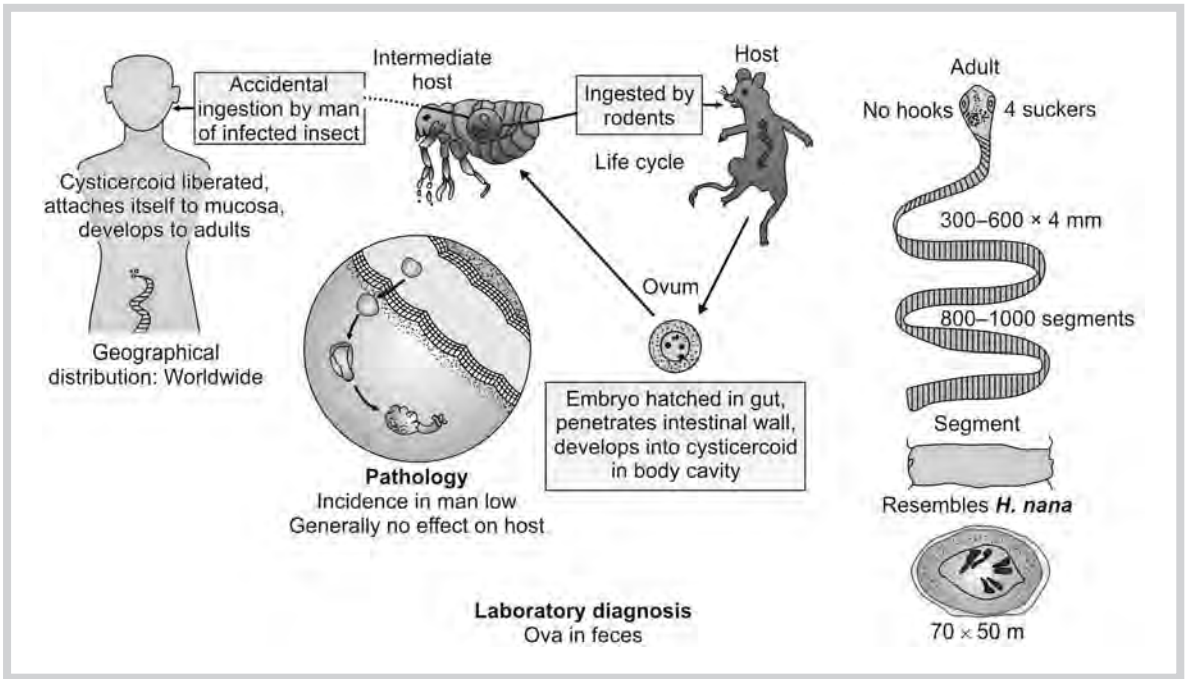
Echinococcus granulosus (Causing Hydatid Disease)



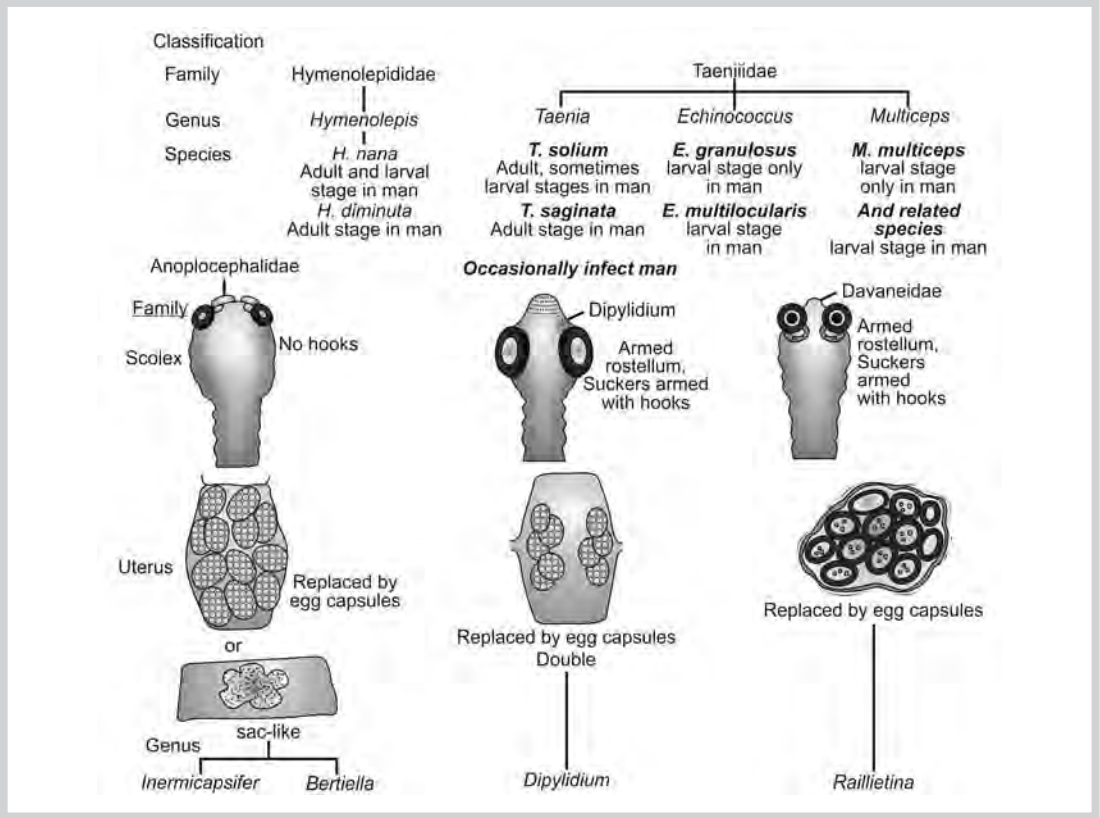
Dwarf Tapeworm Hymenolepis nana

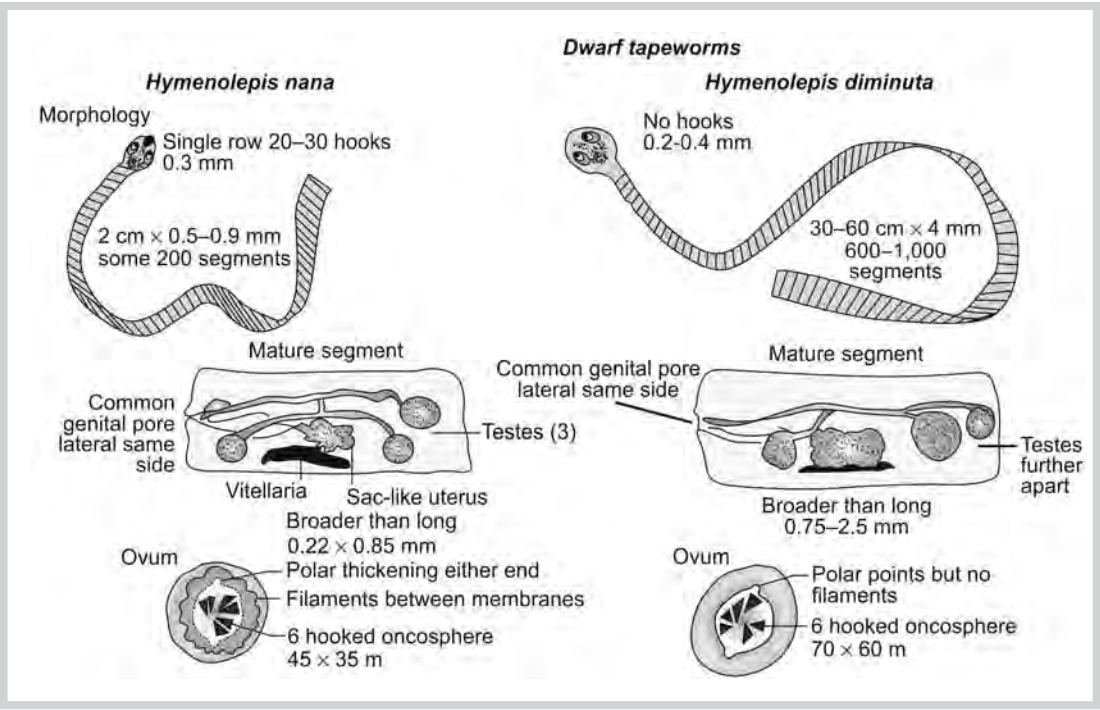


Hymenolepsis diminuta

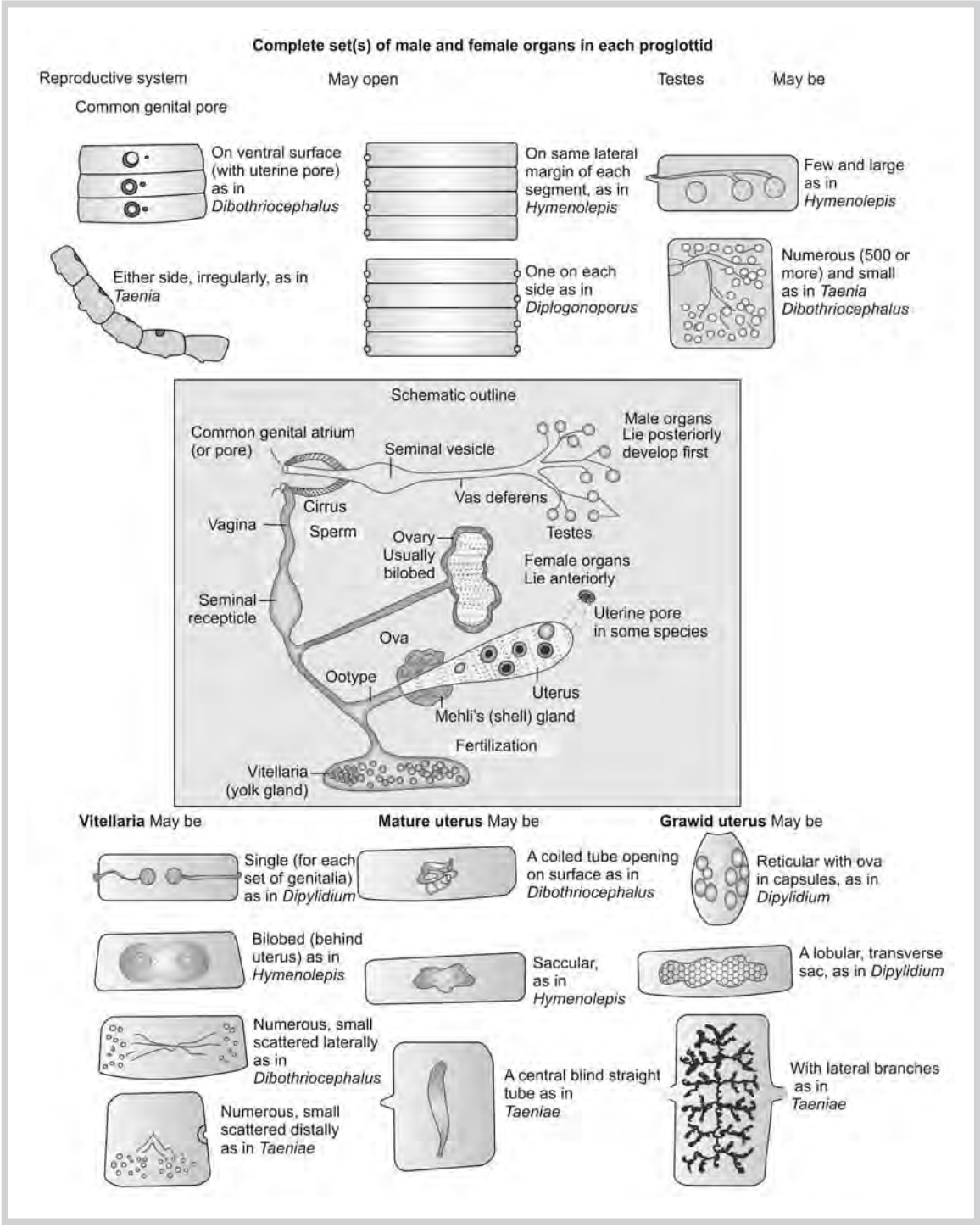


(Cestoda)


Classification
Family
Hymenolepididae
Genus
Hymenolepis
Species
H. nana
Adult and larval stage in man
H. diminuta
Adult stage in man
Taeniidae
Taenia
Echinococcus
Multiceps
T. solium
Adult, sometimes larval stages in man
T. saginata
Adult stage in man
E. granulosus
larval stage only in man
E. multilocularis
larval stage in man
M. multiceps
larval stage only in man
And related species
larval stage in man
Anoplocephalidae
Family
Scolex
No hooks
Occasionally infect man
Dipylidium
Armed rostellum, Suckers armed with hooks
Davaneidae
Armed rostellum, Suckers armed with hooks
Uterus
Replaced by egg capsules
or
sac-like
Genus
Inermicapsifer
Bertiella
Replaced by egg capsules
Double
Dipylidium
Replaced by egg capsules
Raillietina


Dwarf tapeworms
Hymenolepis nana
Hymenolepis diminuta
Morphology
Single row 20–30 hooks
0.3 mm
2 cm × 0.5–0.9 mm
some 200 segments
No hooks
0.2-0.4 mm
30–60 cm × 4 mm
600–1,000 segments
Mature segment
Common genital pore lateral same side
Testes (3)
Vitellaria
Sac-like uterus
Broader than long
0.22 × 0.85 mm
Ovum
Polar thickening either end
Filaments between membranes
6 hooked oncosphere
45 × 35 m
Mature segment
Common genital pore lateral same side
Testes further apart
Broader than long
0.75–2.5 mm
Ovum
Polar points but no filaments
6 hooked oncosphere
70 × 60 m

Cestoda (General Morphology)



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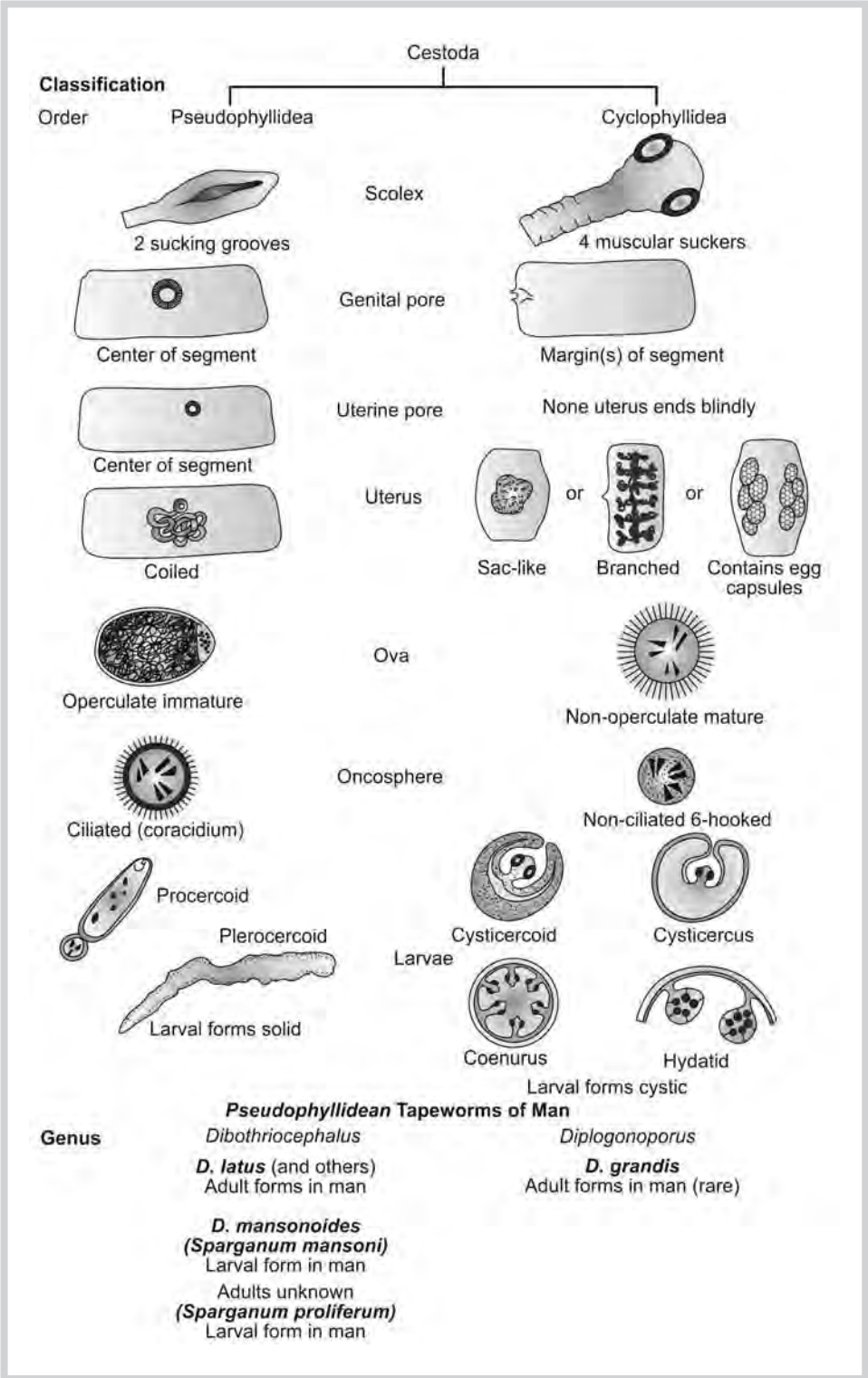

Cestoda
Classification
Order
Pseudophyllidea
Cyclophyllidea
Scolex
2 sucking grooves
4 muscular suckers
Genital pore
Center of segment
Margin(s) of segment
Uterine pore
None uterus ends blindly
Center of segment
Uterus
or
or
Coiled
Sac-like
Branched
Contains egg capsules
Ova
Operculate immature
Non-operculate mature
Oncosphere
Ciliated (coracidium)
Non-ciliated 6-hooked
Procercoid
Cysticercoid
Cysticercus
Plerocercoid
Larvae
Larval forms solid
Coenurus
Hydatid
Larval forms cystic
Pseudophyllidean Tapeworms of Man
Genus
Dibothriocephalus
Diplogonoporus
D. latus (and others)
Adult forms in man
D. grandis
Adult forms in man (rare)
D. mansonoides
(Sparganum mansoni)
Larval form in man
Adults unknown
(Sparganum proliferum)
Larval form in man

Flukes of Man (Tables 8.11 and 8.12)

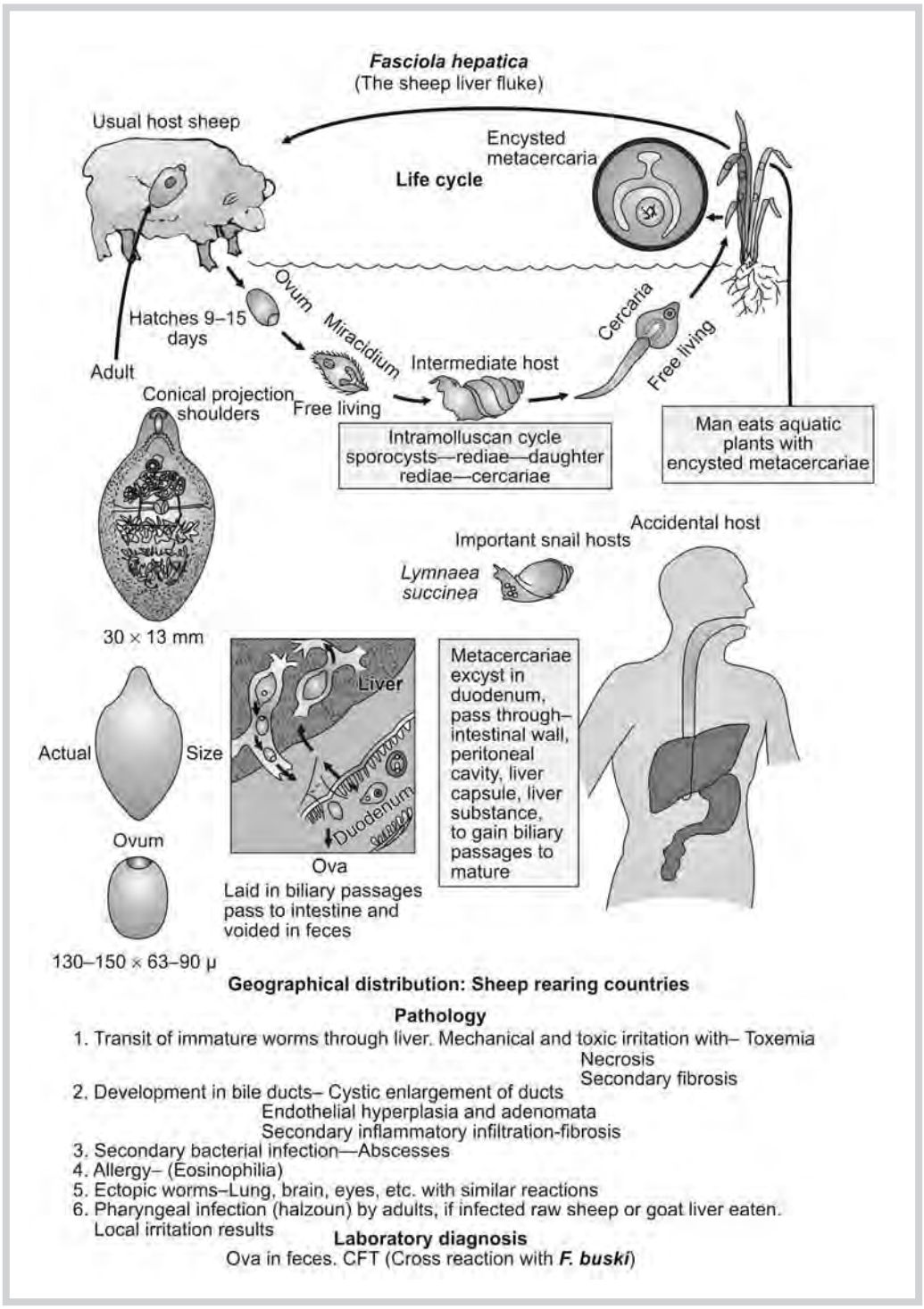
TABLE 8.11: Flukes of man

Parasite and distribution	*Definitive host*			*Intermediate host cycle outside man*	*Reservoir host*
	Enters man	*Habitat*	*Exit*		
Schistosoma haematobium Middle East, Africa, Egypt is classical focus	Cercaria penetrates skin	Vesical and pelvic venous plexuses draining urinary bladder	Terminal spined large egg in urine or feces (rare)	Egg hatches to release swimming miracidium in water. Invades appropriate snail (*Clonorchis* egg ingested by snail)	Man, monkeys
Schistosoma mansoni Africa, Latin America, Carribean Islands	-do-	Branches of inferior mesenteric veins draining rectum and sigmoid colon	Lateral spined large egg in feces	In snail tissues each miracidium becomes a sporocyst, which forms a number of embryos (sporocysts or rediae, depending on species) which in turn produce many cercariae	Man, baboons monkeys, *Possums, wild rats*
Schistosoma japonicum Japan, East Asia, Philippine Islands	-do-	Same as for *S. mansoni* but occurs chiefly in superior mesenteric veins draining small intestine	Round small spined eggs in feces	Cercariae swarm from snail	Man, horses, pigs, sheep, goats, cows, dogs, cats, water-buffaloes, rodents
Fasciolopsis buski East and South Asia	Ingested metacercaria on water plant or other vegetation	Small intestine, attached to intestinal wall		Encyst on water plants *(Fasciola and Fasciolopsis)* or invade fish *(Clonorchis)* crayfish or crab *(Paragonimus)* or directly penetrate human skin *(Schistosoma)*	Man, pigs
Fasciola hepatica Worldwide, sheep and cattle raising areas		Major bile ducts after migrating from intestine through peritoneal cavity, liver capsule, and parenchyma	Egg in bile to feces	-do-	Sheep, cattle, other herbivores, man an accidental host
Clonorchis sinensis South Asia, immigrants in America	Ingested metacercaria in raw fish	Bile ducts migrating from intestine through ampulla of Vater	-do-	-do-	Man, dogs, cats, fish-eating mammals
Paragonimus westermani	Ingested metacercaria in crayfish, crab	Encysted in lungs, pleural and peritoneal cavities, liver, migrating from intestine through peritoneal cavity	Egg in sputum or feces	-do-	Man, wildcats, foxes, wolves, dogs, rats, pigs, weasels

TABLE 8.12: Fluke diseases of man

Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Schistosomiasis or bilharziasis *Schistosoma* *haematobium* *mansoni* *japonicum*	*Initial:* Skin penetration by cercariae producing itching, erythema, petechiae, usually a hypersensitization following repeated exposures. Eosinophilia in later case, with fever *Maturation of worms:* Fever, hepatomegaly with tenderness, edema, diarrhea; eosinophilia variable. *Adult worms:* Systemic and histologic changes mainly due to granulomatous reaction against eggs acting as foreign bodies. Symptoms vary with duration, frequency, and severity of exposure and degree of host reaction, itself related to age, nutrition and concurrent infections. Generally with bowel and vesical disturbances, lasting for months to years. *Chronic disturbances:* Portal hypertension with resulting esophageal varices; liver and intestinal or bladder granulomata and fibrosis Thickening and calcification in bladder wall (*S. haematobium*), thickening of small intestine (*S. japonicum*), prolapse of rectum (*S. mansoni*), loss of gut motility, local tissue and organ dysfunction	*Urine*: Direct smear for eggs, sedimentation and hatching of miracidia in diluted urine for *S. haematobium*, preferably in last portion of urine passed. *Feces*: Direct smear; concentration for *S. mansoni* or *japonicum* eggs. Rectal biopsy and sigmoidoscopy (*S. haematobium*). *Blood*: Precipitin (circumoval or CHR) test
Fasciolopsiasis *Fasciolopsis buski*	Localized inflammation of jejunum or duodenum, followed often by ulceration at sites of worm attachment. Diarrhea with foul-smelling stools; abdominal pain. In severe infections eosinophilia, ascites, anorexia, nausea, vomiting, toxemia, prostration	*Feces*: Direct smear, sedimentation
Fascioliosis *Fasciola hepatica*	Worm migrations cause tissue necrosis and fibrosis. Bile duct damage resembles that for *Clonorchis*. Picture like any biliary derangement (gallbladder, choledochus involvement). Eosinophilia. Infection runs a chronic course of many years	*Feces*: Direct smear, sedimentation. Duodenal drainage; complement fixation, skin tests
Clonorchiasis *Clonorchis sinensis*	Similar hepatic involvement, large numbers of parasites cause diarrhea, jaundice, cachexia, eosinophilia. Proliferation and desquamation of biliary epithelium, dilatation, and thickening of the wall occur, severe symptoms of liver dysfunction, recurring jaundice with hepatomegaly may follow. Long continued chronic course common	Direct smear, sedimentation; eggs in biliary drainage
Paragonimiasis *Paragonimus westermani*	*Lung:* Parasites are embedded, usually in pairs, in subpleural cysts (eggs act as foreign bodies) with inflammation, eosinophilia and fibrous capsule formation. Chronic cough with fever, brown sputum, hemoptysis, severe chest pain, bronchial pneumonia or pleural fluid common. May enter any organ and produce local symptoms, e.g. abdominal pain, diarrhea, CNS involvement. Similar lesions in other tissues.	*Sputum*: Direct smear. *Feces*: Direct smear, sedimentation; complement fixation, etc.

Fasciola hepatica (The Sheep Liver Fluke)



Geographical distribution: Sheep rearing countries

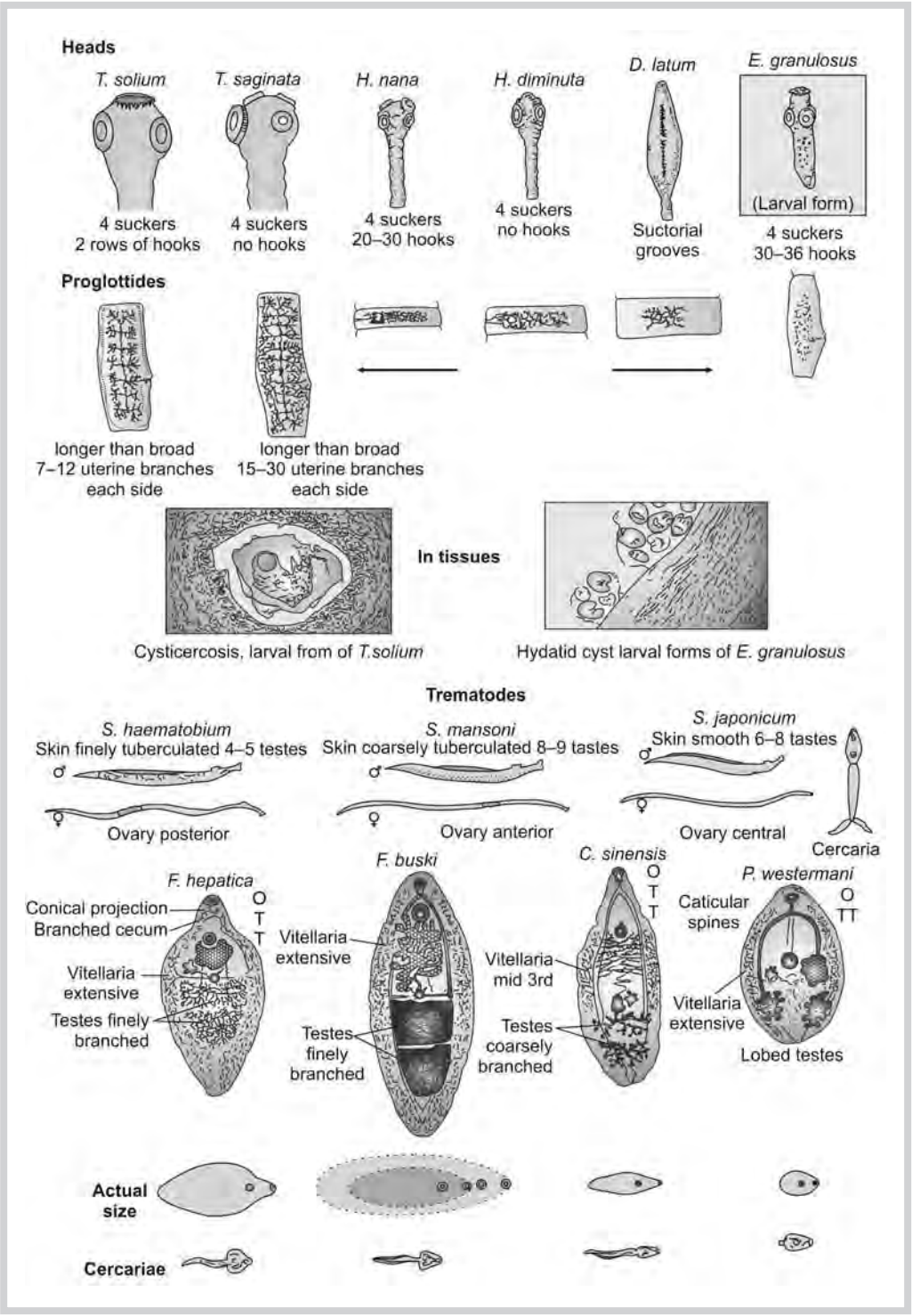
Pathology

1. Transit of immature worms through liver. Mechanical and toxic irritation with– Toxemia
   Necrosis
   Secondary fibrosis
2. Development in bile ducts– Cystic enlargement of ducts
   Endothelial hyperplasia and adenomata
   Secondary inflammatory infiltration-fibrosis
3. Secondary bacterial infection—Abscesses
4. Allergy– (Eosinophilia)
5. Ectopic worms–Lung, brain, eyes, etc. with similar reactions
6. Pharyngeal infection (halzoun) by adults, if infected raw sheep or goat liver eaten.
   Local irritation results

Laboratory diagnosis

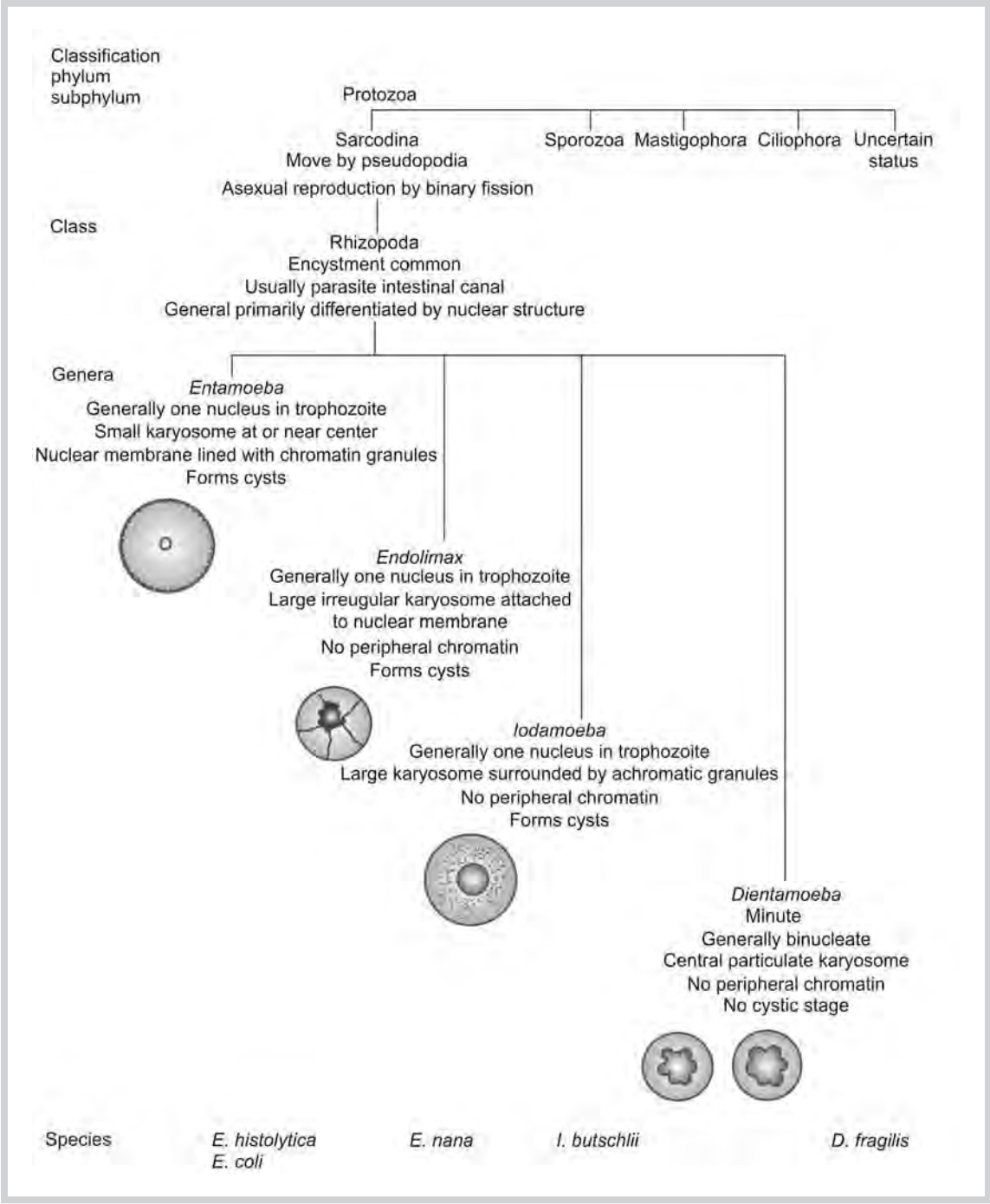
Ova in feces. CFT (Cross reaction with ***F. buski***)

Morphology of Adults and Larvae Cestoda (Contd...)

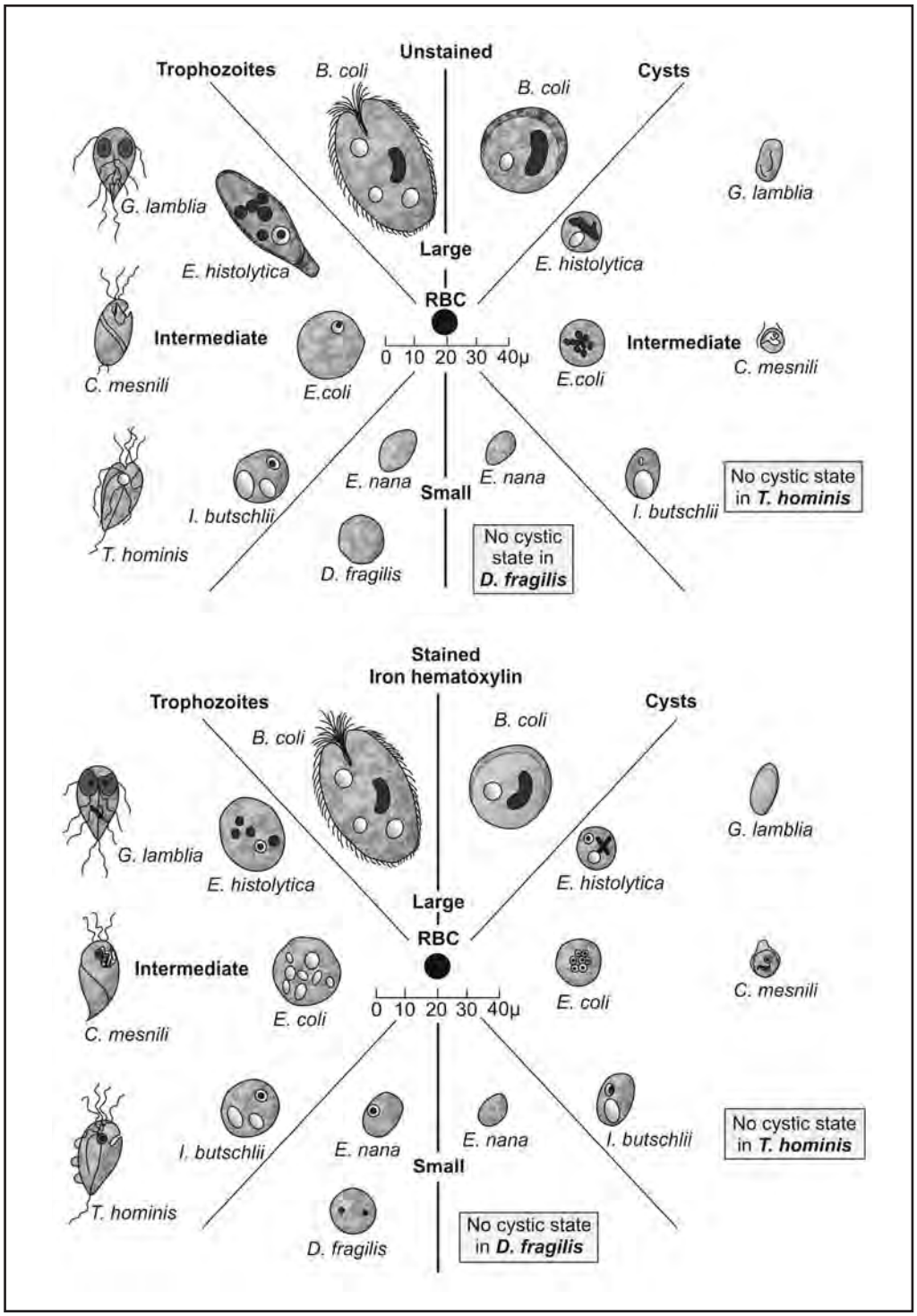


Recapitulation—Parasitology at a Glance

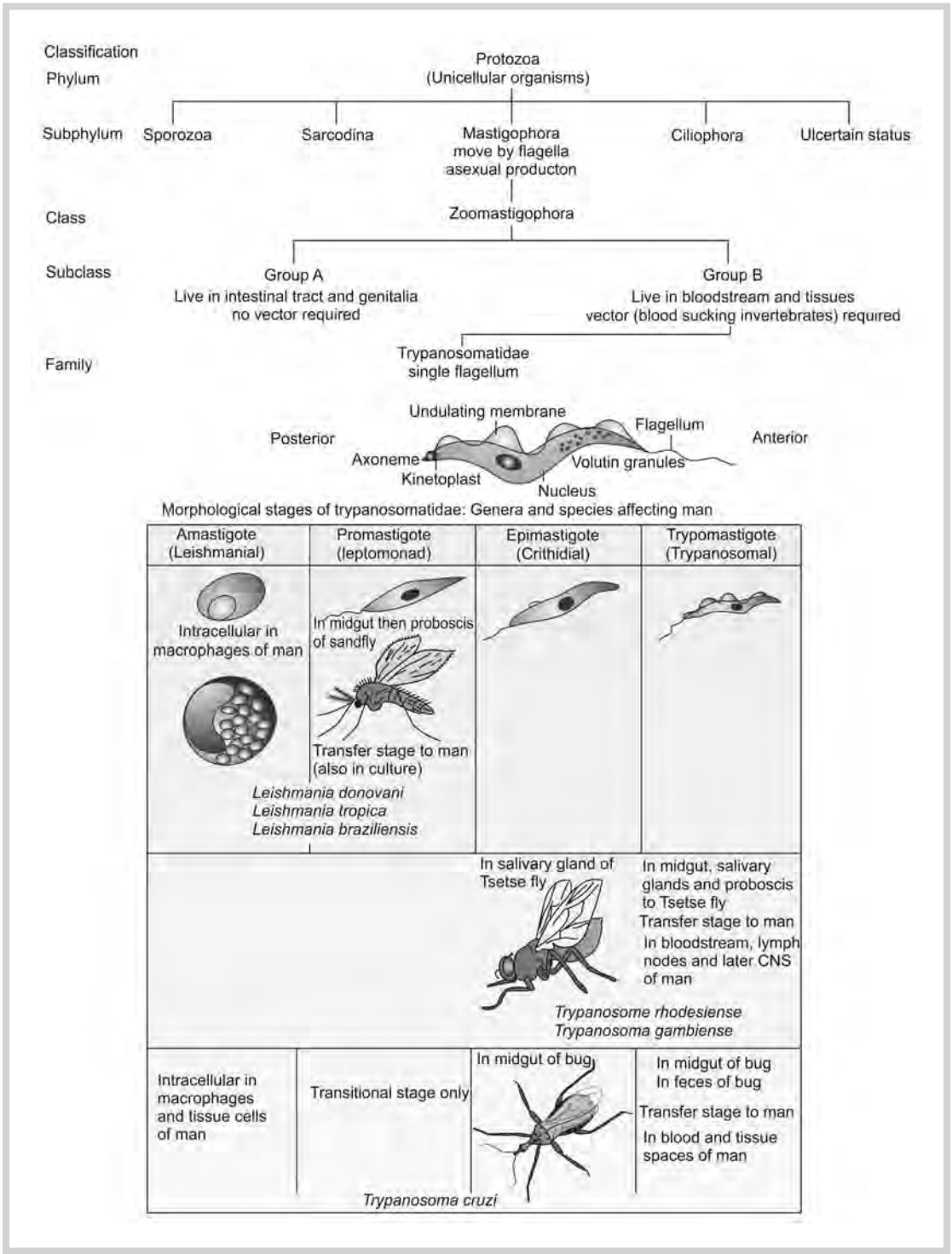
The Amebae of the Intestinal Canal



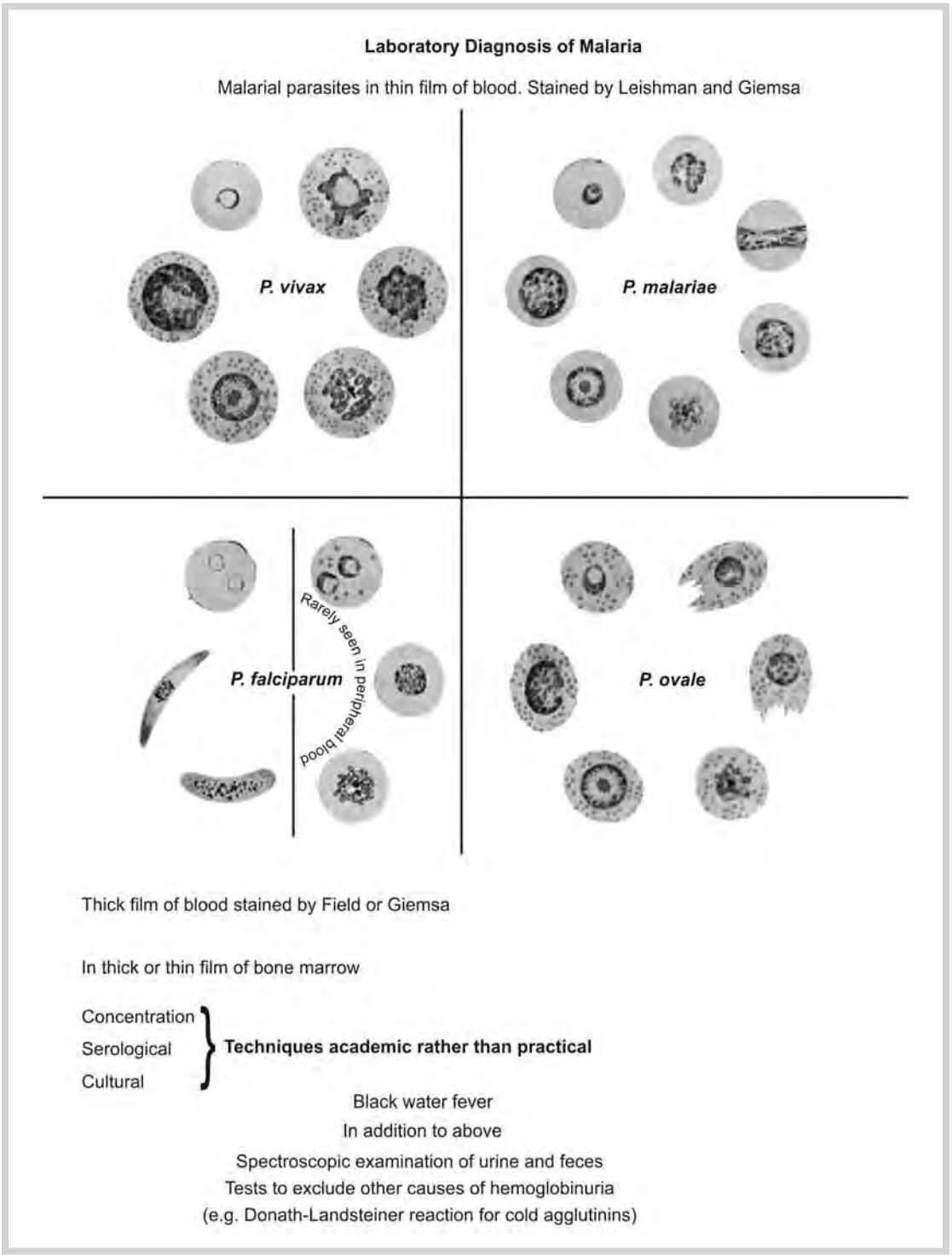
Recapitulation Protozoa Inhibiting the Intestine



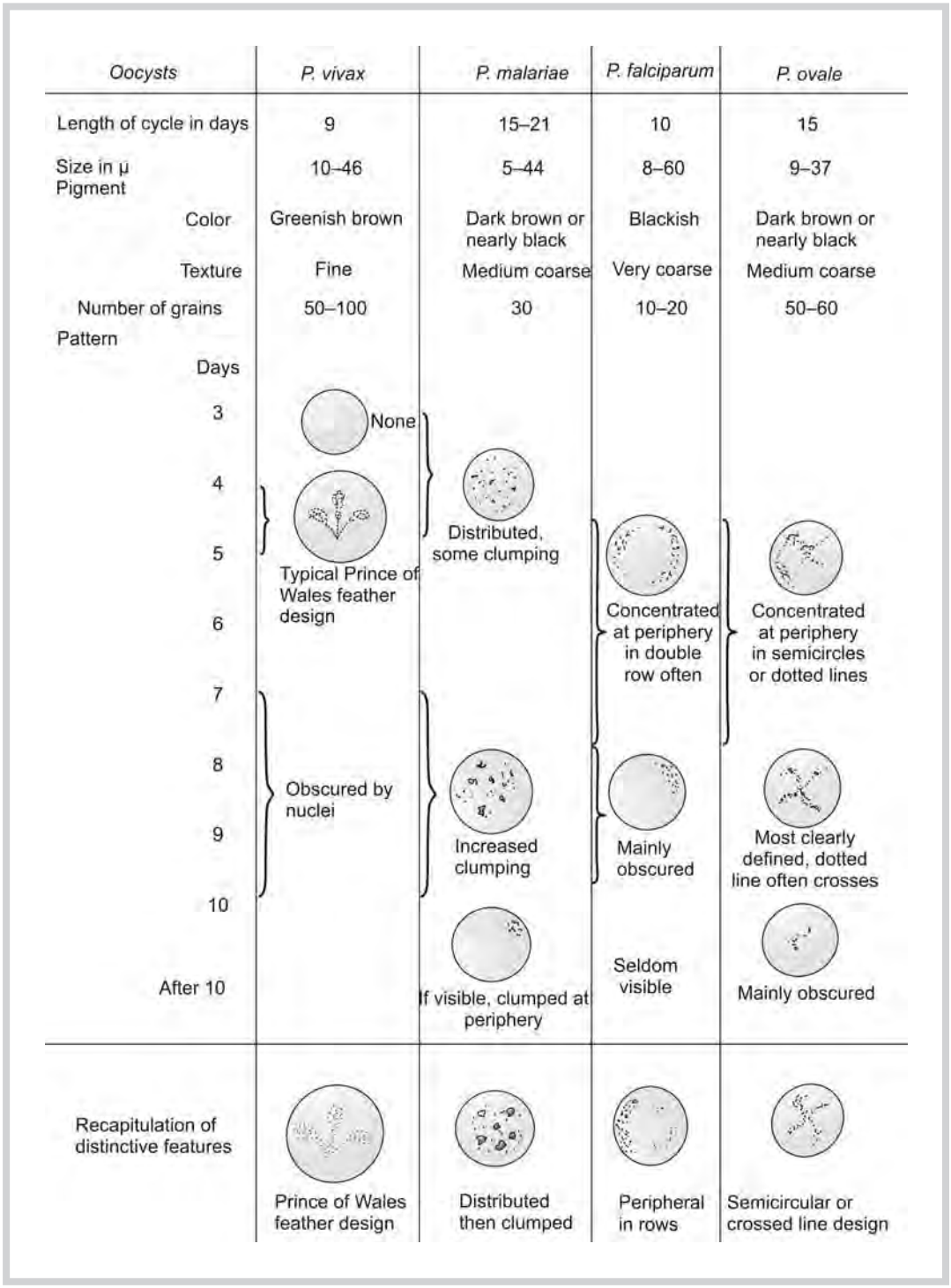
The Body Fluid and Tissue Flagellates (Causing Leishmaniasis and Trypanosomiasis)



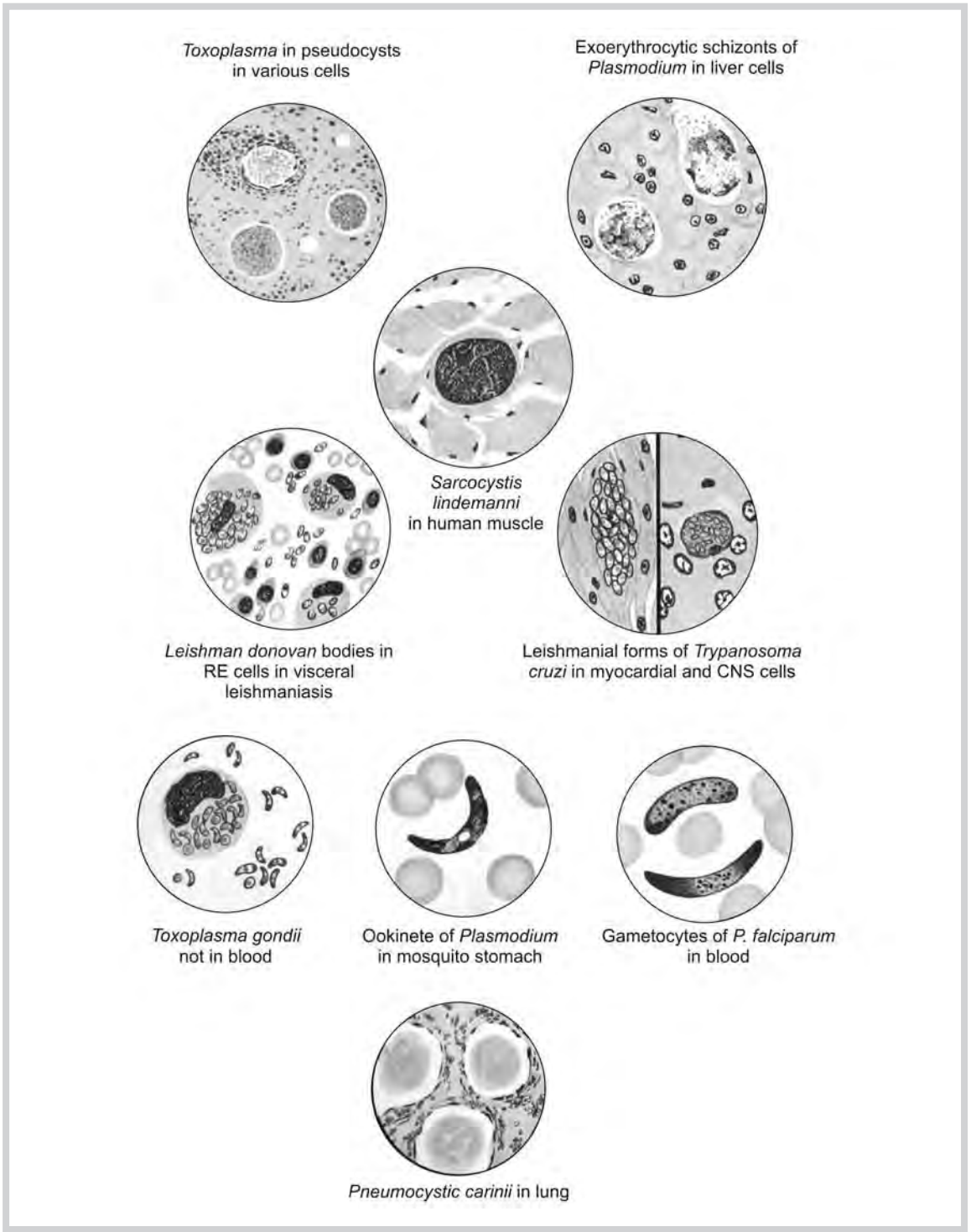
Laboratory Diagnosis of Malaria



Malaria Species Identification in the Mosquito—Pigment in Oocysts



Recapitulation Morphological Differentiation



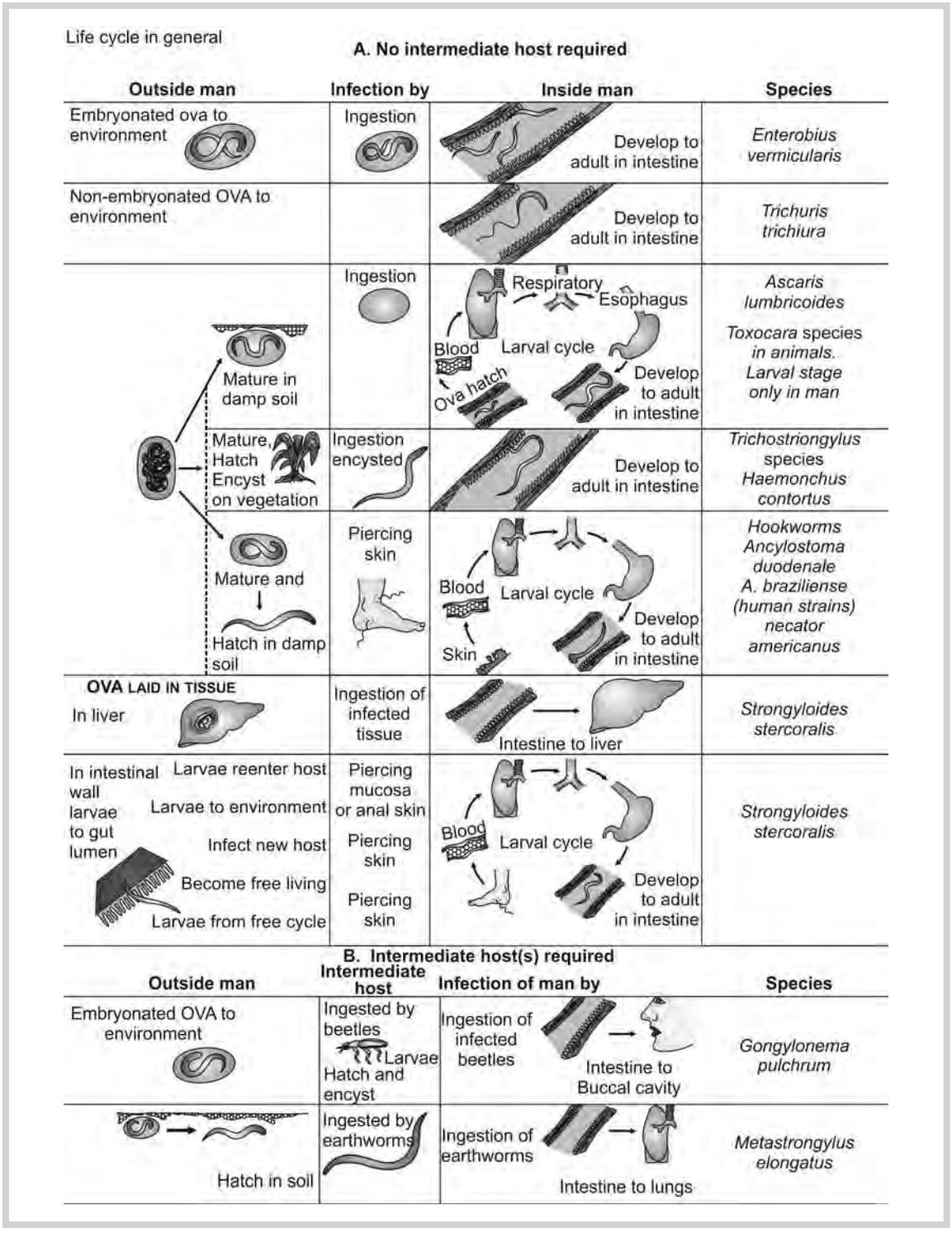
Recapitulation

Recapitulation

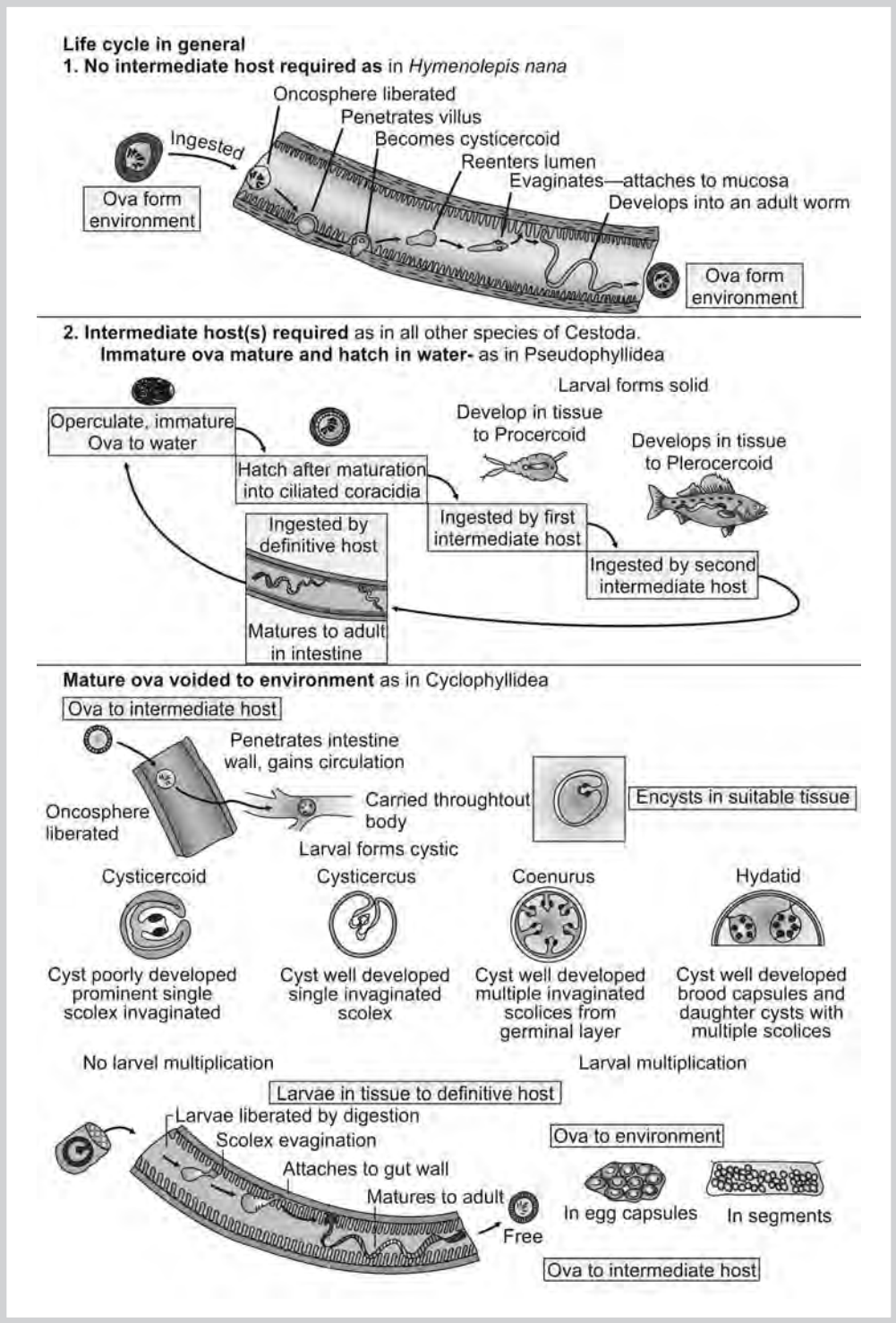
The essential differences between:

	African trypanosolasis (sleeping .sickness)	South American *trypanosomiasis* (Chagas' disease)	*Visceral leishmaniasis* (Kala azar)
Caused by	*Trypanosoma gambiense or Trypanosoma rhodesiense*	*Trypanosoma cruzi*	*Leishmania donovani*
Vector	Glossina species (Tsetse flies)	Triatomidna species (cone-nosed bugs)	Phlebotomus species (sandflies)
Cycle in vector	Anterior station development	Posterior station development	Anterior station development
Stage ingested then	Trypanosomal	Trypanosomal	Leishmanial
	Multiply and move forward Enter salivary glands via duct	Metamorphose to crithidia Multiply move backward	Metamorphose to leptomonad Multiply
	Metamorphose to crithidia multiply		Move backward Block proboscis
	Metamorphose to metacyclic Trypanosomes	Metamorphose to metacyclic Trypanosomes	
	Infective : Injected in saliva	Infective : Passed in bug feces	Infective : Injected from proboscis
Cycle in man			
Form injected	Metacyclic trypanosome	Metacyclic trypanosome	Leptomonad
	None-remain as trypanosomes	Enter histiocytes locally and become leishmanial	Enter histiocytes locally and become leishmanial
Multiplication (all by binary fission)	As trypanosomes in blood and tissue spaces	As leishmanial forms in cells	As leishmanial forms in macrophage cells
Then	Remain in bloodstream as trypanosomes	Carried to regional nodes: some Leishmanial forms infect further histiocytes: some metamorphose through transitional leptomonad and crithidial forms to gain blood as trypanosomes. Do not multiply as such but disseminated, enter further tissue cells, metamorphose to leishmanial forms and multiply	Further dissemination as leishmanial form only
Pathogenesis	Circulating, multiplying trypanosomes cause parasitemia and toxemia damaging tissues	Parasitization and destruction of all type of tissue cell Circulating trypanosomes produce toxemia	Parasitization and destruction RE cell
Pathological effects	Mainly general toxemia lymphadenopathy CNS involvement	General toxemia local functional disability of whichever tissues invaded, especially lymph nodes, heart, CNS	General toxemia from breakdown of RE cells Proliferation of RE cells (mainly spleen, liver, bone marrow, lymph nodes) No CNS involvement
Clinico-pathological correlation	Acute (*T. rhodesiense*) Fever and severe toxemia Chronic (*T. gambiense*) Fever Lymphadenopathy Encephalitis	Fever Enlarged nodes, spleen and liver Protein manifestations depending on localization in tissues especially Cardiac CNS syndromes	Fever Anemia Enlarged liver and spleen

Nematoda


Life cycle in general
A. No intermediate host required
Outside man
Infection by
Inside man
Species
Embryonated ova to environment
Ingestion
Develop to adult in intestine
Enterobius vermicularis
Non-embryonated OVA to environment
Develop to adult in intestine
Trichuris trichiura
Ingestion
Respiratory
Esophagus
Blood
Larval cycle
Ova hatch
Develop to adult in intestine
Ascaris lumbricoides
Toxocara species in animals. Larval stage only in man
Mature in damp soil
Mature, Hatch Encyst on vegetation
Ingestion encysted
Develop to adult in intestine
Trichostrongylus species Haemonchus contortus
Piercing skin
Mature and
Hatch in damp soil
Blood
Larval cycle
Skin
Develop to adult in intestine
Hookworms Ancylostoma duodenale A. braziliense (human strains) necator americanus
OVA LAID IN TISSUE
In liver
Ingestion of infected tissue
Intestine to liver
Strongyloides stercoralis
In intestinal wall larvae to gut lumen
Larvae reenter host
Larvae to environment
Infect new host
Become free living
Larvae from free cycle
Piercing mucosa or anal skin
Piercing skin
Piercing skin
Blood
Larval cycle
Develop to adult in intestine
Strongyloides stercoralis
B. Intermediate host(s) required
Outside man
Intermediate host
Infection of man by
Species
Embryonated OVA to environment
Ingested by beetles
Larvae
Hatch and encyst
Ingestion of infected beetles
Intestine to Buccal cavity
Gongylonema pulchrum
Hatch in soil
Ingested by earthworms
Ingestion of earthworms
Intestine to lungs
Metastrongylus elongatus

Cestoda



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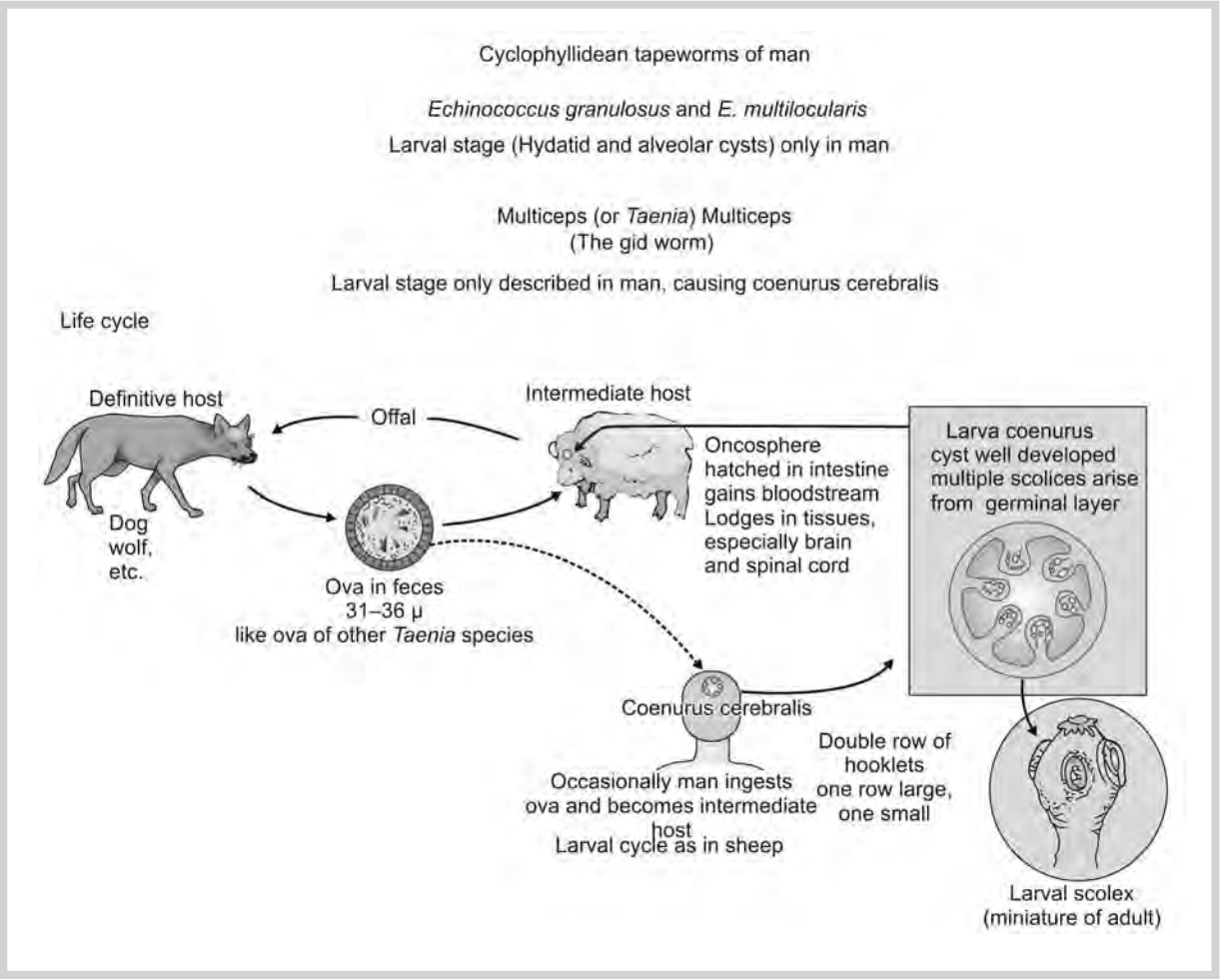
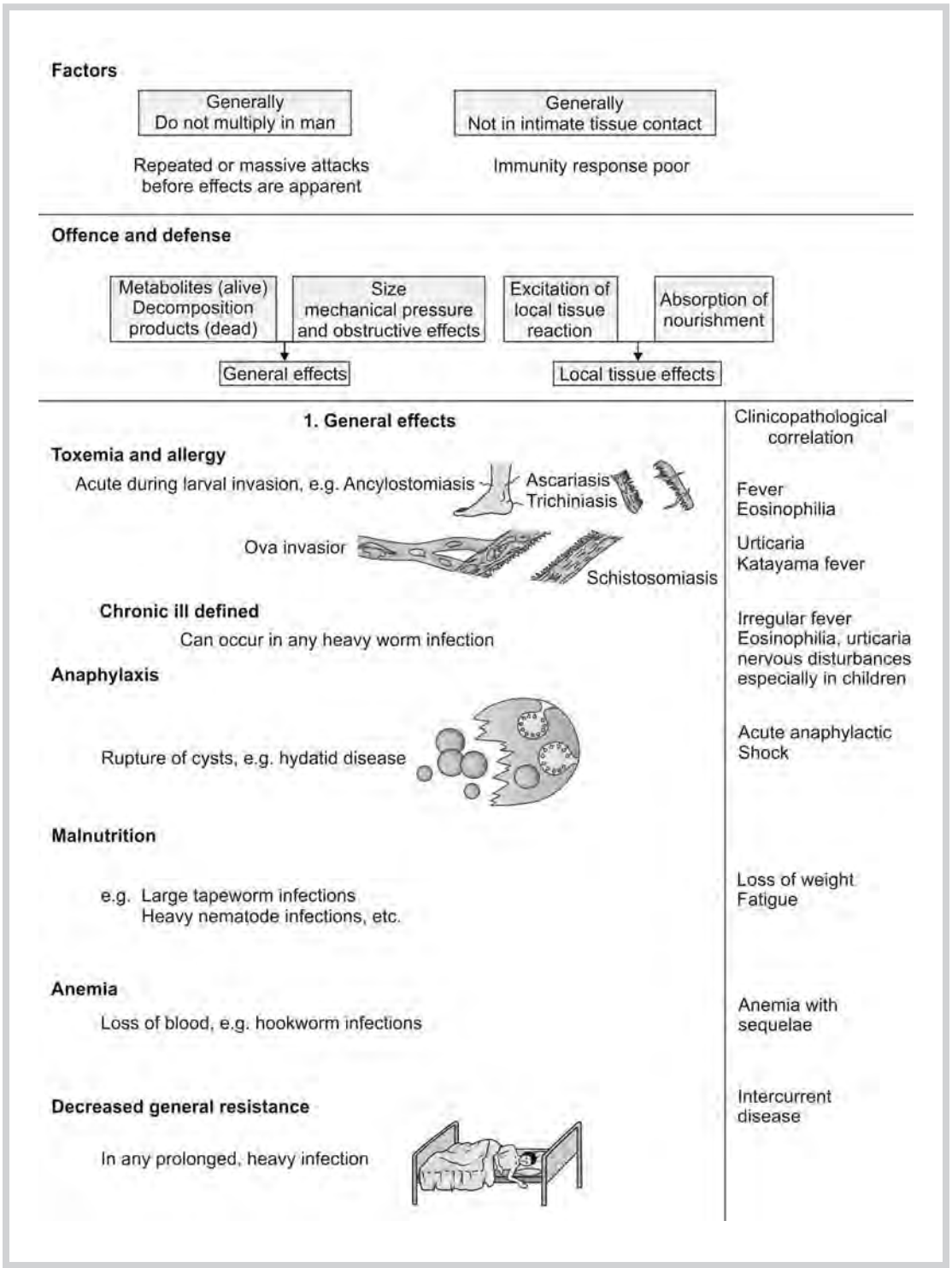

Cyclophyllidean tapeworms of man
Echinococcus granulosus and E. multilocularis
Larval stage (Hydatid and alveolar cysts) only in man
Multiceps (or Taenia) Multiceps
(The gid worm)
Larval stage only described in man, causing coenurus cerebralis
Life cycle
Definitive host
Offal
Intermediate host
Dog
wolf,
etc.
Ova in feces
31–36 μ
like ova of other Taenia species
Oncosphere
hatched in intestine
gains bloodstream
Lodges in tissues,
especially brain
and spinal cord
Larva coenurus
cyst well developed
multiple scolices arise
from germinal layer
Coenurus cerebralis
Occasionally man ingests
ova and becomes intermediate
host
Larval cycle as in sheep
Double row of
hooklets
one row large,
one small
Larval scolex
(miniature of adult)

Recapitulation Pathogenesis and Pathology of Worm Infections



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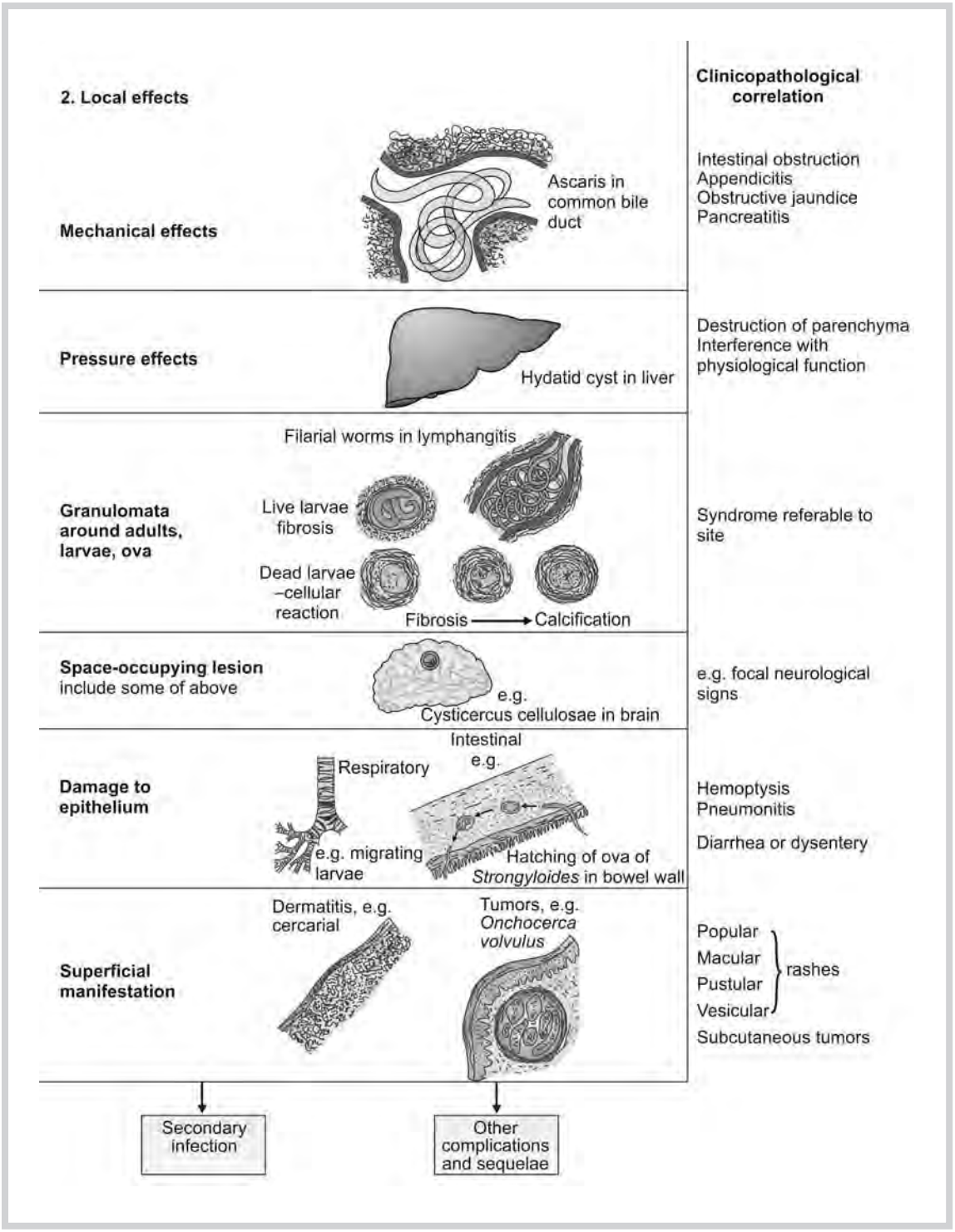
2. Local effects
Clinicopathological correlation
Mechanical effects
Ascaris in common bile duct
Intestinal obstruction
Appendicitis
Obstructive jaundice
Pancreatitis
Pressure effects
Hydatid cyst in liver
Destruction of parenchyma
Interference with physiological function
Filarial worms in lymphangitis
Granulomata around adults, larvae, ova
Live larvae fibrosis
Dead larvae –cellular reaction
Fibrosis → Calcification
Syndrome referable to site
Space-occupying lesion include some of above
e.g.
Cysticercus cellulosae in brain
e.g. focal neurological signs
Damage to epithelium
Respiratory
e.g. migrating larvae
Intestinal
e.g.
Hatching of ova of Strongyloides in bowel wall
Hemoptysis
Pneumonitis
Diarrhea or dysentery
Superficial manifestation
Dermatitis, e.g. cercarial
Tumors, e.g. Onchocerca volvulus
Popular
Macular
Pustular
Vesicular
rashes
Subcutaneous tumors
Secondary infection
Other complications and sequelae

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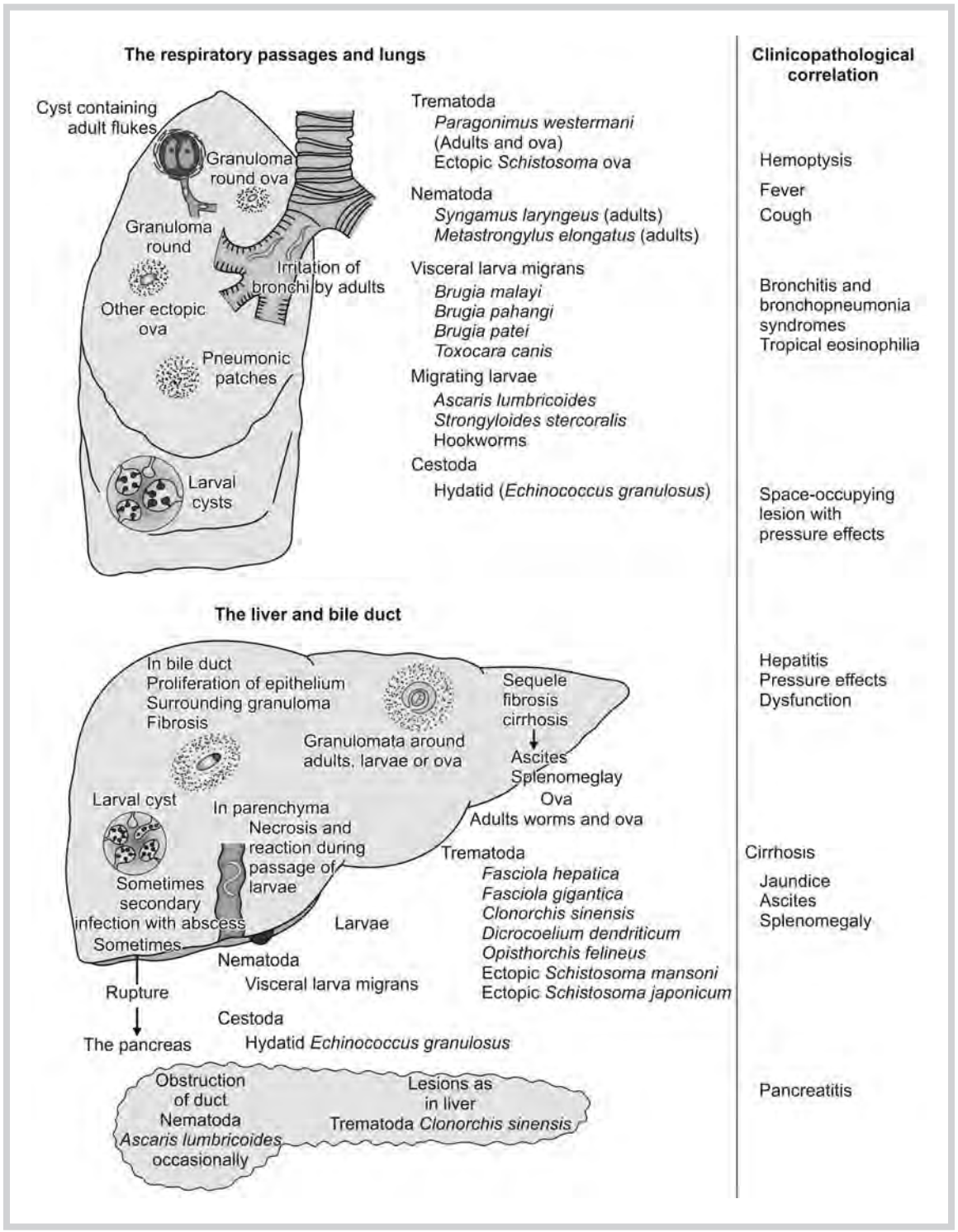
The respiratory passages and lungs
Cyst containing adult flukes
Granuloma round ova
Granuloma round
Irritation of bronchi by adults
Other ectopic ova
Pneumonic patches
Larval cysts
Trematoda
Paragonimus westermani (Adults and ova)
Ectopic Schistosoma ova
Nematoda
Syngamus laryngeus (adults)
Metastrongylus elongatus (adults)
Visceral larva migrans
Brugia malayi
Brugia pahangi
Brugia patei
Toxocara canis
Migrating larvae
Ascaris lumbricoides
Strongyloides stercoralis
Hookworms
Cestoda
Hydatid (Echinococcus granulosus)
Clinicopathological correlation
Hemoptysis
Fever
Cough
Bronchitis and bronchopneumonia syndromes
Tropical eosinophilia
Space-occupying lesion with pressure effects
The liver and bile duct
In bile duct
Proliferation of epithelium
Surrounding granuloma
Fibrosis
Granulomata around adults, larvae or ova
Sequele fibrosis cirrhosis
Ascites
Splenomeglay
Ova
Adults worms and ova
Larval cyst
In parenchyma
Necrosis and reaction during passage of larvae
Sometimes secondary infection with abscess
Sometimes
Rupture
The pancreas
Larvae
Nematoda
Visceral larva migrans
Cestoda
Hydatid Echinococcus granulosus
Trematoda
Fasciola hepatica
Fasciola gigantica
Clonorchis sinensis
Dicrocoelium dendriticum
Opisthorchis felineus
Ectopic Schistosoma mansoni
Ectopic Schistosoma japonicum
Hepatitis
Pressure effects
Dysfunction
Cirrhosis
Jaundice
Ascites
Splenomegaly
Obstruction of duct
Nematoda
Ascaris lumbricoides occasionally
Lesions as in liver
Trematoda Clonorchis sinensis
Pancreatitis

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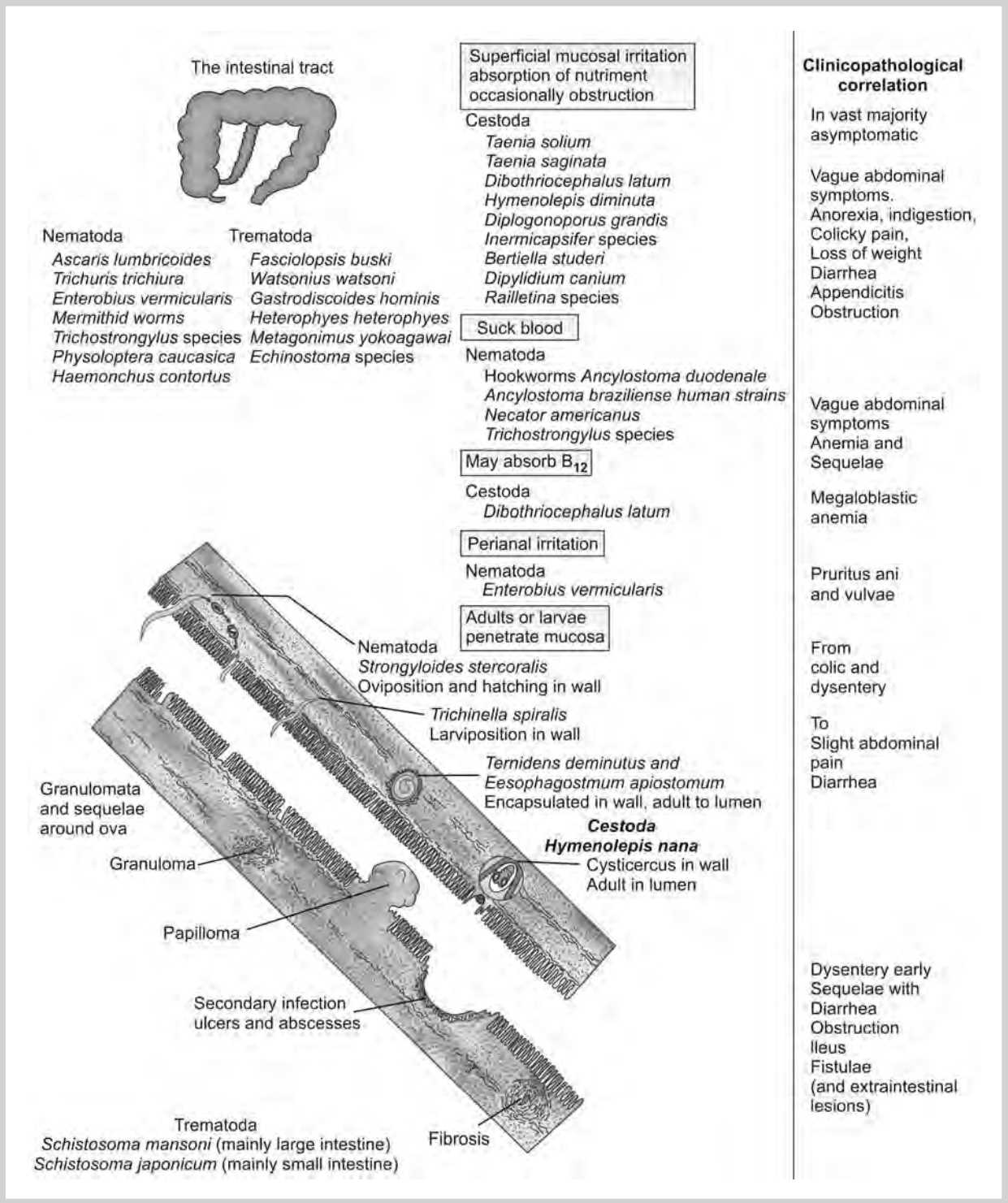
The intestinal tract
Nematoda
Ascaris lumbricoides
Trichuris trichiura
Enterobius vermicularis
Mermithid worms
Trichostrongylus species
Physoloptera caucasica
Haemonchus contortus
Trematoda
Fasciolopsis buski
Watsonius watsoni
Gastrodiscoides hominis
Heterophyes heterophyes
Metagonimus yokoagawai
Echinostoma species
Superficial mucosal irritation
absorption of nutriment
occasionally obstruction
Cestoda
Taenia solium
Taenia saginata
Dibothriocephalus latum
Hymenolepis diminuta
Diplogonoporus grandis
Inermicapsifer species
Bertiella studeri
Dipylidium canium
Railletina species
Suck blood
Nematoda
Hookworms Ancylostoma duodenale
Ancylostoma braziliense human strains
Necator americanus
Trichostrongylus species
May absorb B12
Cestoda
Dibothriocephalus latum
Perianal irritation
Nematoda
Enterobius vermicularis
Adults or larvae
penetrate mucosa
Nematoda
Strongyloides stercoralis
Oviposition and hatching in wall
Trichinella spiralis
Larviposition in wall
Ternidens deminutus and
Eesophagostmum apiostomum
Encapsulated in wall, adult to lumen
Cestoda
Hymenolepis nana
Cysticercus in wall
Adult in lumen
Granulomata
and sequelae
around ova
Granuloma
Papilloma
Secondary infection
ulcers and abscesses
Fibrosis
Trematoda
Schistosoma mansoni (mainly large intestine)
Schistosoma japonicum (mainly small intestine)
Clinicopathological correlation
In vast majority asymptomatic
Vague abdominal symptoms.
Anorexia, indigestion,
Colicky pain,
Loss of weight
Diarrhea
Appendicitis
Obstruction
Vague abdominal symptoms
Anemia and
Sequelae
Megaloblastic anemia
Pruritus ani and vulvae
From colic and dysentery
To
Slight abdominal pain
Diarrhea
Dysentery early
Sequelae with
Diarrhea
Obstruction
Ileus
Fistulae
(and extraintestinal lesions)

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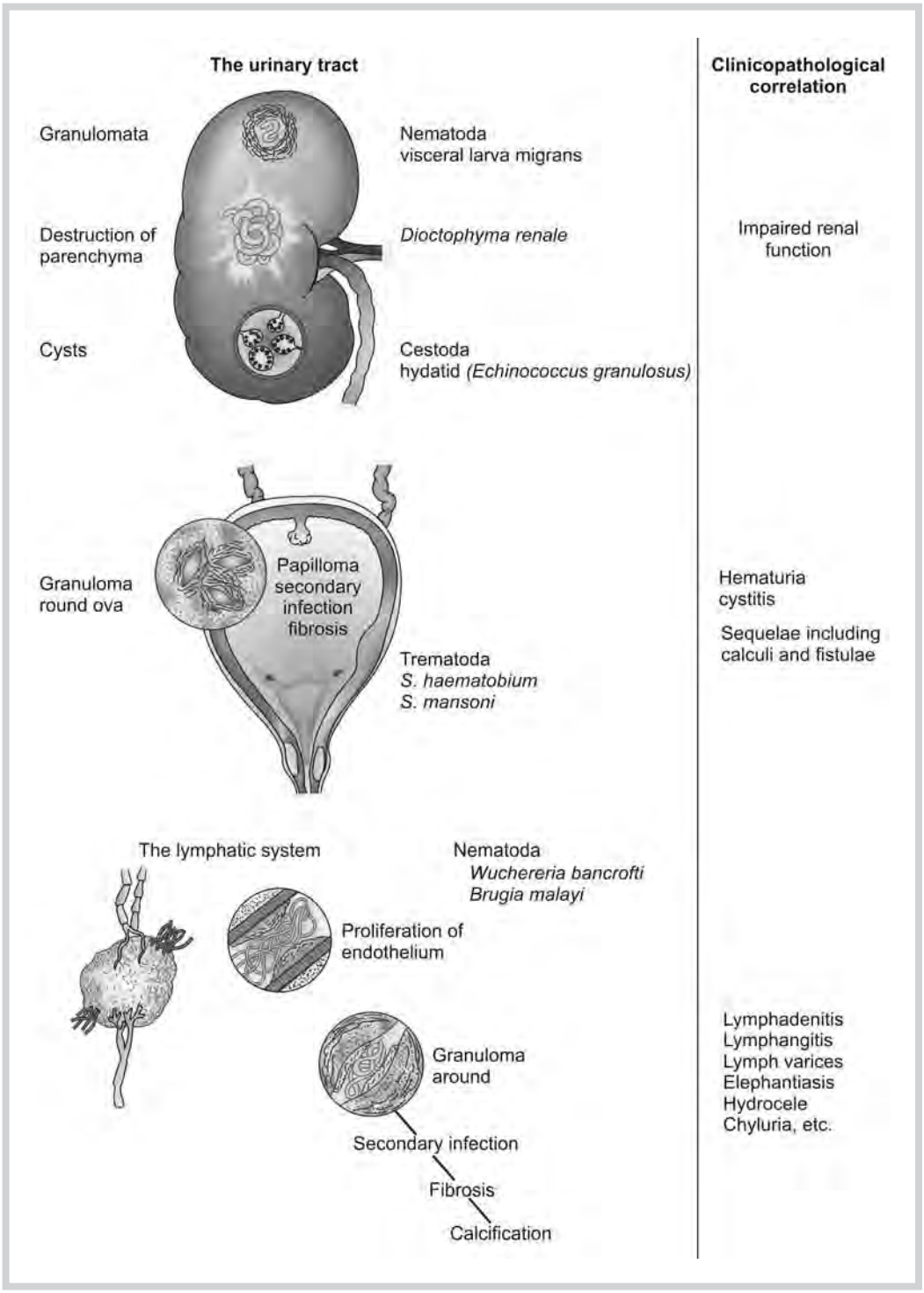
Local Effects of Worm Infections



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The circulatory system	Clinicopathological correlation
Transient in blood	
1. Larvae to pulmonary capillaries	
Nematoda	
Ascaris lumbricoides	Hemoptysis
Strongyloides stercoralis	Pneumonia
Ancylostoma duodenale	sometimes
Ancylostoma braziliense (human strain)	Tropical eosinphilia
Necator americanus	
Dirofilaria species	
Brugia malayi	
Brugia pahangi	
Brugia patei	
2. Larvae to encyst at site of predilection	Severe constitutional symptoms in trichinosis
Nematoda	
Trichinella spiralis	None per se with remainder
Cestoda	
Taenia solium (cysticercus cellulosae)	
Echinococcus granulosus (hydatid)	
Multiceps species *(coenurus)*	
3. Larvae to mature to adults at site of predilection	
Nematoda	None per se
Wuchereria bancrofti	
Brugia malayi	
Trematoda	
Ectopic *Paragonimus westermani*	
Ectopic *Fascioda hepatica*	
4. Ectopic or animal strain larvae to provoke granulomata anywhere (visceral larva migrans)	None per se
Nematoda / **Cestoda**	
Ascaris lumbricoides / *Sparganum mansoni*	None per se
Strongyloides stercoralis / *Sparganum proliferum*	
Toxocara canis (and cati)	
Gnathostoma spinigerum	
5. Ectoic ova to provoke granulomata anywhere	None per se
Nematoda / **Trematoda**	
Schistosoma mansoni / *Heterophyes heterophyes*	
Schistosoma japonicum / *Metgonimus species*	
More permanent in blood	None per se
1. Adults live in circulatory system	
Nematoda / **Trematoda**	
Sometimes *Dirofilaria* species in heart / *Schistosoma haematobium*	
Schistosoma mansoni	
Schistosoma japonicum	
2. Larva circulate in blood	May be responsible for some general constitutional symptoms
Nematoda	
Wuchereia bancrofti	
Brugia malayi	
Loa Loa	
Dipetalonema sterptocerca	
Dipetalonema perstans	
Mansonella ozzardi	Myocarditis and heart failure
Dirofilaria species	
In myocardium larvae provoking granulomata and myocarditis	
Nematoda	
Trichinella spiralis	
Occasionally other ectopic larvae to ova	

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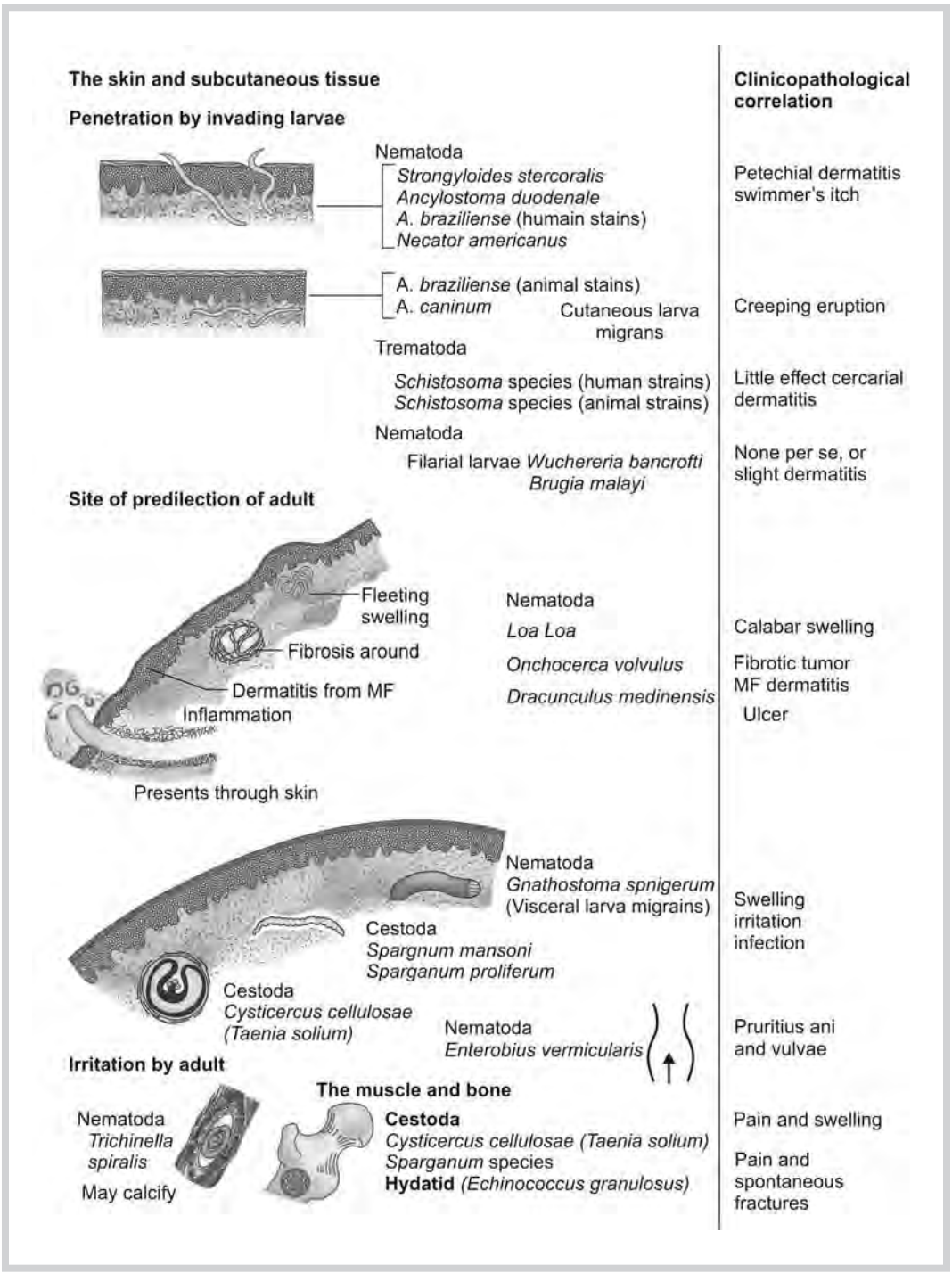
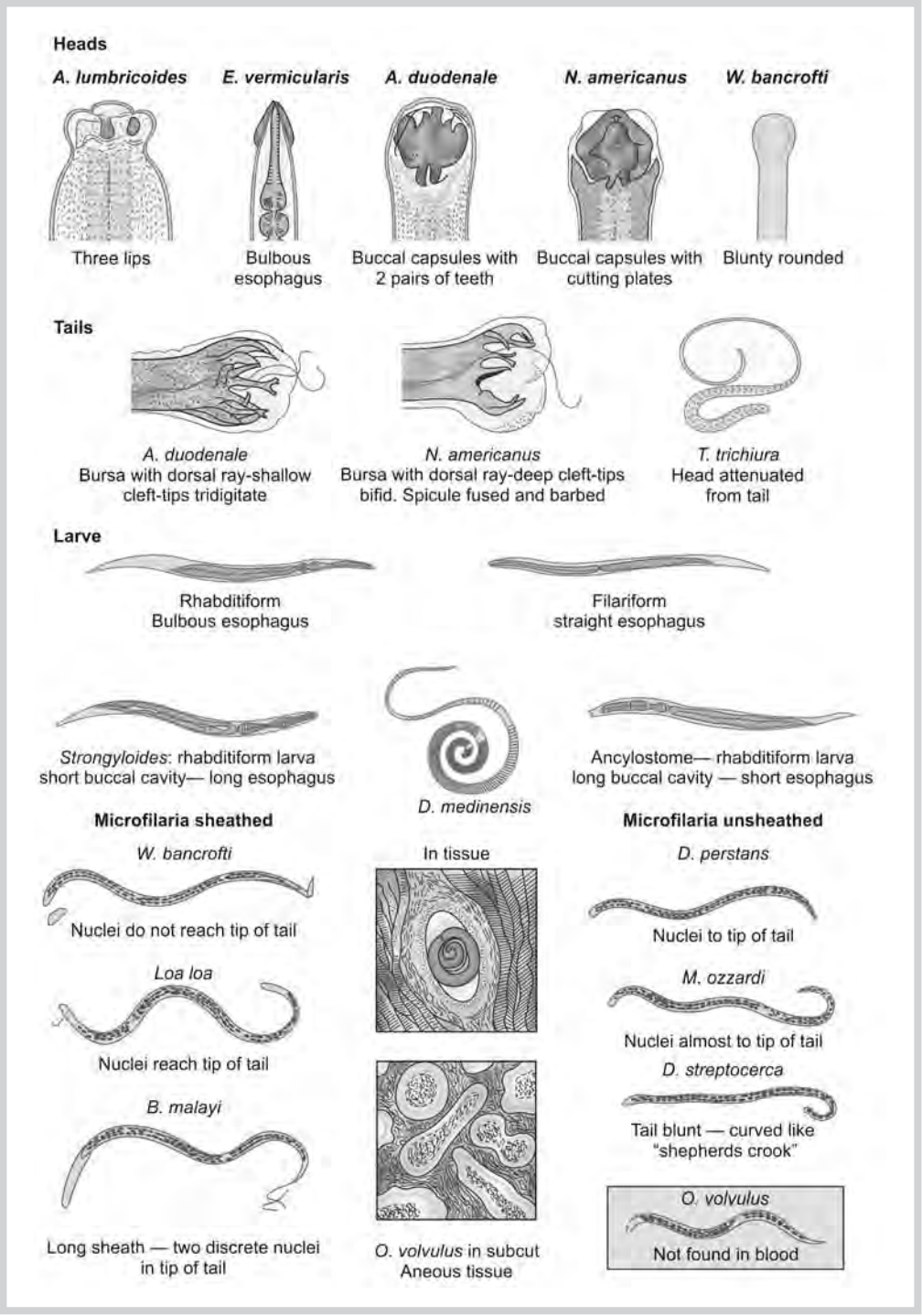

The skin and subcutaneous tissue
Clinicopathological correlation
Penetration by invading larvae
Nematoda
Strongyloides stercoralis
Ancylostoma duodenale
A. braziliense (humain stains)
Necator americanus
Petechial dermatitis swimmer's itch
A. braziliense (animal stains)
A. caninum
Cutaneous larva migrans
Creeping eruption
Trematoda
Schistosoma species (human strains)
Schistosoma species (animal strains)
Little effect cercarial dermatitis
Nematoda
Filarial larvae Wuchereria bancrofti
Brugia malayi
None per se, or slight dermatitis
Site of predilection of adult
Fleeting swelling
Fibrosis around
Dermatitis from MF
Inflammation
Presents through skin
Nematoda
Loa Loa
Onchocerca volvulus
Dracunculus medinensis
Calabar swelling
Fibrotic tumor
MF dermatitis
Ulcer
Nematoda
Gnathostoma spnigerum
(Visceral larva migrains)
Cestoda
Spargnum mansoni
Sparganum proliferum
Cestoda
Cysticercus cellulosae
(Taenia solium)
Swelling
irritation
infection
Nematoda
Enterobius vermicularis
Pruritius ani and vulvae
Irritation by adult
The muscle and bone
Nematoda
Trichinella spiralis
May calcify
Cestoda
Cysticercus cellulosae (Taenia solium)
Sparganum species
Hydatid (Echinococcus granulosus)
Pain and swelling
Pain and spontaneous fractures

Morphology of Adults and Larvae Nematodes



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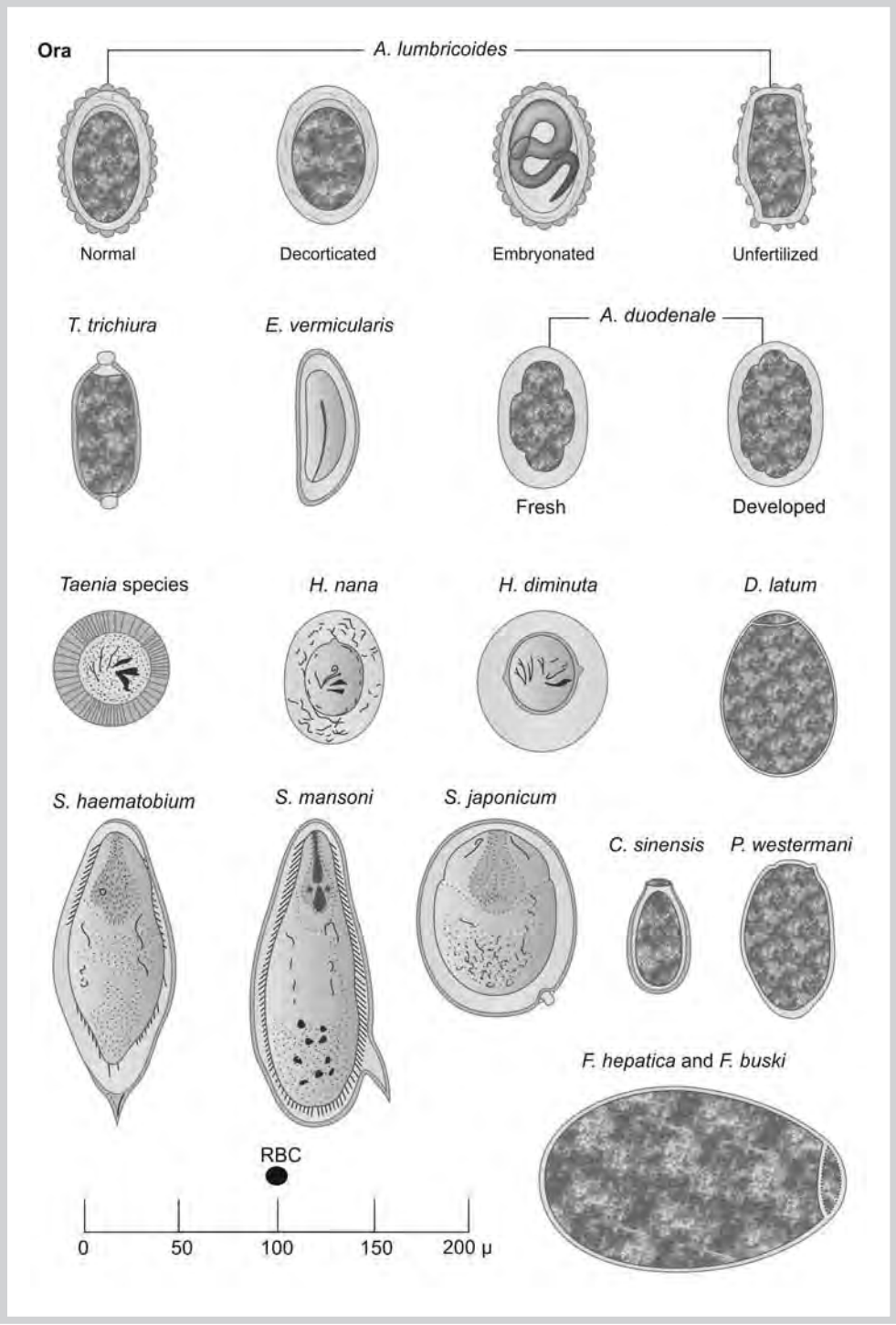
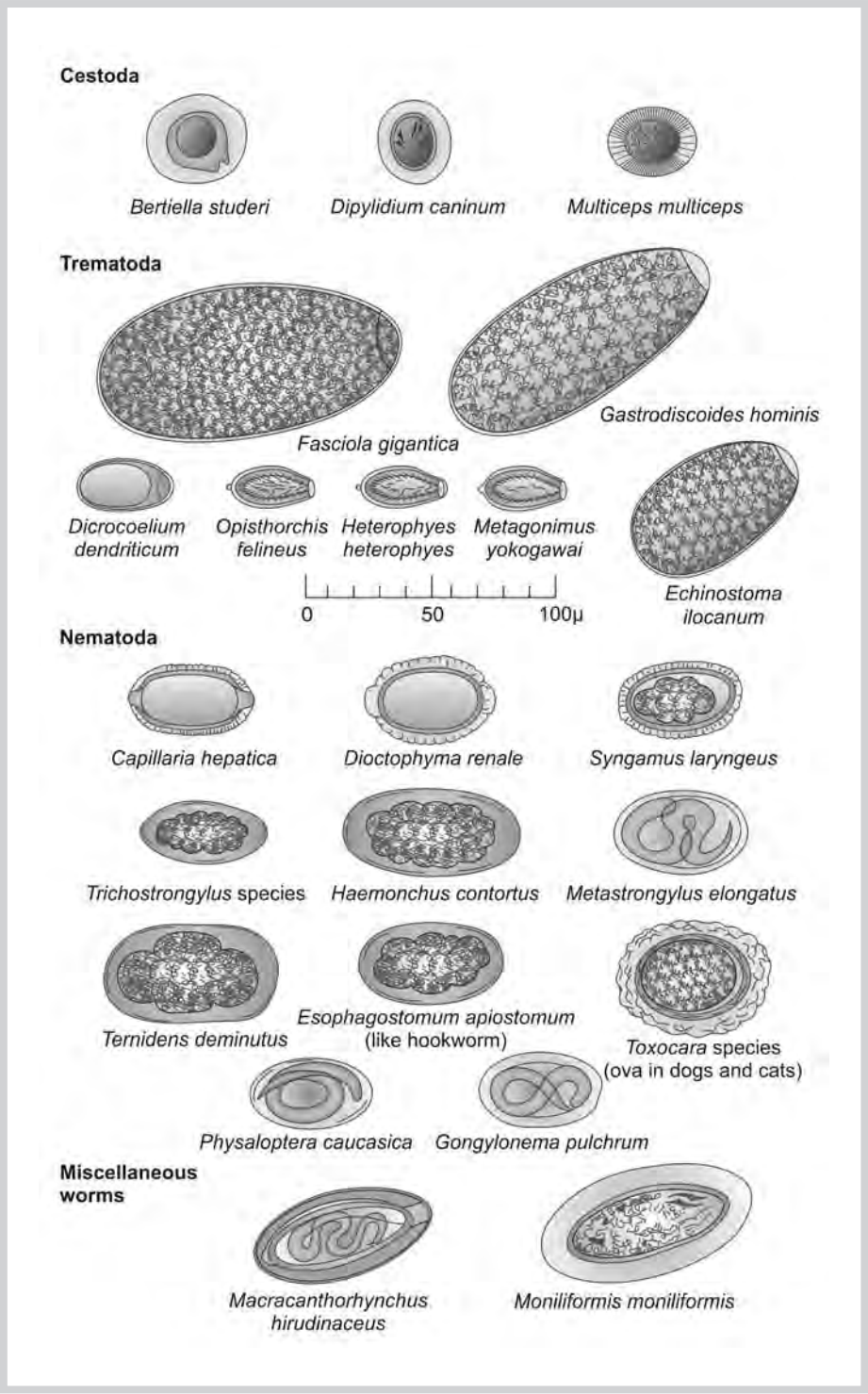

Ora
A. lumbricoides
Normal
Decorticated
Embryonated
Unfertilized
T. trichiura
E. vermicularis
A. duodenale
Fresh
Developed
Taenia species
H. nana
H. diminuta
D. latum
S. haematobium
S. mansoni
S. japonicum
C. sinensis
P. westermani
F. hepatica and F. buski
RBC
0
50
100
150
200 μ

Ova of the Less Common or Less Important Worms



LABORATORY EXAMINATION FOR PARASITES

Preservation and Shipment of Specimens to be Examined for Trophic and Encysted Protozoa

(PVA) Polyvinyl Alcohol Method of Brooke and Goldman

Excellent for ameba, especially if permanent slides are required. For collection of field specimens and for mass surveys, the Merthiolate-Iodine-Formalin (MIF) method is recommended.

1. *Reagent*—Modified Schaudinn's solution is prepared by mixing 5 mL of glacial acetic acid, 1.5 mL of glycerol and 93.5 mL of Schaudinn's solution (2 parts saturated aqueous mercuric chloride and 1 part 95% ethyl alcohol).

   Heat the above solution to 75°C; while stirring, slowly add 5 g polyvinyl alcohol (PVA) powder. This final solution should be clear and free of lumps after cooling. It is used at room temperature, and lasts several months.
2. *Preparation of specimens*
   a. One specimen should be sent without above fixative to be used for detection of protozoan cysts and helminthic ova. This specimen can be used for temporary or permanent smears or for concentration procedures.
   b. Second specimen is prepared by thoroughly mixing with 3 or more parts of fixative in a small vial. To prepare slides, a small amount of this fecal mixture (recent or months old) is spread thinly over about one-third of the slide. After drying for 3 or more hours at 37°C or overnight at room temperature, the smear is stained by the iron hematoxylin method. This procedure is particularly suitable for the preservation and staining of the trophic forms of intestinal protozoa. To obtain satisfactory stained fecal smears containing cystic stages, the excess clear PVA solution is decanted from the vial and a small amount of the remaining fixed fecal material is placed on a piece of facial tissue or toilet paper. The excess PVA solution is allowed to absorb for 5–10 minutes, leaving a moist fecal residue. Gently scrape up a small amount of the residue with the sharp edge of a broken applicator stick and smear with gentle brushing strokes on a slide. Drop smear immediately into 70% alcohol to which iodine has been added to produce a portwine color, and stain by the iron hematoxylin method. To ensure satisfactory preparation of both types smears are recommended.

Merthiolate-Iodine-Formalin (MIF)

Method of Sapero and Lawless

This is now the standard method for mass surveys, collection of bulk material, or parasitologic field studies. Fecal specimens are fixed and stained immediately, and can be examined at any time within several months of collection. This method is particularly good for protozoa.

1. *Reagents*: MIF stock solution is made from 250 mL distilled water, 200 mL of tincture of merthiolate, 25 mL of formaldehyde, 5 mL of glycerol. Store in brown bottle. Lugol's solution (5% iodine in 10% potassium iodide in distilled water), not over 1 week old, forms the second solution.
2. *Preparation of specimens*: For each specimen to be collected, have ready 2.35 mL MIF stock solution in a Kahn test tube with cork stopper and 0.15 mL Lugol's solution in a second Kahn tube with a rubber stopper. Combine the 2 solutions just before adding the fecal specimen. Break up about 0.25 g feces into the combined solution, mix thoroughly, and stopper well (may be examined immediately on a slide, 1 drop fecal preparation and 1 drop distilled water; or stored in a well-stoppered tube, where the stain will be retained for several months).

   Routine Stool Examination and Concentration Methods have been Dealt with Elsewhere (Previous Chapter)

Negative Stain Direct Fecal Smear Examination

Prepare normal fecal smear in saline to which 1–2 drops of 1% isotonic eosin are added. Background material and dead parasites turn uniformly pink. Stain will not penetrate living trophozoites, causing them to stand out markedly as clear, translucent organisms against a pink background. A rapid and useful procedure.

Isotonic eosin, 1%, and brilliant cresyl blue, 0.2%, makes a good vital stain, causing living material to appear as shiny pale blue-green objects on the pink background.

Kato Cellophane Thick Smear Technique (Kato and Miura, 1954)

This method permits rapid examination of a large number of samples (up to 70/h) for eggs of the common helminths. It is not suitable for protozoa or minute helminths or for highly fibrous or gaseous samples.

Materials

1. Wettable cellophane of medium thickness (40–50 μm), cut in strips 22 × 30 mm.
2. Glycerin malachite green solution: 100 mL pure glycerin, 100 mL water, 1 mL 3% aqueous malachite green.

The cellophane strips should be soaked in the glycerin mixture for at least 24 hours before use.

Procedure

1. Place 50 to 60 mg feces (4 mm cube) on a clean slide.
2. Cover with a glycerin-soaked cellophane strip, press to spread feces in an even layer. Feces need not be spread to all areas of cellophane; a circumference equal to the width of the strip is sufficient.
3. Allow to stand at room temperature for 1 hour (or 20–30 minutes at 40°C in a dry incubator). This dries and clears the specimen. (Do not over dry, as gas bubbles will form and air cells will surround the eggs).
4. Examine the entire film under low poor magnification.

Heidenhain's Iron Hematoxylin Staining Method for Intestinal Protozoa

Iron hematoxylin is generally accepted as the most reliable stain for nuclear detail in amebae and for accurate laboratory diagnosis. It also provides a permanent stain preparation. However, it requires critical care and individual slide destaining (to be done carefully), a major handicap in routine handling of specimens by clinical laboratories. The trichrome stain has therefore, superseded iron hematoxylin staining for routine diagnosis, though for critical definition, iron hematoxylin is still unsurpassed. Both iron hematoxylin and trichrome can be used for either PVA-fixed or nonpreserved material.

Staining Solutions

1. *Schaudinn's fixative*—described earlier.
2. *Hematoxylin stain* (stock solution)—dissolve 100 g hematoxylin powder in 100 mL absolute ethanol. Let stand several weeks for maturation. For use, 5 mL of ripened hematoxylin is added to 95 mL distilled water.
3. *Mordant*—dissolve 5 g ferric ammonium sulfate in 10 mL distilled water and filter.

Procedure

1. Make thin fecal smear on a clean glass slide with toothpick, applicator, or stiff haired paste brush.
2. Before drying occurs, immerse slide in Schaudinn's fluid with acetic acid added, heated to 45°C. Fix for 5–15 minutes at this temperature or for 30 minutes at room temperature (omit this step for PVA-fixed specimens).
3. Staining in Coplin jars:

70% alcohol	15 minutes
70% iodine alcohol	5 minutes
70% alcohol	2 minutes
50% alcohol	2 minutes
Tap water (running)	2 minutes
Distilled water	Rinse
5% aqueous iron-alum (mordant)	5 minutes at 30°C
Distilled water (2 changes)	Rinse
0.5 % hematoxylin	10 minutes
Differentiate in 1% aqueous iron-alum	** Usually 3–5 minutes
Tap water (running)	15–20 minutes
50% alcohol	2 minutes
70% alcohol	2 minutes
95% alcohol	5 minutes
Isopropyl alcohol	2 changes of 5 minutes
Carbol-xylol	5 minutes
Xylol (2 changes)	5 minutes each

Mount in xylol-balsam, xylol-Damar or DPX mountant with a cover glass of No. l thickness.

Gomori's Trichrome Stain

Preferred permanent stain for routine diagnosis.

Staining Solution

To 100 mL distilled water, add 0.6 g chromotrope 2 R, 0.3 g light green SF, 0.7 g phosphotungstic acid, and 1 mL glacial acetic acid.

Procedure

Prepare slides as for hematoxylin staining. (Omit steps 1 and 2 for PVA-fixed specimens).

1. Immerse in Schaudinn's fixative	30 minutes at room temperature
2. 70% alcohol (wash)	15 minutes
3. 70% iodine alcohol	10 minutes
4. 70% alcohol (wash)	2 changes of 5 minutes
5. Trichrome stain	20 minutes
6. Acidified 90% alcohol (1% acetic acid)	2 dips
7. 95% alcohol	2 changes of 5 minutes
8. Carbol-xylene	2 minutes
9. Xylene	2 minutes
10. Mount immediately as for hematoxylin preparations.	

Cultivation of Intestinal Protozoa

Numerous types of special media have been developed for the cultivation of the intestinal amebae and flagellates as well as for the ciliate *Balantidium coli*. Among these are Boeck-Drbohlav-Locke-egg serum medium, Nelson's egg yolk infusion-liver extract medium, and Dobelle's medium which contains serum, egg albumin and starch. The parasite (*E. histolytica)* grows in 24 to 36 hours at 37°C. The morphological character of the colony is quite typical of the species.

The cultivation of free-living juveniles of *Ancylostoma duodenale* and *Strongyloides stercoralis* is done on sterilized sand or charcoal paste. Samples of soil or feces containing ova are mixed with equal quantity of sterilized fine sand or animal charcoal and water to make a thick paste. The paste is kept on a filter paper in a petri dish and covered with lid. It is kept at 25 to 30°C for several days. The free-living juveniles collect in the water of condensation.

Urine

Collect urine in a clean, dry container, avoiding fecal contamination. Study centrifuged sediment. Trophozoites of *Trichomonas vaginalis,* unhatched eggs of *Schistosoma haematobium,* and intact scolices or hooklets of *Echinococcus granulosus* may appear in the urine. Viable *S. haematobium* eggs will hatch only after dilution of urine.

Sputum

Examine for parasites as a direct smear under a cover glass. If the sputum is thick, bloody, or pus laden, mix with equal volume 1–2% sodium hydroxide. Stir, let settle, and study sediment. Larvae of *Strongyloides stercoralis,* scolices of *Echinococcus granulosus*, eggs of *P. westermani*, or migrating nematode larvae may be present in the sputum. Charcot-Leyden crystals and eosinophils can also be observed in wet mounts or after wet fixation and staining.

Gastric Washings

For night-swallowed sputum will often yield *Paragonimus* eggs or migrating nematode larvae better than will sputum or feces (owing to less detritus).

Duodenal Aspirates

These are useful for nematode eggs and are particularly helpful for eggs of *Clonorchis* and other bile dwelling parasites.

Spinal Fluid

Examine centrifugate directly under the microscope. Make smears, stain with Giemsa's stain and examine. Culture some of the sediment, as for blood. Inoculate guinea pigs or mice, if necessary.

Vaginal Secretions

Wet preparation may be examined directly for *Trichomonas vaginalis.* Cultures can also be made.

Graham Cellulose Tape Technique for Diagnosis of Enterobiasis

With the help of a tongue depressor, press the adhesive side of a small strip or loop of cellulose tape (e.g. Scotch tape) over the anal and perianal surfaces, preferably at night. Then place tape with adhesive side down in a drop of toluene on a microscopic slide. Examine for eggs or worms which have adhered to the tape.

Blood

Combined Thin and Thick Films

Making Films

Cleanse finger, ear lobe, heel, or toe (of children) thoroughly with spirit and allow to dry. Prick skin deeply to cause a few drops of blood to flow freely. On one end of a meticulously clean slide free of fingerprints or oil film, make a thin smear as for a blood count. For the preparation of the thick film, deposit a large drop of blood at the other end of the slide and spread it out evenly with the corner of another slide to a diameter of about 20 mm. The film should not be too thick since it may crack and peel when dry. Dry the slide in a flat position so that the distribution of blood will be even. Protect from dust and insects, avoid excessive heat. Allow to dry in air for at least 8–12 hours or for 2 hours in an incubator at 37°C. Keep free from dust or contamination with excreta of flies or roaches. Stain as soon as practicable, freshly stained material gives distinctly superior results.

Staining Films

Fix thin filmed end of slide in methanol for 2–3 minutes. *The thick films must not be fixed.* Immerse slides for 30 minutes in a mixture of 1 drop of concentrated Giemsa's stain to each mL of phosphate-buffered distilled water (pH 7.2). Wash off with buffered water and dry in air. Examine with oil immersion objective. Hematoxylin or methylene blue

stain is preferred for microfilariae since nuclear detail and sheaths (if present) stain more distinctly.

Concentration of Microfilariae

Hemolyze 10 mL or more of citrated blood with 50 mL of a 2% solution of a saponin in physiologic saline. Centrifuge and examine the sediment. Chylous urine and scrotal aspirates should always be centrifuged to attempt recovery of microfilariae.

Bone Marrow Smear

For a proper diagnosis of kala-azar, bone marrow smear obtained by sternal puncture is essential. The Leishman's stain or Giemsa's stain are used for this purpose. Microscopic examination under oil immersion shows the presence of intracellular oval bodies containing blue-stained cytoplasm and pink-stained rod-like kinetoplast.

Serological Tests

Serum samples obtained from the patients are used for following tests in certain parasitic infections:

1. *Precipitation:* It is done for trichinelliasis, hydatid disease and cysticercosis.
2. *Flocculation test*: Slide flocculation tests are useful for trichinelliasis and schistosomiasis.
3. *Complement fixation test*: This test is used in several parasitic infections for detecting complement fixing antibodies, but this is of special value in cases of schistosomiasis, trichinelliasis, hydatid disease, nonpulmonary paragonimiasis, and toxoplasmosis.
4. *Fluorescent antibody test (FAT)*: This test is applied to demonstrate the presence of parasites or their specific antibodies in tissue or in the blood of the patient, e.g. *Toxoplasma gondii* and *Entamoeba histolytica*.
5. ELISA tests now are available for most parasitic infestations.
6. Immunochromatography technique based kits.

Intradermal Tests

These are done to demonstrate allergic state in certain parasitic diseases. The antigen of the suspected parasite is injected intradermally and in positive cases, wheal or induration results. These tests are useful for hydatid disease (Casoni's test), trichinelliasis, filariasis, chronic schistosomiasis and toxoplasmosis.

Casoni's Test

It was first developed by Casoni in 1911. Intradermal injection of 0.2 mL on flexor aspect of right arm of a fresh sterile hydatid fluid (sterilized by Seitz filter) produces within half an hour in all positive cases a large wheal (5 cm in diameter) with multiple pseudopodia, it fades in an hour.

A delayed reaction appears after 18 to 24 hours characterized by edema and induration 5–6 cm surrounding the site of injection. A negative reaction does not exclude echinococcal infection. The test usually becomes positive 8–12 weeks after infection and remains positive after surgical removal of cyst from the patient.

Hydatid fluid from human cases (removed operatively) or from animals (obtained from a slaughter house) is used as an antigen. While 0.2 mL of antigen is injected to one arm, sterile normal saline 0.2 mL is injected in the other arm for control.

Muscle Biopsy for Trichinella spiralis

Small pieces of deltoid, biceps, or gastrocnemius muscle are removed from the vicinity of their tendinous attachment under local anesthesia.

1. Microscopically examine small pieces of muscle compressed between 2 glass slides for encysting or encysted larvae.
2. Digest muscle in artificial gastric juice (pepsin and hydrochloric acid) and examine sediment for motile larvae.

CHAPTER 9

Clinical Hematology

The blood consists of a fluid of complicated and variable composition, the plasma, in which are suspended erythrocytes (red blood cells—RBCs), leukocytes (white blood cells—WBCs) and platelets. By using an anticoagulant, the formed elements can be separated from plasma. When blood coagulates, the fluid that remains after separation of the clot is called serum. Serum = Plasma-fibrinogen. The techniques of hematology are concerned mainly with the cellular formed elements of blood, their number or concentration, the relative distribution of various types of cells and the structural or biochemical abnormalities that promote disease.

WAYS OF OBTAINING BLOOD

Capillary or Peripheral Blood

For hematologic exercises, venous blood obtained from a vein is better. However, for total and differential blood counts and for hemoglobin estimation, blood can be taken by pricking:

(i) The lobe of the ear, (ii) the palmar surfaces of the tip of the finger, (iii) in infants, from the plantar surface of the (a) heel or (b) the great toe. The puncture should be about 3 mm deep. An edematous or a congested part should not be used. If the area to be punctured is cold and cyanotic, warm it by massaging or else erroneous results may be obtained. Clean the site with spirit or alcohol, let dry and puncture. Wipe off the first drop of blood, never press out blood. Having obtained the requisite amount of blood, let the patient apply slight pressure over the area with sterile swab (Fig. 9.1).

Venous Blood (Venipuncture)

Reassure the patient about what is to be done. Inspect the veins, use a tourniquet if needed (Fig. 9.2). Use a syringe of a size according to the amount of blood required. Needles of gauge less than 22 should be used and be 1 to 1½ inches long. Instead of a tourniquet, one can use a sphygmomanometer cuff, apply pressure that is midway between systolic and diastolic pressure. Ask the patients

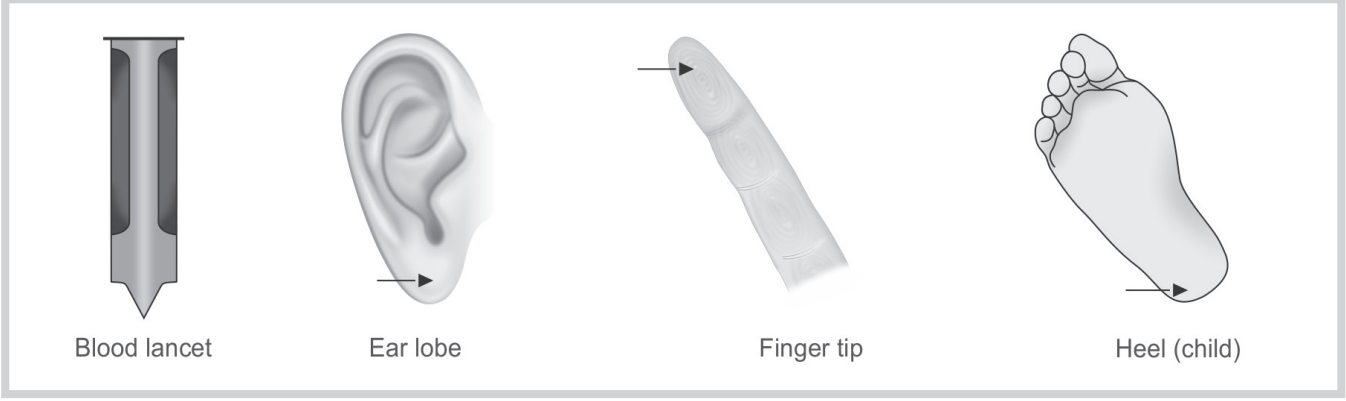


FIG. 9.1: Sites for obtaining-blood skin puncture

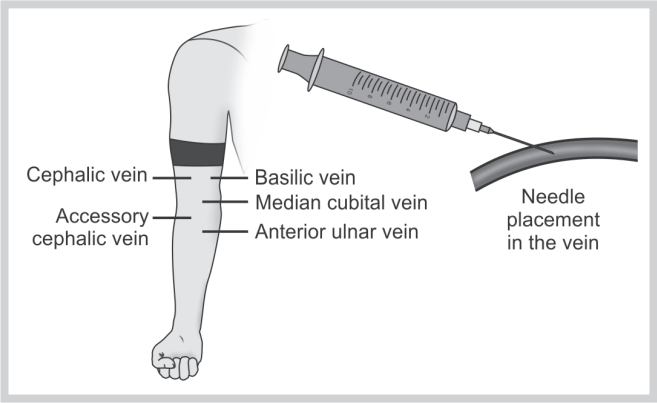


FIG. 9.2: Sites for obtaining blood by venipuncture from forearm

to open and close his fist several times. Take aseptic precautions, and puncture the vein. Sometimes, it may be difficult to obtain blood at the first instance, in such a case, first the skin around the vein is punctured and then the vein is punctured, the moment needle enters the vein, blood flows back into the syringe. If still blood has not been obtained, withdraw the needle and in many instances blood would be obtained. Having withdrawn blood, loosen the tourniquet and ask the patient to open his fist. Let the patient apply a sterile gauze piece with gentle pressure over the area. The patient should apply pressure gently with three fingers. Why three fingers? Simply because the site of skin puncture is not the same as the site of venipuncture. Make sure that the bleeding has stopped before the patient leaves. In infants, blood may be secured from femoral or external jugular vein. Wash the syringe after dispensing the blood in the right container. Transfer blood from syringe into the container gently (not through the needle). Ideally use disposable syringes and needles.

Complications

Immediate

Hematoma and syncope. Hematomas can be avoided by applying adequate gentle pressure at the site and for syncope a physician should be brought to take care. In any case, let the patient lie down if he or she is already not and raise the foot end. Some patients with a bleeding tendency may overbleed.

Late Local Complications

Thrombosis of the vein. Rarely thrombophlebitis may occur. Apply *Thrombophob*® if it happens. Seek professional medical help.

Late General Complications

Transmission of serum hepatitis, AIDS by using contaminated needle and syringe.

Clinical Alert

1. If oozing from the puncture site is difficult to stop, elevate area and apply a pressure dressing. Stay with the patient until bleeding stops.
2. Never draw blood for any laboratory test from the same extremity that is being used for IV fluids, IV medications or blood transfusion.
3. In patients with leukemia or agranulocytosis and in others with lowered resistance, the finger prick and ear lobe puncture are more likely to cause infection than venipuncture. If capillary sample is necessary in these patients, the cleansing agent should remain in contact with the skin for 7 to 10 minutes. Alcohol is not bactericidal, povidone-iodine (*Betadine*®) is the cleansing agent of choice on leukemic patients.

ANTICOAGULANTS

On letting blood stand, it clots after some time. Anticoagulants are agents that prevent clotting when mixed with blood in an appropriate proportion. The common pathway of the clotting mechanism is represented as under:

$$\text{Prothrombin} \xrightarrow[Ca^{++}]{\text{Thromboplastin}} \text{Thrombin}$$

$$\text{Fibrin} \longleftarrow \text{Fibrinogen}$$

(insoluble) Thrombin + Ca^{++} (soluble)

Fibrin with blood cells with entrapped constituents comprise a clot.

The thromboplastin released by damaged tissue, or platelets converts inactive prothrombin into active thrombin in the presence of calcium ions. Thrombin converts soluble fibrinogen into insoluble fibrin clot in the presence of calcium ions. Anticoagulants commonly used for hematological investigations are as follows:

EDTA

Ethylenediamine tetra-acetic acid (EDTA), disodium or potassium salt. EDTA acts by chelating calcium and preserves cellular element better than oxalates. Eight (8) mg of the salt is enough for anticoagulating 3 to 4 mL of blood.

— EDTA can be used for hemograms, ESR (Wintrobe's method), platelet count, DLC and peripheral smear examination.

Advantages of EDTA

- Cellular morphology is preserved better, even 2–3 hours after blood collection
- As platelet clumping is prevented, EDTA is a better anticoagulant for platelet counts
- EDTA 2K salt is recommended for CBC, is more water soluble (1.5 ± 0.25 mg/mL of blood).

Disadvantages of EDTA

- When in excess, EDTA shrinks RBCs and leukocytes. If in excess of 2 mg/mL:
  - PCV is significantly reduced
  - MCHC is proportionately increased
  - Platelets swell and disintegrate, therefore, a fallaciously high platelet count may be obtained
  - It cannot be used for coagulometry applications.

Making EDTA Bulbs

Four (4) grams of disodium or dipotassium salt is added to 100 mL of deionized water. About 0.2 mL of this solution is added to chemically clean vials, the vials are later kept in an incubator or hot air oven till complete liquid dries up (may take 1–2 hours at 60–80°C). White layer of anticoagulant can be seen at the bottom. This bulb is for 3–4 mL of blood, as it contains 8 mg of EDTA per vial.

Oxalates

Oxalates act by chelating calcium, and calcium oxalate is formed as insoluble precipitate. These are used for blood chemistry and hematocrit.

- *Potassium oxalate* (2–3 mg/mL of blood) but it may cause shrinkage of cells. Not used anymore.
- *Double oxalate* used for ESR and hematocrit. Potassium oxalate and ammonium oxalate are used together in a ratio of 2:3, this is done to counter the swelling effect of ammonium oxalate and shrinking effect of potassium oxalate on the RBCs.
  - Double oxalates can be used for
    - Hemoglobin, TLC, RBC count, ESR by Wintrobe's method and PCV estimation.
  - *Disadvantages*
    - Leukocytic morphology is not well preserved and hence not suitable for peripheral smear studies
    - The calcium chelated is precipitated in calcium oxalate, which is a toxic substance, it is never to be used for blood banking applications.

Making Double Oxalate Bulbs

Prepare double oxalate solution as follows:

1. Potassium oxalate 1.6 g.
2. Ammonium oxalate 2.4 g.
3. Deionized water 100 mL. Mix well, 0.2 mL of the solution will contain 8 mg of the oxalates, which prevent clotting of about 3 to 4 mL of blood.

Trisodium Citrate

Trisodium citrate is used for ESR and some coagulation studies. This too acts by chelating calcium. For ESR, ratio is 1:4; while for coagulation studies ratio is 1:9. 1 part of 3.8% trisodium citrate and 4 or 9 parts of blood respectively.

Heparin

Heparin (powder or liquid) acts by inhibiting thrombin and other stages of clotting factor activation.

Special Anticoagulants

Special anticoagulants include ACD (acid-citrate dextrose) used in blood banking and fluoride and oxalate for sugar estimations. Other blood banking anticoagulants are also used. Wherever possible, the necessary tests, investigations and preparation of blood films should be done immediately

If this is not possible, refrigerate the sample at 4°C. Before taking blood from the venous blood containers, invert them gently several times (60) or else unacceptable deterioration in precision may ensue.

Anticoagulated Blood Storage and Blood Cell Morphology

Peripheral Smears

Peripheral smears (anticoagulated or direct blood used and stored at 25 ± 5°C). Unfixed smears.

- Up to 60 minutes: No worthwhile notable change
- Up to 3 hours, few changes may be visible
- Up to 12–18 hours: Neutrophils are affected
- Lobes may get separated
- Cytoplasmic borders may appear ragged with small intracytoplasmic vacuolation.

- Mononuclear cells may show cytoplasmic vacuolation, nuclear disintegration or budding
- RBCs do not change for up to 6 hours at room temperature (25 ± 5°C) but longer periods may cause progressive crenation and sphering.

EDTA Blood

- On storing EDTA blood, the following changes may occur:
  - MCV increases
  - Osmotic fragility increases
  - Sedimentation rate gradually decreases
  - TLC and platelet counts decrease
  - Reticulocyte count decreases within 6 hours
  - Hemoglobin remains unchanged if the sample does not get infected.

Diagnostic Alerts

- Perform all investigations as soon as the blood sample is taken
- Never freeze the sample. On storing the sample at 4°C, the deterioration rate slows down
- Perform all counts within 2 hours of blood collection
- Excessive EDTA in the sample will significantly lower TLC within 1–2 hours
- Leukocytic degenerative changes will affect automated differential counts
- A refrigerated sample must always be brought to room temperature before being used. All samples must be mixed gently, preferably by rotation, for at least 2 minutes before testing.

BLOOD COLLECTION SYSTEM

- Whatever be the reason for obtaining blood, in the interest of the patient and your own interest, it is ideal and necessary to use sterile disposable blood collection systems, viz. disposable syringes or the Vacutainers. These are meant for single use and are to be discarded (never to be used again). Relatively new in our country, but established all over the world and being used for decades, is the Vacutainer blood collection system manufactured by *Becton Dickinson* (BD). Other makes/brands are also available.
- The Vacutainer system consists of a needle, a needle holder and a glass/plastic vacuum tube instead of the syringe barrel and plunger. Once the vein is punctured, the Vacutainer tube appropriately in contact with the needle, the requisite quantity of blood flows automatically into the Vacutainer tube so that the need to pull the plunger out is obviated. Vacutainer is simple to use, quicker, cleaner and safer. It offers— leakproof tubes, standardization of specimen quality at high level, opportunity to rationalize laboratory procedures and innovative, high technology tubes. Appropriate anticoagulants, and other additives are preadded in appropriate quantities so that all that is required is clean venipuncture. Containers also available for collecting blood from infants with the help of a skin puncture, these are called microtainers. The blood so collected is adequate for micro or dry chemistries. From a single venipuncture, blood can be collected in separate vacutainers (for different purposes—EDTA or oxalate-fluoride or citrate vacutainers) meant for different purposes and very easily identified by the color of their caps. The vacutainer system is a cleaner system, as blood does not come in contact with atmosphere as it flows straight from the vein through the sterile needle into the sterile tube. The process of transferring blood from syringe to different bulbs is eliminated. Contamination from fallen blood is entirely removed. The incidence of hemolysis is significantly reduced because the major cause of it—the transfer of blood from the syringe to container—is eliminated. In hematology, because of the instant contact between blood and anticoagulant minimizes microclot formation. Furthermore, all vacutainer tubes are sterile, guaranteeing the biological integrity of the sample—a particularly important factor with ESR determinations and coagulation studies, which can be seriously distorted by microbial growth in the citrate solution. The laboratory personnel derive the maximum benefit (by use of vacutainers) though the physician is assisted only indirectly in the form of quality reports. Also available are vacutainer culture systems, where blood is injected into the culture media directly without even coming in contact with atmosphere (Fig. 9.3).
- Table 9.1 gives color codes for the Vacutainer systems and in all cases different volume containers are available—from 2 to 15 mL.

BD Vacutainer® Order of Draw for Multiple Tube Collections

Designed for Your Safety

Reflects change in CLSI recommended order of draw (H3-A5, Vol 23, No 32, 8.10.2)

TABLE 9.1: Vacutainer color codes and their usage

Stopper color code	*Additive*	*Used for*	*Remarks*
1. Gray and red	Inert polymer Barrier material tubes	Chemistry	Serum separation
2. Yellow and red	None	Chemistry	Clot activator tubes
3. Red	None	Chemistry	Silicone coated interior
4. Red/orange green	None	Chemistry	-
5. Yellow	None	Chemistry	Silicon-coated interior
6. Royal blue	None/heparin/EDTA	Toxicology and nutrition studies, e.g. trace elements, heavy metals, etc.	
7. Brown	Sodium heparin	Lead determination	
8. Yellow black	Thrombin (NIH)	For stat procedures as thrombin hastens clotting and therefore quick serum separation	
9. Gray	Sodium fluoride/ Iodoacetic lithium potassium oxalate- NaF/EDTA-NaF/ Thymol NaF	For glucose estimation	
10. Green	Sodium heparin Lithium heparin Ammonium heparin	For chemistry or cytology	
11. Blue	Sodium citric acid	For coagulation studies	
12. Lavender	EDTA	For hematology studies	
13. Yellow	ACD solution A ACD solution B ACDP solution Alsever's solution	For blood banking For blood banking	
14. Green	Na heparin	LE cell preparation	Prelabeled
15. Gray/Black	Gives citrate Blood a ratio of 1:4	For ESR estimation by Westergren's method	Prelabeled
16. Blue	Ammonium oxalate and potassium oxalate in a ratio of 6:4	For ESR estimation by Wintrobe's methods	Prelabeled
17. Yellow	Sodium polyane tholesulfonate 0.35% in 0.85% sodium chloride	For microbiology	Prelabeled

Closure Color	**Collection Tube**	**Mix by Inverting**
BD Vacutainer ® Blood Collection Tubes (glass or plastic)		
	• Blood Cultures – SPS	8 to 10 times
	• Citrate Tube (Fig. 9.3)	3 to 4 times
or	• BD Vacutainer® SST™ Gel Separator Tube	5 times
	• Serum Tube (glass or plastic)	5 times (plastic) none (glass)
	• BD Vacutainer® Rapid Serum Tube (RST)	5 to 6 times
or	• BD Vacutainer® PST™ Gel Separator Tube With Heparin	8 to 10 times
	• Heparin Tube	8 to 10 times
or	• EDTA Tube	8 to 10 times
	• BD Vacutainer® PPT™ Separator Tube K2EDTA with Gel	8 to 10 times
	• Fluoride (glucose) Tube	8 to 10 times

Contd..

Note: Always follow your facility's protocol for order of draw

Handle all biologic samples and blood collection "sharps" (lancets, needles, luer adapters and blood collection sets) according to the policies and procedures of your facility. Obtain appropriate medical attention in the event of any exposure to biologic samples (for example, through a puncture injury) since they may transmit viral hepatitis, HIV (AIDS), or other infectious diseases. Utilize any built-

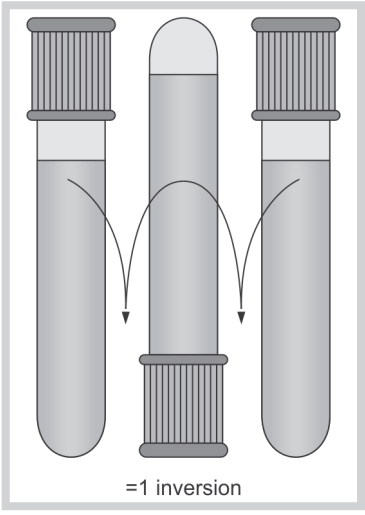


FIG. 9.3: Mixing of anticoaguted tubes

in used needle protector if the blood collection device provides one. BD does not recommend reshielding used needles, but the policies and procedures of your facility may differ and must always be followed. Discard any blood collection "sharps" in biohazard containers approved for their disposal.

When using a winged blood collection set for venipuncture and a coagulation (citrate) tube is the first specimen tube to be drawn, a discard tube should be drawn first. The discard tube must be used to fill the blood collection set tubing's "dead space" with blood but the discard tube does not need to be completely filled. This important step will ensure proper blood to additive ratio. The discard tube should be a nonadditive or coagulation tube.

HEMOGLOBIN

Hemoglobin (Hb) is the main constituent of the RBCs and carries out the important function of transportation of oxygen from lungs to various parts of the body. To a lesser extent, it transports back carbon dioxide from the body to the lungs. When fully saturated, each gram of hemoglobin holds approximately 1.34 mL of oxygen. The red cell mass of an adult contains approximately 600 g of hemoglobin, capable of carrying 800 mL of oxygen.

Hemoglobin Estimation: Sahli's Method: (Sahli's Hemoglobinometer) (Fig. 9.4)

This is based on conversion of hemoglobin to acid hematin, which has brown color. Fill hemoglobin tube till 20 mark with N/10 HCl. To this, add blood sucked till the specific mark (20 μL) on the hemoglobin pipette and wait for 5–45 minutes. During this time keep stirring the mixture of acid-blood in the tube. Add distilled water until a match is obtained with the brown glass standard (comparator) provided. Read the lower level of fluid meniscus on g% side of the tube.

Report hemoglobin in g/100 mL of blood. If hemoglobin is less than 2 g%, take double the quantity of blood and divide the result by 2. If hemoglobin concentration is extremely high dilute blood with equal amount of normal saline, take the reading and multiply by 2. This method, however, does not estimate carboxyhemoglobin, methemoglobin and sulfhemoglobin. Non-hemoglobin substances (protein, lipids) in plasma and cell stroma may influence the color of blood diluted with acid. It is, therefore, not a very satisfactory method.

Cyanmethemoglobin Method

(Drabkin's solution and the standard available from Coral Clinical Systems, Goa)

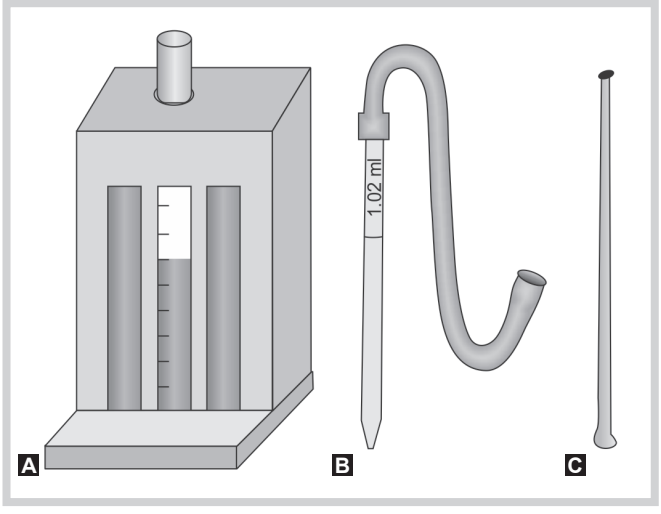
Drabkin's Reagent

In 1000 mL of deionized water are mixed:

- Potassium ferricyanide: 400 mg
- Potassium dihydrogen phosphate: 280 mg
- Potassium cyanide: 100 mg
- Nonidet (non ionic detergent): 1 mL.

This reagent can be stored in a polythene container. Concentrated stock solutions can also be prepared and diluted accordingly when needed.

Pipette carefully and take care not to discard cyanide solutions into sinks or receptacles containing acid (to prevent formation of hydrocyanic acid). To 5 mL of



FIGS. 9.4A TO C: (A) Sahli's hemoglobinometer; (B) Hb pipette; (C) Stirrer

Drabkin's solution, add 20 μL of blood. Mix well. Read in a photocolorimeter at 540 nm (green filter).

For this procedure, certified standard hemoglobin solution may be obtained from reputable laboratory supply firms. By diluting the known standard hemoglobin solution, a graph (linear) may be obtained by plotting the known Hb concentration against the colorimetric optical density reading so that in future the corresponding hemoglobin value can directly be read off from the calibration curve after knowing the optical density of a particular unknown blood sample.

Sheard-Sanford Oxyhemoglobin Method

Mix 20 mL of 0.1% sodium carbonate and 0.1 mL of blood or aliquots of these (e.g. 4 mL diluent for 20 μL blood); read optical density in photometer at 540 nm within 30 minutes. Photometer calibration should be based on blood iron determination or oxygen capacity determination.

Other Methods

Alkali Hematin Method

It does not estimate fetal hemoglobin and is no longer used in routine hemoglobinometry.

Gasometric Method

Van Slyke's oxygen capacity method. It is an indirect method, which estimates the amount of hemoglobin from the amount of oxygen it absorbs. This method is very complicated for routine clinical work.

Specific Gravity Method

The normal specific gravity of blood ranges from 1.048 to 1.066. The average for men is 1.057 and for women it is 1.053. From specific gravity of the unknown sample, its hemoglobin is calculated. This is a very rapid and an uncomplicated method and finds its main use in screening potential blood donors for anemia.

Chemical Methods

Obsolete. Hemoglobin is estimated by finding the iron content.

Sodium Lauryl Sulfate Method

(*Available* from coral clinical systems, Goa)

This is a KCN-free reagent where SLS substitutes KCN. The color complex formed is SLS-Hb which is read at 540 nm. The greatest advantage being that *all forms of Hb are converted.* The method is relatively free from interferences due to lipemia and presence of WBCs. Linearity is superior to that of Cyanmeth Hb method.

Normal Hemoglobin Values

Men	15.5±2.5 g/dL	14–18 g%
Women	14.0±2.5 g/dL	11.5–16.5 g%
Infants full term cord blood	16.5±3.0 g/dL	13.5–19.5 g%
Children, 1 year	12.0±1.0 g/dL	11.0–13.0 g%
Children 10-12 years	13.0±1.5 g dL	11.5–14.5 g%

According to current WHO specification for males 13.2 g/dL and for females up to 11.7 g/dL Hb are said to be normal. For children from 3 months to puberty, 10.7 g/dL is said to be normal Hb level.

ANEMIA

It is defined as reduction in the concentration of hemoglobin in the peripheral blood below the normal for the age and sex of the patient.

Diurnal variations: Hb values are highest in the morning and lowest in the evening. A change in the Hb must be 1.5 g% or more to be considered definitely significant.

Causes of Anemia

1. Blood Loss

- Acute post-hemorrhagic anemia
- Chronic post-hemorrhagic anemia.

2. Impaired Red Cell Formation

a. Disturbance of bone marrow due to deficiency of substances essential for erythropoiesis

- Iron deficiency anemia
- Megaloblastic macrocytic anemias due to deficiency of vitamin B_{12} or folic acid
- Anemia associated with scurvy.

b. Disturbance of bone marrow functions not due to deficiency of substances essential for erythropoiesis

- Anemia associated with
  - Infection
  - Renal failure
  - Liver disease
  - Disseminated malignancy
- Aplastic anemia
- Anemia associated with bone marrow infiltration, e.g. leukemia, malignant lymphoma, multiple myeloma, myelosclerosis
- Anemia associated with myxedema and hypopituitarism
- Sideroblastic anemias
- Congenital dyserythropoietic anemias.

3. Increased Destruction of Red Cell (Hemolytic) Anemia

- Hemolysis due to corpuscular defects (intracorpuscular or intrinsic abnormality)
- Hemolytic anemia due to abnormal hemolytic mechanisms (extracorpuscular or extrinsic defect).

Polycythemia

Hemoglobin value for the age and sex of the patient is called polycythemia. Of course, one has to refer to other parameters as well.

Polycythemia (erythrocytosis) refers to:

- Increase in Hb
  - Above 18 g% in males
  - Above 16.5 g% in females. In addition, there is:
- Increase in red cell count:
  - Above 6 million/cu mm in males
  - Above 5.5 million/cu mm in females.
- Increase in hematocrit (PCV)
  - Above 55% in males
  - Above 47% in females.

Causes

Primary

Polycythemia vera (neoplastic).

Secondary

1. Associated with hypoxia
   a. Cardiovascular disease, usually congenital resulting in significant venous admixture.
   b. Pulmonary disease resulting in:
      - Impaired gas perfusion
      - Perfusion of poorly aerated lung
      - Pulmonary arteriovenous fistula.
   c. High altitude residence.
   d. Hypoventilation associated with obesity (Pickwickian syndrome).
   e. Hemoglobin variants with increased affinity for oxygen.
   f. Heavy smoking.
   g. Methemoglobinemia (rarely).
2. Due to inappropriate erythropoietin increase in:
   a. Benign/malignant tumors of:
      - Kidney
      - Liver
      - CNS
      - Uterus
      - Ovary.
   b. Renal disease (besides malignancies)
      - Hydronephrosis
      - Vascular impairment
      - Cysts.
3. Associated with adrenocortical steroids or Androgens.
   a. Adrenal hypercorticism (all types)
   b. Virilizing tumors
   c. Androgens used therapeutically (rarely corticoids).
4. Associated with chronic chemical exposure
   a. Nitrites, sulfonamides, other substances producing methemoglobin and sulfhemoglobin.
   b. Cobalt, shellac components, various alcohols.
5. Relative
   a. Stress or spurious polycythemia
   b. Dehydration: water deprivation, vomiting
   c. Plasma loss: burns, enteropathy.

HEMATOCRIT/PACKED CELL VOLUME (PCV)

Definition

Hematocrit is the volume of red cells expressed as a percentage of the volume of whole blood in the sample. The venous hematocrit is almost same as that obtained from a skin puncture. Dried heparin, EDTA or double oxalate are satisfactory anticoagulants.

Methods

1. Using Wintrobe's tube.
2. Using microhematocrit capillaries.

Wintrobe's Tube

Fill the Wintrobe's tube till the 100 mark on top with a Pasteur pipette ensuring that there are no air-bubbles in the blood column. Centrifuge this tube for 15 minutes at 3500 rpm (or longer at lower speeds) until packing is complete. After centrifuging, the blood is separated into 3 layers, a column of red blood cells at the bottom, a narrow middle layer—buffy coat of WBCs and platelets and the topmost fluid column of plasma. The percentage of the height of the column of blood occupied by packed red cells constitutes the hematocrit. Roughly, the hematocrit value is three times the hemoglobin concentration.

Sources of Error

1. Inadequate mixing of blood.
2. Irregularity of the bore of the tube.
3. Incomplete packing.

Microhematocrit

This method is in common use in most well-equipped laboratories. Capillary tubes coated with anticoagulant can be filled with blood obtained from finger puncture or from a venipuncture or with blood already anticoagulated. One end of the filled capillary tube is sealed with sealing wax (e.g. Plasticine) or the empty end is sealed with heat. The sealed tube is centrifuged for 3 minutes in a special

high-speed centrifuge. By reading the packed cell height and the total height of the entire specimen, the hematocrit can be determined. Special reading devices are available.
Values: If the red cells are of normal size (normocytic), and the red cell count is 5 million, the hematocrit is about 45%.
Men—Range 42–52% Average = 47%
Women—Range 37–47% Average = 42%.

Interpretation

Causes of reduced hematocrit—causes of anemia. Causes of raised hematocrit—causes of polycythemia.

If packed cell volume has been determined by Wintrobe's tube, one can obtain some more information.

Buffy coat: A buffy coat of thickness 1 mm approximately corresponds to a total leukocyte count of about 10,000. Absent or minimal buffy coat implies leukopenia, a thickness more than 1 mm implies leukocytosis. In addition, in sub-leukemic leukemia, a film can be made from the buffy coat where a greater concentration of WBCs will be available, and identification of atypical cells would become easier and less time consuming. Another advantage is for performing LE cell or phenomenon test, for which also WBCs can be picked up from the buffy coat. The platelets form a very thin layer above the white cells, the coat is pinkish white but is of no use clinically, one has to do platelet counts if necessary.

Plasma layer: The topmost layer of plasma can give important clues by observing its color. Its normal color is pale yellow or straw.
Yellow—jaundice
Pink—hemolysis
Creamy white—hyperlipidemia, especially chylomicrons
Brown—methemalbuminemia.

BLOOD CELL COUNTS

White Blood Cell (WBC)

A white cell count (TLC) estimates the total number of white cells in a cubic millimeter of blood. It is important in the diagnosis of disease, especially when accompanied by a differential white cell count.

The diluting fluid: WBC diluting fluid contains a weak acid to lyse the RBCs and a stain for staining the nucleus of WBCs, e.g. Turke's fluid.

Glacial acetic acid 1.5 mL 1% aqueous solution of gentian violet 1.0 mL distilled water 98.0 mL

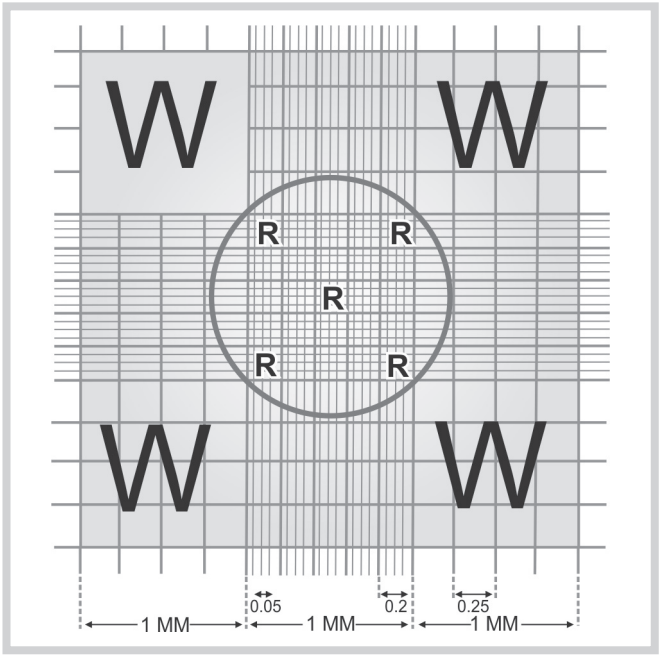


FIG. 9.5: Platelet counting area count the cells in the 25 squares inside the large center square circle

(A pinch of thymol may be added to the diluting fluid to prevent growth of moulds).

Counting Chamber

The chamber normally used for cell counts is the improved Neubauer's chamber which has an area of 9 mm^2 and a depth of 0.1 mm.

Other counting chambers can also be used including the Burker's chamber which has the same area and depth as the improved Neubauer (Fig. 9.5) and the Fuchs-Rosenthal ruled chamber which has the same area but a double depth of 0.2 mm.

Methods

Using a WBC pipette (Fig. 9.6) of a hemacytometer, draw well mixed venous blood or capillary blood and fill till the 0.5 mark. Clean the tip of the tube. Now draw WBC diluting fluid till the 11 mark (or to 0.38 mL of diluting fluid add 0.02 mL of blood with a Hb pipette).

1. Mix the fluid and blood mixture gently, avoiding bubbling.
2. Place the coverslip on the counting chamber at the right place.

Shake the fluid-blood mixture and transfer the mixture using a fine bore Pasteur pipette on to the counting chamber (called charging the chamber), taking care that the mixture

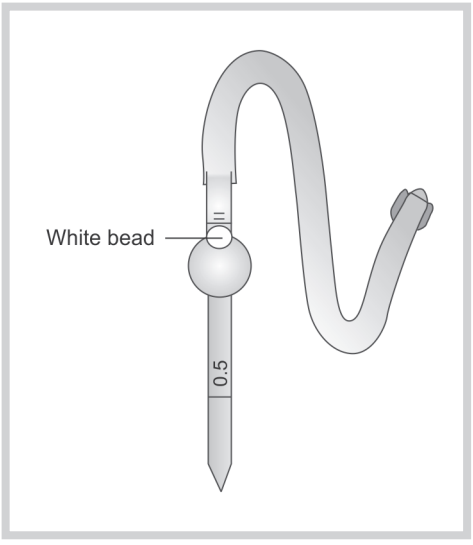


FIG. 9.6: WBC pipette

does not overflow. If it does overflow, wash and dry the chamber to be recharged again.

Allow the cells to settle to the bottom of the chamber for 2 minutes. See that fluid does not get dried up (For preventing this, take a petridish, place a wet filter paper at its bottom, now place the charged chamber gently and close off the dish for about 2 minutes).

For counting, clean the under part of the chamber if it was left in the petridish and place it on the stage of the microscope. Using 10X or low power objective, count the WBCs uniformly in the four larger corner squares (as indicated in the diagram). Cells present on the outermost lines should be counted on one side and those present on the line opposite should not be counted.

Calculate the number of cells per cubic millimeter of blood as follows:

$$= \frac{\text{Cells counted} \times \text{blood dilution} \times \text{chamber depth}}{\text{Area of chamber counted}}$$

$$= \text{number of cells counted} \times \frac{20 \times 10 \text{ (depth factor)}}{4}$$

= number of cell counted × 50

(Dilution factor is 20 for there is no mixing of cells till first 1 mark of the WBC pipette, hence 0.5 parts of blood are present in 10 parts of the diluting fluid dilution factor, then, is 10/0.5 = 20).

Falsely high counts occur due to:

1. Blood taken from an area where there was hemoconcentration.
2. Not wiping away the blood on the outside of tip of the pipette.
3. Blood drawn above the mark in the pipette (happens usually when the rubber mouthpiece is too short).
4. Diluting fluid not taken till the requisite mark.
5. Improper mixing.
6. Uneven distribution in the counting chamber.
7. Extraneous material present (yeast, dirt, etc.).
8. Errors in calculation.

Falsely low counts occur due to:

1. Dilution of the blood with tissue fluid due to edema or squeezing.
2. Delay in counting (this does not affect RBCs as much as WBCs, which are reduced by about 15% in 24 hours).
3. Blood not drawn up to the requisite mark.
4. Diluting fluid taken in excess of the requisite mark.
5. Saliva in the mouthpiece running into the upper end of the pipette causing further dilution.
6. Improper mixing.
7. Uneven distribution in the counting chamber.
8. Uniform systematic counting not done.
9. Cells lost through hemolysis (in RBC count-for instance).
10. Errors in calculation.
11. Clumping of cells or coagulation of the blood.

Causes of raised and reduced leukocyte counts would be presented with differential leukocyte counts, since raised or lowered total counts are usually accompanied by abnormal differential counts.

Correcting the white cell count for nucleated red cells: From peripheral smear, find out the number of nucleated red cells per 100 WBCs counted.

Calculation

$$\frac{\text{Number of nucleated RBCs}}{100 + \text{number of nucleated RBCs}} \times \text{TLC}$$

= Nucleated RBC/cu mm

Corrected count = TLC–Nucleated RBC count.

Red Blood Cell (RBC)

Diluting Fluid

This should be isotonic so that RBCs are not hemolyzed. Normal saline can be used but it may cause crenation of the RBCs and allow rouleaux formation.

One can use:

1. Sodium citrate 3 g
   Formalin 1 mL
   Distilled water to 100 mL
   (Cheap and good) Or
2. Hayem's fluid
   Mercuric chloride 0.5 g

Sodium chloride 1.0 g
Sodium sulphate 5.0 g
Distilled water to 200 mL.

(Needs to be made frequently and in hyperglobulinemia one may set precipitation of protein so RBC clumping may occur. Mercuric chloride acts as an antiseptic).

Method

Draw blood to the 0.5 mark in the RBC pipette (Fig. 9.7). Wipe tip clean and draw diluting fluid to the 101 mark. Shake for 3 minutes. Charge the chamber. Count the RBCs using 40X objective in the 80 smallest squares as indicated in the diagram of the chamber.

RBC count

$$= \frac{\text{No. of cells counted} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Area counted}}$$

Where Dilution is 1 in 200, Depth is 1/10 mm

Area counted is $\frac{80}{400} = \frac{1}{5}$ mm^2

$$\frac{\text{Number counted} \times 200 \times 10}{1/5}$$

$$= \text{Number counted} \times 10{,}000$$

Interpretation

RBC counts are low in anemia and high in polycythemia, the causes of these have already been discussed.

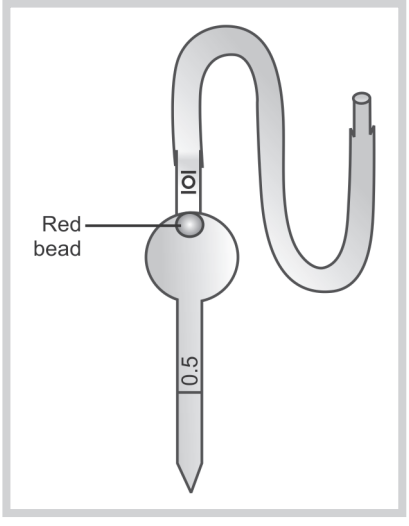


FIG. 9.7: RBC pipette

Platelets

Preferably, use venous blood for platelet counts. Finger prick may cause clumping of platelets. In small children, however, this clumping can be prevented by thinly smearing Vaseline over the area to be punctured (make sure that there has been no clotting of blood).

The blood is diluted in 1% ammonium oxalate stored refrigerated at 4°C which hemolyzes the RBCs (prepared by dissolving 1 g of ammonium oxalate in 100 mL of distilled water).

Method

- Fill blood and diluent (in this case 1% ammonium oxalate) as described for the RBC count and using the RBC pipette. If platelet count is low, a WBC pipette can be used instead
- Charge the chamber with the help of the pipette employed
- Using 40X objective with reduced condenser aperture, count the platelets in the same squares as indicated for RBC counting
- Calculate as (if RBC pipette used)

$$\frac{\text{Cells counted} \times \text{blood dilution} \times \text{chamber depth factor}}{\text{Area of chamber counted}}$$

$= N \times 200 \times 5 \times 10$
$= N \times 10000$

However, if a WBC pipette is employed, the appropriate formula and method should be used. Platelet counts are made in the small 5 RBC squares only.

Platelet count = N × 20 × 5 × 10 or N × 1000
Normal platelet counts = 1.5–3.5 lakhs/cu mm.

Rees-Ecker Method for Platelet Count

Various components of the diluting fluid used have various functions, e.g. citrate prevents coagulation while formalin fixes the platelets and prevents their clumping together. Here, no attempt is made to lyse RBCs. Platelets are identified by their size, shape and dark color. Brilliant cresyl blue (the dye used) provides the background during cell counting. This dye does not stain the platelets and, therefore, is not essential for the counting procedure.

Diluting Fluid

Consists of:

Trisodium citrate	3.8 g
Neutral formaldehyde	0.2 mL
Brilliant cresyl blue	0.1 g
Deionized water	100 mL

Dissolve the ingredients in 100 mL volumetric flask, filter, centrifuge, transfer to a well-stoppered bottle and keep at 2–8°C (refrigerate). This fluid if not contaminated will stay good indefinitely. Filter aliquot of the diluting fluid immediately before use.

All glassware must be scrupulously clean. Dirt or dust particles can resemble and may be counted as platelets.

Procedure

- Take 3.98 mL of diluent (freshly filtered) into a test tube
- Add to the diluent 0.02 mL (20 µL) of well-mixed anticoagulated blood. With the help of a Sahli pipette, wipe out the outer tip of the pipette before dilution. Wash out the contents in the pipette into the diluent tube 3–4 times
- Immediately mix the diluent with the specimen for at least 5 minutes or so
- Employ the Sahli's pipette for charging either side of the chamber
- Keep the charged hemocytometer inside a moist chamber (can be a petri dish with a moistened or wet filter paper — on which the chamber can be kept). Let stay for about 15 minutes. This permits the platelets to settle down, and the moistened chamber does not allow evaporation of the fluid
- Place the hemocytometer on the stage of the microscope, focus the RBC counting area under low magnification. Now move to the corner square of the red cell area carefully to high dry objective
- Platelets are bluish and must be distinguished from debris. They are oval, round, or comma-shaped, refractile bodies that vary in size normally from 1 to 5 microns.
- Count the platelets in the finely ruled center area (1 mm^2) of each side of the chamber. Take the average counts of two sides. (In the new improved Neubauer ruling, there are 25 small squares and each of these contain 16 smallest squares. The area covered by the 25 squares is equal to 1 mm^2).

Platelet count/mL or cu mm =

$$\frac{\text{Number of platelets counted} \times \text{dilution}}{\text{Volume of fluid}}$$

Where,

volume of fluid for the 1 sq mm area = $1 \times 0.1 = 0.1$ mL (cu mm)

Dilution = 200

So platelet count/cu mm =

$$\frac{\text{Number of platelets counted} \times 200}{0.1}$$

$$= \text{Number of platelets counted} \times 2000$$

Rough Estimation of Platelet Count from Stained thin Smear

A well-prepared peripheral blood smear can be used to check the results of direct counting. Determine the ratio of platelets to red cells on a thin blood smear used for differential leukocyte count. If the average number of platelets is 8 to 25 in 10 fields, it is reported to be adequate, and if it is 0 to 5, it is reported as inadequate.

Causes of Thrombocytopenia

1. *Causes of platelet production failure:* Selective megakaryocyte depression:
   - Drugs
   - Chemicals
   - Viral infections.

   Part of general bone marrow failure:
   - Aplastic anemia
   - Leukemia
   - Myelosclerosis
   - Marrow infiltration, e.g. in carcinoma, lymphoma
   - Multiple myeloma
   - Megaloblastic anemia.
2. *Increased destruction of platelets*
   - Acute or chronic ITP (idiopathic thrombocytopenic purpura)
   - Secondary immune thrombocytopenia (post-infection, SLE, CLL, and lymphomas).
3. *Abnormal distribution of platelets*
   - Splenomegaly.
4. *Dilutional loss*
   - Massive transfusion of old blood to bleeding patients.

Raised Platelet Count (Thrombocytosis)

Can occur as a part of generalized myeloproliferative disorder, e.g. CML or following acute hemorrhage.

ERYTHROCYTE INDICES

These can be calculated from:

a. Hematocrit,
b. Hemoglobin concentration, and
c. Red cell counts.

The Mean Cell Volume (MCV)

$$\text{MCV} = \frac{\text{Packed cell volume}}{\text{Red cell count per liter}} \times 10^{15} \text{ fl}$$

Normal Values

Adults 76–96 fl.

Infants, full term cord blood average 106 fl.

Children, 1 year 76–87 fl.
Children 10–12 years 76–93 fl.
MCV is reduced in microcytic anemias
MCV is raised in macrocytic anemias

The Mean Cell Hemoglobin (MCH)

$$\text{MCH} = \frac{\text{Hemoglobin in g/L}}{\text{Red cell count per mL}} \text{ pg.}$$

Normal MCH in adults is from 27 to 32 pg.
MCH is reduced in hypochromic anemias.

The Mean Cell Hemoglobin Concentration (MCHC)

$$\text{MCHC} = \frac{\text{Hb in g\%}}{\text{PCV}} \times 100 = 31\text{–}35 \text{ g\%}$$

This too, is low in hypochromic anemias.

COMPLETE BLOOD COUNT (CBC)

Normal values

	Normal range, unit	*SI units*
Hematocrit (HCT)		
Adult females	37–47%	0.37–0.47 L/L
Pregnant		
Trimester 1	35–46%	0.35–0.46 L/L
Trimester 2	30–42%	0.30–0.42 L/L
Trimester 3	34–44%	0.34–0.44 L/L
Postpartum	34–44%	0.34–0.44 L/L
Adult males	40–54%	0.40–0.54 L/L
Children		
Newborn	42–68%	0.42–0.68 L/L
3 months	29–54%	0.29–0.54 L/L
1 year	29–41%	0.29–0.41 L/L
3 years	31–44%	0.31–0.44 L/L
10 years	34–45%	0.34–0.45 L/L
Hemoglobin (HGB)		
Adult females	12–16 g/dL	7.4–9.9 mmol/L
Pregnant		
Trimester 1	11.4–15.0 g/dL	7.1–9.3 mmol/L
Trimester 2	10.0–14.3 g/dL	6.2–8.9 mmol/L
Trimester 3	10.2–14.4 g/dL	6.3–8.9 mmol/L
Postpartum	10.4–15.0 g/dL	6.4–9.3 mmol/L
Adult males	14.0–18.0 g/dL	8.7–11.2 mmol/L

Contd..

Contd...

	Normal range, unit	*SI units*
Panic low level	<5 g/dL	<3.1 mmol/L
Panic high level	>18 g/dL	>11.2 mmol/L
Children		
Newborn		
Day 1	15.5–24.5 g/dL	9.6-15.2 mmol/L
Days 2–3	19.0 g/dL	11.8 mmol/L
Days 4–8	14.3–22.3 g/dL	8.9–13.8 mmol/L
Days 9–13	16.5 g/dL	10.2 mmol/L
2–8 weeks	10.7–17.3 g/dL	6.6–10.7 mmol/L
3–5 months	9.9–15.5 g/dL	6.1–9.6 mmol/L
6–11 months	11.8 g/dL	7.3 mmol/L
1–2 years	9.0–14.6 g/dL	5.6–9.0 mmol/L
3–9 years	9.4–15.5 g/dL	5.8–9.6 mmol/L
10 years	10.7–15.5 g/dL	6.6–9.6 mmol/L
11–15 years	13.4 g/dL	8.3 mmol/L
Panic levels	<5g/dL	<3.1 mmol/L
	>18 g/dL	>11.2 mmol/L
Red Blood Cell (RBC) Count		
Adult females	4.0–5.5 million/μL	4.0–5.5 × 10^{12}/L
Pregnant		
Trimester 1	4.0–5.0 million/μL	4.0–5.0 × 10^{12}/L
Trimester 2	3.2–4.5 million/μL	3.2–4.5 × 10^{12}/L
Trimester 3	3.0–4.9 million/μL	3.0–4.9 × 10^{12}/L
Postpartum	3.2–5.0 million/μL	3.2–5.0 × 10^{12}/L
Adult males	4.5–6.2 million/μL	4.5–6.2 × 10^{12}/L
Children		
Newborn		
Day I	4.1–6.1 million/μL	4.1–6.1 × 10^{12}/L
Days 2–8	5.1 million/μL	5.1 × 10^{12}/L
Days 9–13	5.0 million/μL	5.0 × 10^{12}/L
2–8 weeks	3.8–5.6 million/μL	3.8–5.6 × 10^{12}/L
3–5 months	3.8–5.2 million/μL	3.8–5.2 × 10^{12}/L
6–11 months	4.6 million/μL	4.6 × 10^{12}/L
1–2 years	3.6-5.5 million/μL	3.6–5.5 × 10^{12}/L
3 years	4.5 million/μL	4.5 × 10^{12}/L
4 years	4.0–5.2 million/μL	4.0–5.2 × 10^{12}/L
5 years	4.6 million/μL	4.6 × 10^{12}/L
6–10 years	4.7 million/μL	4.7 × 10^{12}/L
11–15 years	4.8 million/μL	4.8 × 10^{12}/L

Contd..

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	Normal range, unit	*SI units*
Mean Cell Volume (MCV)		
Adults	82–98 μ^3	82–98 fl
Children		
Newborn		
Day 1	106 μ^3	106 fl
Days 2–3	105 μ^3	105 fl
Days 4–8	103 μ^3	103 fl
Days 9–13	98 μ^3	98 fl
2–8 weeks	90 μ^3	90 fl
3 months	82 μ^3	82 fl
4–5 months	80 μ^3	80 fl
6–11 months	77 μ^3	77 fl
1 year	78 μ^3	78 fl
2 years	77 μ^3	77 fl
3 years	79 μ^3	79 fl
4–10 years	80 μ^3	80 fl
11–15 years	82 μ^3	82 fl
Mean Cell Hemoglobin (MCH)		
Adults	26–34 pg	1.61–2.11 fmol
Children		
Newborn		
Day I	38 pg	2.36 fmol
Days 2–3	37 pg	2.30 fmol
Days 4–8	36 pg	2.23 fmol
Days 9–13	33 pg	2.05 fmol
2–8 weeks	30 pg	1.86 fmol
3 months	28 pg	1.73 fmol
4–5 months	27 pg	1.67 fmol
6–11 months	26 pg	1.61 fmol
1–2 years	25 pg	1.55 fmol
3 years	26 pg	1.61 fmol
4–10 years	27 pg	1.67 fmol
11–15 years	28 pg	1.73 fmol
Mean Cell Hemoglobin Concentration (MCHC)		
Adults	31–38%	19.2–23.58 mmol/L
Children		
Newborn		
Day 1	36%	22.34 mmol/L
Days 2–8	35%	21.72 mmol/L
Days 9-13	34%	21.10 mmol/L

Contd..

Contd...

	Normal range, unit	*SI units*
2–8 weeks	33%	20.48 mmol/L
3–5 months	34%	21.10 mmol/L
6–11 months	33%	20.48 mmol/L
3 years	35%	21.72 mmol/L
1–2 years	32%	19.86 mmol/L
4–15 years	34%	21.10 mmol/L
White Blood Cell (WBC) Count		
Adult females	4500–11,000/mL	4.5–11.0 × 10^9/L
Pregnant		
Trimester 1	6600–14,100/mL	6.6–14.1 × 10^9/L
Trimester 2	6900–17,100/mL	6.9–17.1 × 10^9/L
Trimester 3	5900–14,700/mL	5.9–14.7 × 10^9/L
Postpartum	9700–25,700/mL	9.7–25.7 × 10^9/L
Adult males	4500–11,000/mL	4.5–11.0 × 10^9/L
Children		
Newborn	9000–30,000/mL	9.0–30.0 × 10^9/L
3 months	5700–18,000/mL	5.7–18.0 × 10^9/L
1 year	6000–17,500/mL	6.0–17.5 × 10^9/L
3 years	5700–16,300/mL	5.7–16.3 × 10^9/L
10 years	4500–13,500/mL	4.5–13.5 × 10^9/L
White Blood Cells Differential		
Granulocytes		
Segmented		
Neutrophil (Segs)	54–62%	0.54–0.62
Adults	3800/µL or mm^3	3800 × 106/L
Children		
Birth	8400/µL or mm^3	8400 × 10^6/L
12 hours	12,100/µL or mm^3	12,100 × 10^6/L
24 hours	8870/µL or mm^3	8870 × 10^6/L
1 week	4100/µL or mm^3	4100 × 10^6/L
2 weeks	3320/µL or mm^3	3320 × 10^6/L
1–2 months	2750/µL or mm^3	2750 × 10^6/L
4 months	2730/µL or mm^3	2730 × 10^6/L
6 months	2710/µL or mm^3	2710 × 10^6/L
8 months	2680/µL or mm^3	2680 × 10^6/L
10 months	2600/µL or mm^3	2600 × 10^6/L
12 months	2680/µL or mm^3	2680 × 10^6/L
2 years	2660/µL or mm^3	2660 × 10^6/L
4 years	3040/µL or mm^3	3040 × 10^6/L
6 years	3600/µL or mm^3	3600 × 10^6/L

Contd..

Contd...

	Normal range, unit	*SI units*
8–14 years	3700/μL or mm^3	3700 × 10^6/L
15 to 20 years	3800/μL or mm^3	3800 × 10^6/L
Band Neutrophils (Bands)	3–5%	0.03–.05
Adults	620/μL or mm^3	620 × 10^6/L
Children		
Birth	2540/μL or mm^3	2540 × 10^6/L
12 hours	3460/μL or mm^3	3460 × 10^6/L
24 hours	2680/μL or mm^3	2680 × 10^6/L
1 week	1420/μL or mm^3	1420 × 10^6/L
2 weeks	1200/μL or mm^3	1200 × 10^6/L
1 month	1150/μL or mm^3	1150 × 10^6/L
2 months	1100/μL or mm^3	1100 × 10^6/L
4–10 months	1000/μL or mm^3	1000 × 10^6/L
12 months	990/μL or mm^3	990 × 10^6/L
2 years	850/μL or mm^3	850 × 10^6/L
4 years	710/μL or mm^3	710 × 10^6/L
6 years	670/μL or mm^3	670 × 10^6/L
8 years	660/μL or mm^3	660 × 10^6/L
10 years	645/μL or mm^3	645 × 10^6/L
12-14 years	640/μL or mm^3	640 × 10^6/L
15-20 years	620/μL or mm^3	620 × 10^6/L
Eosinophils	1–4%	0.01–0.04
Adults	200/μL or mm^3	200 × 10^6/L
Children		
Birth	400/μL or mm^3	400 × 10^6/L
12-24 hours	450/μL or mm^3	450 × 10^6/L
l week	500/μL or mm^3	500 × 10^6/L
2 weeks	350/μL or mm^3	350 × 10^6/L
1 month-l yr	300/μL or mm^3	300 × 10^6/L
2 years	280/μL or mm^3	280 × 10^6/L
4 years	250/μL or mm^3	250 × 10^6/L
6 years	230/μL or mm^3	230 × 10^6/L
8-20 years	200/μL or mm^3	200 × 10^6/L
Basophils	(M) 0.75%	0–0.0075
Adults	40/μL or mm^3	40 × 10^6/L
Children		
Birth-24 hours	100/μL or mm^3	100 × 10^6/L
1 week-8 years	50/μL or mm^3	50 × 10^6/L
10–20 years	40/μL or mm^3	40 × 10^6/L

Contd..

Contd...

	Normal range, unit	*SI Units*
Monocytes	2–10%	0.02–0.1
Adults	300/μL or mm^3	300 × 10^6/L
Children		
Birth	1050/μL or mm^3	1050 × 10^6/L
12 hours	1200/μL or mm^3	1200 × 10^6/L
24 hours-1 week	1100/μL or mm^3	1100 × 10^6/L
2 weeks	1000/μL or mm^3	1000 × 10^6/L
1 month	700/μL or mm^3	700 × 10^6/L
2 months	650/μL or mm^3	650 × 10^6/L
4 months	600/μL or mm^3	600 × 10^6/L
6–8 months	580/μL or mm^3	580 × 10^6/L
10–12 months	550/μL or mm^3	550 × 10^6/L
2 years	530/μL or mm^3	530 × 10^6/L
4 years	450/μL or mm^3	450 × 10^6/L
6 years	400/μL or mm^3	400 × 10^6/L
8–12 years	350/μL or mm^3	350 × 10^6/L
14 years	380/μL or mm^3	380 × 10^6/L
16–18 years	400/μL or mm^3	400 × 10^6/L
20 years	380/μL or mm^3	380 × 10^6/L
Lymphocytes	20–40%	0.20–0.40
Adults	2500/μL or mm^3	2500 × 10^6/L
Children		
Birth–12 hrs	5500/μL or mm^3	5500 × 10^6/L
24 hours	5800/μL or mm^3	5800 × 10^6/L
1 week	5000/μL or mm^3	5000 × 10^6/L
2 weeks	5500/μL or mm^3	5500 × 10^6/L
1 month	6000/μL or mm^3	6000 × 10^6/L
2 months	6300/μL or mm^3	6300 × 10^6/L
4 months	6800/μL or mm^3	6800 × 10^6/L
6 months	7300/μL or mm^3	7300 × 10^6/L
8 months	7600/μL or mm^3	7600 × 10^6/L
10 months	7500/μL or mm^3	7500 × 10^6/L
12 months	7000/μL or mm^3	7000 × 10^6/L
2 years	6300/μL or mm^3	6300 × 10^6/L
4 years	4500/μL or mm^3	4500 × 10^6/L
6 years	3500/μL or mm^3	3500 × 10^6/L
8 years	3300/μL or mm^3	3300 × 10^6/L
10 years	3100/μL or mm^3	3100 × 10^6/L
12 years	3000/μL or mm^3	3000 × 10^6/L
14 years	2900/μL or mm^3	2900 × 10^6/L

Contd..

Contd...

	Normal range, unit	*SI units*
16 years	2800/µL or mm^3	2800×10^6/L
18 years	2700/µL or mm^3	2700×10^6/L
20 years	2500/µL or mm^3	2500×10^6/L
Platelets		
Adults	150,000–400,000/ µL or mm^3	$150–400 \times 10^9$/L
Panic/Levels	<30,000/µL or mm^3	$<30 \times 10^9$/L
	>l,000,000/µL or mm^3	$>1000 \times 10^9$/L
Children		
Newborn	100,000–300,000/ µL or mm^3	$100–300 \times 10^9$/L
3 months	260,000/µL or mm^3	260×10^9/L
1–10 years	250,000/µL or mm^3	250×10^9/L
Panic/Levels	<20,000/µL or mm^3	$<20 \times 10^9$/L
	> 1,000,000/µL or mm^3	$>1000 \times 10^9$/L

Complete Blood Count (CBC)

CBC includes—TLC, DLC, RBC count, hemoglobin, hematocrit, RBC indices, platelet count and a peripheral smear examination.

ERYTHROCYTE SEDIMENTATION RATE (ESR)

This is the rate at which erythrocytes sediment on their own weight when anticoagulated blood is held in a vertical column, it is expressed as the fall of RBCs in mm at the end of first hour (starting point—when the tube or pipette was filled with blood).

Methods

Westergren's Method

1. Westergren's pipette (open at both ends) is about 30 cm long with a bore diameter of about 2.5 mm (Fig. 9.8).
2. The lower 20 cm are marked from 0 (top) to 200 (bottom).
3. Anticoagulant used is 3.8% trisodium citrate solution. One part of anticoagulant is added to 4 parts of blood.
4. The pipette accepts about 1 mL of blood. Fill the pipette by sucking till the 0 mark and clamp it vertically in the Westergren's rack.
5. Read the upper level of red cells exactly after 1 hour.

This is a better method than Wintrobe's since the reading obtained is magnified as the column is lengthier.

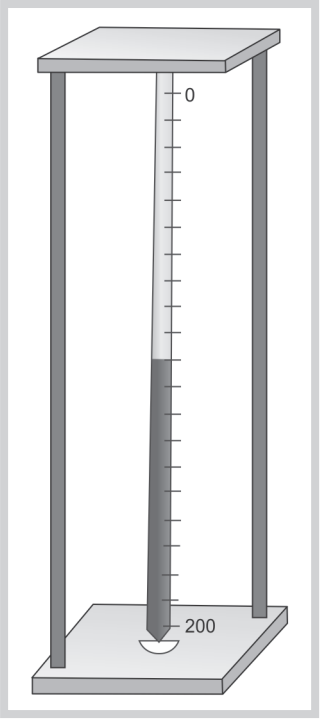


FIG. 9.8: Westergren's ESR pipette with stand

Normal Values

Males—0 to 5 mm at the end of 1st hour. Females—0 to 7 mm at the end of 1st hour.

Wintrobe's Method

1. The Wintrobe's tube (Fig. 9.9) is about 11 cm long, bore diameter is 2.5 mm and the bottom 10 cm are graduated.
2. Graduations are from zero (top) to hundred (bottom) for ESR and zero (bottom) to hundred (top) for PCV.
3. EDTA blood is used and the tube is filled till zero mark on top with the help of a Pasteur pipette.
4. Set it up vertically and read exactly after 1 hour.
5. As has already been said that this tube can also be used for PCV estimation.

Normal Values

Males—0 to 9 mm at the end of 1st hour. Females—0 to 20 mm at the end of 1st hour.

Microsedimentation (Landau) Method

Capillary blood can be taken.

Materials Required

1. 5.0 g/dL sodium citrate solution.
2. Landau pipette: This pipette resembles RBC pipette. It is graduated from 0 to 50 mm.

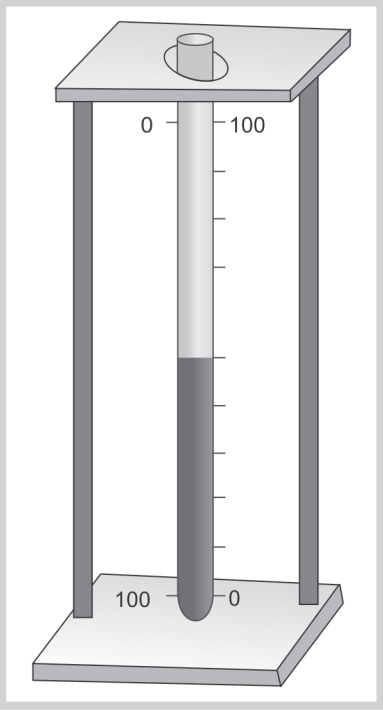


FIG. 9.9: Wintrobe's ESR tube with stand

3. Landau pipette stand.
4. Suction device for drawing blood into the pipette.
5. Capillaries for blood collection.

Procedure

1. Attach Landau pipette to the suction device.
2. Draw 5.0 g/dL citrate up to first line on the stem.
3. Now draw blood by suction device up to second mark on the stem (avoid air bubbles).
4. Wipe excess blood on the external side of the pipette.
5. Draw citrate solution and blood into the bulb of the pipette. Mix the contents thoroughly.
6. Force back the mixture into the stem of the pipette.
7. Set the upper level of the mixture of the zero mm mark at the top.
8. Detach the suction device.
9. Place the pipette in vertical position on the stand, set time to one hour.
10. Note the reading (the distance red cells have fallen or the extent of plasma column) after one hour.

Normal Values

Male: 0–5 mm after Ist hour. Female: 0–8 mm after Ist hour.

Zeta Sedimentation Rate (ZSR)

The zeta sedimentation rate (ZSR) is performed using a special small-bore capillary tube that is filled with blood and span for 3–4 minutes in a centrifuge called Zetafuge® (Beckman Coulter). This centrifuge alternately compacts and disposes the RBCs under standardized centrifugal force. The tube is then read on a special reader to obtain a value called the Zetacrit, which represents the percentage of sedimented erythrocytes. The Zetacrit value is divided into the hematocrit value (also a percentage) and the result in the ZSR expressed as percentage.

The ZSR's advantages are that it is rapid, correct for anemia, and requires only a small blood sample which is desirable for pediatric patients. However, a special centrifuge and reader are needed to perform the test.

Many other automated systems/devices are also available.

Sources of Error for any ESR Method

1. Improper anticoagulant.
2. Tube not vertical, an inclination of 3° raises ESR by almost 30%.
3. Dirty tube.
4. Bubbles caused by too vigorous mixing.
5. Hemolysis may modify ESR.
6. Prolonged storage of blood after withdrawing it, the test should be performed within 3 hours.
7. Pipette/tube kept on a vibrating surface (vibration prevents rouleaux formation).

Interpretation of ESR

The value of ESR is that it indicates the possible presence of organic disease, or to follow the course of disease. Its main use is as a prognostic tool. It is used as a diagnostic criterion (minor) in rheumatic fever only.

Rapid ESR is Found in

1. In any chronic infection, e.g. tuberculosis (maximum in miliary tuberculosis), has prognostic value.
2. Any extensive inflammation, cell destruction or toxemia.
3. Pregnancy, after the second month.
4. Puerperium, returns to normal within 2 months.
5. Active myocardial infarction (rapid rise).
6. Acute myocardial infarction (rapid rise).
7. Active rheumatoid arthritis (not much elevated in osteoarthritis).
8. Nephrosis (low blood albumin, anemia).
9. All types of shock.
10. Active syphilis (moderate acceleration).
11. Postoperative states (for variable periods).
12. Any active infectious disease, acute or chronic.
13. Salpingitis, appendicitis (often normal), due to absorption of purulent necrotic material.

14. Infected, necrotic or malignant tumors.
15. Liver disease (depends upon blood proteins).
16. Menstruation (slight acceleration).

Slow ESR is Usually Seen in

1. Newborn infants.
2. Polycythemia.
3. Congestive heart failure.
4. Allergic states.
5. Sickle cell anemia (poikilocytosis).

Factors that Play a Role in ESR

1. Plasma Factors

- An accelerated ESR is favored by elevated levels of fibrinogen, and to a lesser extent, of globulins (α and β globulins are more effective than γ globulin)
- These plasma factors cause increased formation of rouleaux which due to more weight sediment more rapidly than do single cells
- Albumin retards sedimentation
- Extreme increase in plasma viscosity slows down ESR
- Cholesterol accelerates and lecithin retards the ESR.

2. Red Cell Factors

- Anemia is responsible for accelerated ESR. The change in erythrocyte-plasma ratio favors rouleaux formation
- Microcytes sediment more slowly and macrocytes somewhat more rapidly than normocytes. The sedimentation rate is directly proportional to the weight of the cell aggregate and inversely proportional to the surface area
- Poikilocytosis retards ESR because abnormal shape hampers rouleaux formation.

3. Anticoagulants

- Sodium citrate and EDTA do not effect ESR but oxalates and heparin may.

Stages in ESR

1. First 10 minutes—is the period of aggregation. Rouleaux formation occurs at this stage and sedimentation is slow.
2. Next 40 minutes—is period of fast settling, during this period rate of fall is constant.
3. Last 10 minutes—is the final period of packing.

Interfering Factors

1. The blood sample should not be allowed to stand for more than 2 hours before the test is started because rate will increase.
2. In refrigerated blood, the sedimentation rate is greatly increased. Refrigerated blood should be allowed to return to room temperature before the test is performed.
3. Factors leading to reduced rates:
   - High blood sugar
   - High albumin level
   - High phospholipids
   - Decreased fibrinogen level of the blood in newborns
   - Certain drugs (see below).
4. *Drugs*
   a. That increase ESR levels:
      - Dextran
      - Methyldopa
      - Methysergide
      - Oral contraceptives
      - Penicillamine
      - Theophylline
      - Trifluperidol
      - Vitamin A.
   b. Those that decrease levels:
      - Ethambutol
      - Quinine
      - Salicylates
      - Drugs that cause a high blood glucose level (cortisone and ACTH).

BLOOD FILM EXAMINATION

Preparation of a Thin Blood Film

A thin blood film is made by spreading a drop of blood evenly across a clean grease free slide, using a smooth edged spreader.

Making of Spreaders (Fig. 9.10)

- Select a slide which has smooth edges
- Using a glass cutter and a ruler, mark off 4 equal divisions, each measuring 19 mm
- Break off at each division to give 4 spreaders
- Readymade spreaders are available.

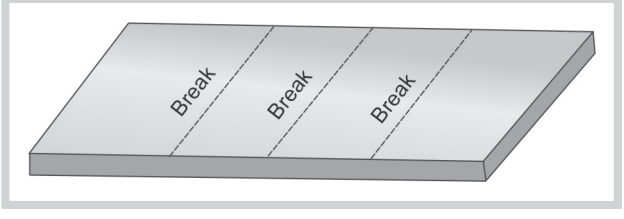


FIG. 9.10: Making a spreader

For anemic blood, a rapid smearing is needed; whereas for thick concentrated blood, smearing should be done slowly. A well-spread smear shows no lines extending across or downwards through the film and the smear should be tongue shaped (Figs 9.11A and B).

Making Thick Smears

While the thin smears are used for describing blood cells, the thick smears are used for detecting malarial parasites and microfilariae. A large drop of blood is taken on the center of a slide and with the aid of a needle or slide corner spread the drop over ½ an inch square area. When dry, the thickness should be such that printed matter can be seen through it.

Fixing of Blood Films

Before staining, the blood films need to be fixed with acetone-free methyl alcohol for ½ to 1 minute in order to prevent hemolysis when they come in contact with water while staining them with aqueous (water-based) stains or when water has to be added subsequently. Alcohol denatures the proteins and hardens the cell contents. For Wright's stain and Leishman's stain, no prefixation is required as these contain acetone-free methyl alcohol; but for Giemsa's stain, prefixation is a must because the alcohol content is only 5% in the ready-to-use stain.

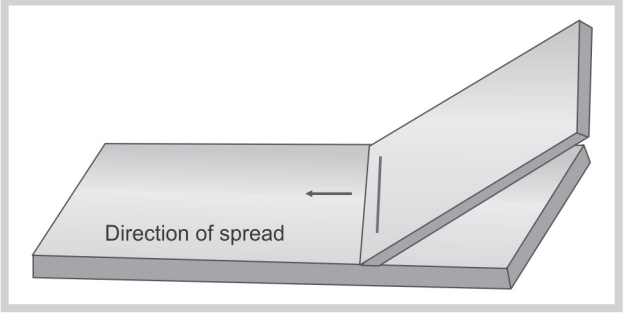


FIG. 9.11A: Direction of spread

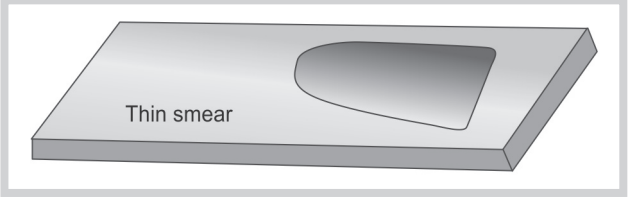


FIG. 9.11B: A thin peripheral blood smear

Staining of Blood Films

Blood cells have structures that are acidophilic and some basophilic structures, so they vary in their reaction (pH). The nuclei are basophilic and stain blue. The highly basophilic (acidic) basophil granules also stain blue. Hemoglobin (being basic) stains acidophilic or red.

Stains that are made up of combinations of acid and basic dyes are called Romanowsky stain and various modifications are available, e.g. Wright's, Leishman's, Giemsa's, and Jenner's stains. Most use methylene blue as the basic stain, though toluidine blue is used in some. Most use eosin as the acid stain, though Azure I and Azure II are also used.

The dried film can stay for a couple of days in hot dry weather, but gets bad if they are not fixed in hot and humid climate that exists in India.

It is best to use neutral distilled water for diluting the stain. Stale distilled water becomes acidic after absorbing CO_2 from atmosphere. If the distilled water is alkaline RBCs stain a dirty bluish green color, the parts of WBC which should stain blue will be slightly purplish, the granules of eosinophils bluish or greenish instead of pink and granules of neutrophils overstained. If the water is acidic RBCs stain bright orange and nuclei of the white cells a very pale color.

The ideal pH is 6.8 and in order to maintain this buffered distilled water is used. Buffer water is a solution which tends to keep its original pH even on addition of small amount of alkali or acid (Buffer tablets ready for use, to be dissolved in distilled water).

Buffer Solution used in the Laboratory

Solution No. I

NaOH (sodium hydroxide) 8 g.

Distilled water 1000 cc.

Solution No. II

KH_2PO_4 (Potassium dihydrogen phosphate) 27.2 g. Distilled water 1000 cc.

Take 23.7 cc of solution I, add to it 50 cc of solution II, add 20 cc of the above mixed solution to 1000 cc of distilled water. This has a pH of 6.8.

Stain Preparation and Staining

Wright's Stain

Wright's stain (powder) 0.2 g.

Acetone free methyl alcohol 100 cc.♥

Let stand this solution for a few days.

If the WBC granules do not stand out clearly, try out a 0.25 or 0.3% solution.

Method

Cover the slide with stain for 1–2 minutes taking care that it does not dry on the slide. Now dilute this with equal amount of buffer water (if the stain is ripe, a scum or film with a metallic sheen will form on the surface of the diluted stains on the slide). The diluted stain is allowed to act for 3–5 minutes and then flooded off with buffer or tap water. The stain should never be poured off or a precipitate of the stain will be deposited on the slide. Should this occur, it can sometimes be removed by flooding the slide with undiluted stain for 10–15 seconds and then washing it off again by flooding the slide once more with buffered water.

Leishman's Stain

Powdered Leishman's stain 0.15 g.

Acetone-free methyl alcohol 133 mL.

All the stain should be dissolved (better if the stain crystals are well ground before), keep the stain in a glass stoppered bottle. Do not filter.

Method

Like that for Wright's stain but with double dilution of the buffer water; (i) Pour few drops (about 8) on the slide. Wait for 2 minutes, (ii) Add double the amount (16 drops) of buffered water. Mix by rocking and not by blowing and wait for 7–10 minutes, (iii) The stain is flooded off with distilled water and this should be complete in 2-3 seconds. Longer washing will remove stain, and (iv) Stand in a rack to drain and air dry. A fan will expedite the process.

Giemsa's Stain

Giemsa powder 0.3 g Glycerin 25.0 mL Acetone-free methyl alcohol 25.0 mL.

This makes stock solution and before use, it has to be diluted by adding 1 mL (stain) to 9 mL of buffered distilled water.

Method

The blood film is fixed with methyl alcohol for 3–5 minutes and dried. Pour on diluted stain and keep for 15 minutes or longer. Wash off with tap water or neutral distilled water and dry.

Staining of Thick Films

Thick films have to be dehemoglobinized before staining with one of the previously mentioned stains. The slide is kept in distilled water for 10 minutes, then taken out, dried and stained with any of the stains already mentioned. They must not be fixed before staining, or the water will not hemolyze the cells. The stains commonly used are Field's stain and Simeon's stain.

Field's Stain

Field's stain A

Methylene blue	0.8 g
Azure I	0.5 g
Disodium hydrogen phosphate (anhydrous)	5.0 g
Potassium dihydrogen phosphate anhydrous	6.25 g
Distilled water	500 mL

Field's stain B

Eosin (yellow eosin, water soluble) 1.0 g

Disodium hydrogen phosphate (anhydrous) 5.0 g

Potassium dihydrogen phosphate (anhydrous) 6.25 g

Distilled water 500 mL.

Grind all solids well and dissolve in the said solvent, keep the stains for 4 hours for ripening and filter before use. Keep the stains in covered jars. The depth of the solution should be about 3 inches, the level should be maintained by adding more of the stain solution.

Method

1. Dip the film for one second in solution A.
2. Remove from solution A and immediately rinse by waving very gently in clean water for a few seconds, until the stain ceases to flow from the film and the glass of the slide is free from stain.
3. Dip for one second in solution B.
4. Rinse by waving gently for 2–3 seconds in clean water.
5. Place vertically in a rack to drain and dry.

Simeon's Modification of Boye's and Sterenal's Method

This stain can be used instead of Leishman's or Wright's stain when methyl alcohol is not available to prepare them.

Solution I

Eosin pure 1 g

Distilled water 1000 mL.

Solution II

a. Medicinal methylene blue 1 g dissolves, distilled water 75 mL completely.
b. Potassium permanganate 1.5 g dissolves, distilled water 75 mL completely.
    1. Mix (a) and (b) in a flask. A massive precipitate is formed.
    2. The flask is kept in a water bath at boiling point for half an hour during which time the precipitate redissolves.
    3. Filter. The stain is now ready for use, it needs no further dilution.

Method for Staining Thin Films

1. Fix the smear by immersion into rectified spirit—1 minute.
2. Rinse with tap water—4 seconds.
3. Immerse into solution I—10 seconds.
4. Rinse with tap water—4 seconds.
5. Immerse into solution II—15 seconds.
6. Rinse with tap water—4 seconds.
7. Immerse again into solution I—5 seconds.
8. Rinse with tap water—4 seconds.
9. Allow to dry in an upright position.

Procedure for Staining Thick Smears

1. Dehemoglobinize by immersion into tap water, if necessary.
2. Immerse in Sterenel's blue (solution II)—6 seconds.
3. Wash in tap water.
4. Immerse in eosin solution (solution I)—12 seconds.
5. Wash in tap water, allow it to dry in air. Examine under microscope. The stains are useful for screening purposes.

Mounting and Preservation of Films

Unstained films cannot be preserved well. Due to hardening of plasma, they do not stain well after some time. Stained films if left unmounted tend to fade away rapidly. Canada balsam should not be used as it decolorizes the smear. Gurr's neutral mounting medium is quite satisfactory. Use only thin coverslips for mounting.

RAPID DIAGNOSTICS

Automation in Hematology

Coulter Principle

The **Coulter principle** states that particles pulled through an orifice, concurrent with an electrical current, produce a change in impedance that is proportional to the size of the particle traversing the orifice. The Coulter principle was named for its inventor, Wallace H Coulter.

Wallace was an electrical engineer by training with a passion for radio technology. During the Second World war, Wallace joined the US Navy. While working on a technique to detect submarines using sonar, he frequently detected large echos where no submarines were operating. In an attempt to determine the source, Wallace lowered a series of small bottles with remote trap doors to various depths. The bottles were constructed such that the remote door could be opened and shut at predetermined depths, filling the bottle with seawater from that depth. The source of the false echos turned out to be high concentrations of plankton. In order to count the number of plankton cells per milliliter of seawater accurately and reproducibly, Wallace created a device that would become the basis for the Coulter principle.

The device consisted of a dual chambered container whose two sides were separated by a thin membrane. A small hole in the membrane called an aperture was the only connection between the two chambers. Electrodes from a battery were placed in the chambers, positive on one side and negative on the other. An ohmmeter was connected to the circuit so as to measure the resistance to the flow of current (impedance) from one electrode, through the orifice, and to the other electrode. Both chambers were filled with seawater from the trap bottles. Then one of the two chambers was partially drained, forcing seawater to flow from the opposite chamber, through the orifice to balance the level of liquid in the two sides. As the seawater passed through the orifice so did the plankton cells, which created momentary changes in impedance that were seen on the ohmmeter. By counting the number of impedance pulses per unit of seawater, Wallace's device was able to count the number of plankton particles.

This technology found commercial success in the medical industry where it revolutionized the science of hematology. Red blood cells, white blood cells and platelets make up the majority of the formed elements in the blood. The average salinity of human blood is very close to that of seawater, and mixture of salt (NaCl) and water with the same salinity as seawater is said to be isotonic with whole blood. When whole anticoagulated human blood is diluted with isotonic saline, the Coulter principle can be applied to count and size the various cells that make up whole blood. The first commercial application of the Coulter principle to hematology came in 1954 with the release of the Coulter Counter Model A (developed by Wallace and brother Joseph R. Coulter). Within a decade, literally, every hospital laboratory in the United States had a Coulter Counter, and today every modern hematology analyzer depends in some way on the Coulter principle.

The Basics of Hematology Analyzers in a Nutshell

Hematology cell counters continue to provide an ever-broader scope of capabilities. Technologies that were leading edge a few years ago, such as reticulocyte enumeration, are now routine. Methods that heretofore required much manual manipulation—such as CD4 counts—can now be incorporated as part of the random-access CBC specimen stream on instruments such as the Abbott Cell-Dyn series. Food and Drug Administration approval of quantitative nucleated red blood counts on several instruments now permits automated handling of patients with a variety of pathologic states.

For 25 years, the Holy Grail in the automated counting of the WBC differential has been the enumeration/quantification of immature granulocytes. This debate continues with clinical colleagues who insist they must have a manual differential because they want to know if "bands" are numerous. It does not faze them that study after study demonstrates that the "band count" is terribly imprecise and non-reproducible. At least one manufacturer has submitted applications to the FDA for clinical use of the "immature granulocyte" channel. This advance has great potential for the precise and accurate quantitation of immature granulocyte forms (the collective total of promyelocytes, myelocytes, and metamyelocytes). Ironically, the clinical significance of automated immature granulocyte counts is difficult to measure at present, since the existing literature is heavily weighted toward only band counts and not extended immature granulocyte counts. We do hope to see these immature granulocyte counts take hold and, finally, eliminate the use of the manual band count.

Bayer's reticulocyte hemoglobin measurement is useful in the early diagnosis of iron deficiency and in monitoring response to treatment.

Another interesting new channel is hematopoietic progenitor cells, or HPCs, available on the Sysmex XE-2100. In some settings, this will permit stem cells to be quantitated (for example, in an apheresis product) without requiring a direct CD34 study on a flow cytometer. This study is based on differential membrane lipid content. HPCs have lower membrane lipid content than mature leukocytes and are preserved after treatment with a lysing agent.

With increasing routine automation of assays that previously required the use of flow cytometers, we may see flow cytometers redirected to more in-depth analyses of cell structure and function—the emerging field of cytomics.

The rate-limiting step on the introduction of new diagnostic modalities is no longer a matter of how quickly the technology can be developed, licensed, and deployed. Far more important is how quickly medical practitioners embrace the new technologies and incorporate them into their routines.

Those selecting hematology instruments can no longer base their decisions solely on the lowest-price instrument. Medical considerations should and may dominate. Perhaps the patient mix requires a parameter that is available only on certain instruments, for example, Operational considerations may be paramount—reliable, high-throughput, easy-to-use instrumentation may be more crucial than having all the newest parameters on a more difficult-to-use instrument. The fiscal effect of eliminating flow cytometry for high-volume studies, such as CD4 or CD34, may outweigh a higher cost-per-test on CBCs.

- Three-dimensional VCS technology provides the highest sensitivity, specificity and efficiency in abnormality detection
- Compact, bench top analyzer saves valuable laboratory space
- 75 samples-per-hour throughout maximizes productivity
- Detailed reports and histograms for operator review
- Automatic calibration and Zero-routine-maintenance maximizes uptime
- Data management system stores up to 5,000 patient records
- Closed vial sampling, automatic cap piercing and probe wipe minimize biohazards
- Walkaway automation frees up valuable operator time
- Positive patient ID makes sample tracking easy
- No routine maintenance
- Built-in Quality Assurance ensures accuracy.

Coulter MAXM and MAXM AL Hematology Flow Cytometry Systems (Fig. 9.12)

The MAXM is the easiest hematology system to learn and operate. It features walkaway operation, positive patient identification, automatic calibration, auto-probe wipe, single-operator interface and continuous computer monitoring of system performance. Best of all, the MAXM requires no routine daily maintenance. Your staff is free to handle more complex tasks. The optional Autoloader allows walkaway operation. Load 25 bar-coded samples and then just walk away. The MAXM automatically analyzes both patient samples and controls-providing automatic printouts of the finished reports at a throughput of up to 75 samples per hour unsupervised.

Coulter MAXM AL together with Coulter STKS and the Coulter GEN-S™ System offer the only fail-safe

FIG. 9.12: Coulter MAXM

sample management system with positive patient ID and monitoring of sample integrity both pre and postsampling. This means peace of mind for you, no reports incorrectly distributed because of short samples and no mix-up in the identity of the patient.

Instrument Specifications

Parameters
- White blood cell count
- Lymphocyte % and #
- Monocyte % and #
- Neutrophil % and #
- Eosinophil % and #
- Basophil % and #
- Red blood cell count
- Hemoglobin concentration
- Hematocrit
- Mean corpuscular volume
- Mean corpuscular hemoglobin
- Mean corpuscular hemoglobin concentration
- Red cell distribution width
- Platelet count
- Mean platelet volume.

Throughput
- 75 samples per hour
- 30 samples per hour for Retics.

Sample Requirements
- 185 µL primary mode
- 125 µL secondary sample mode
- 50 µL predilute mode.

Patient Result Storage
- 1,000 sets plus sample analysis screen displays
- 5,000 sets plus all sample analysis screen displays for Retic units.

Barcode Symbology
- Code 39®
- Codabar
- Interleaved 2 of 5
- Code 128.

0–24 Hours Sample Stability
- Near-native state analysis of WBC using four reagents that are safe to use and discard
- Printouts via standard graphics printer with optional color kit, or single ticket printer.

High Efficiency through Comprehensive Flagging
Instrument-defined suspect abnormalities (User defined abnormalities).
- Definitive flags
- High and low laboratory action limits
- RBC morphology Gradient.

DEVELOPMENT OF BLOOD CELLS AND SITES OF BLOOD FORMATION

Normal Sites

Fetus: Less than 2 months—yolk sac. *From 2–7 months:* Liver, with minimal hemopoiesis in spleen.

After 3 months: Hemopoiesis starts in bone marrow. *Full-term infant:* Bone marrow is the only site for production of granulocytes and monocytes. Occurs mainly in the spleen, lymph nodes and other lymphoid tissues, though liver and bone marrow produce these in much less numbers.

After birth: Same as above except that the monocytes are provided by the bone marrow, spleen and lymphoid tissues contribute minimally.

Abnormal Sites

Extramedullary hemopoiesis (myeloid metaplasia): In certain disorders the fetal, organs revert to their old function supported by the reticulum cells, which retain their potential hemopoietic activity. This occurs when bone marrow cannot any further fulfil the requirements or demand imposed upon it, e.g. in:
- Growing children with hemolysis
- Myelosclerosis
- Secondary carcinoma of the bone.

Development of Blood Cells (Flow chart 9.1)

Blood formation has to undergo three stages:
1. Multiplication of precursor cells (1% of all marrow cells are in dividing phase).
2. Gradual maturation (both structural and functional).
3. Release into the peripheral circulation. The exact release mechanism is ill understood, granulocytes achieve this by their motility and RBCs by diapedesis.

Erythropoiesis (Fig. 9.13)

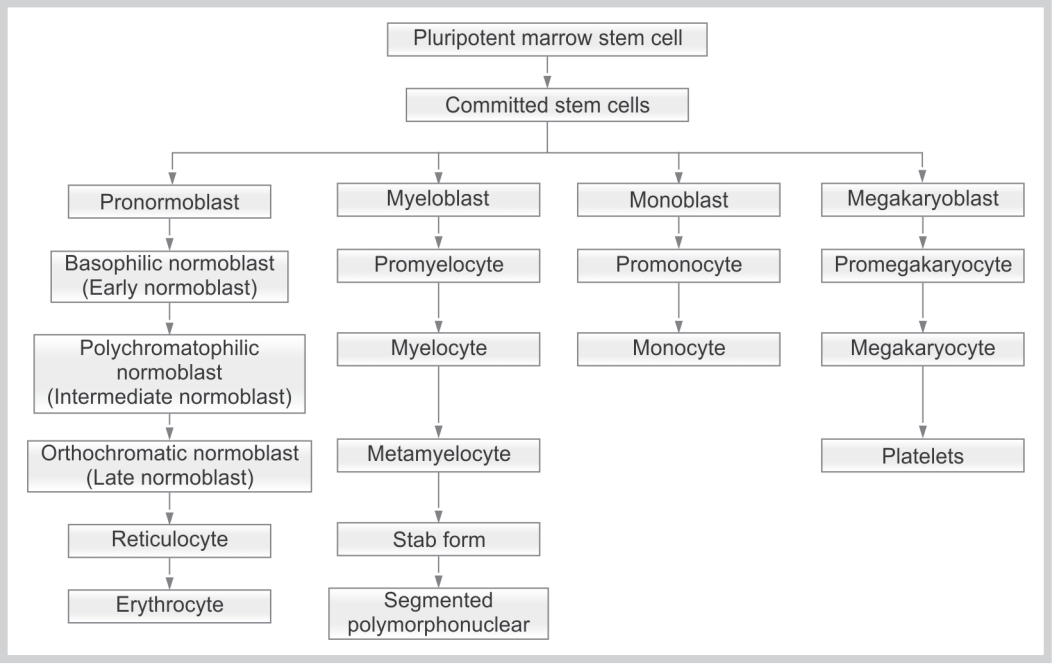
Erythroblast is a nucleated red cell.

Normoblast implies normal (reaction) erythropoiesis.

Normoblastic maturation involves:
- Reduction in cell size
- Ripening of cytoplasm, i.e. hemoglobinization.

Maturation time from pronormoblast to RBC is 7 days. Mitotic division occurs till the intermediate normoblast stage.

FLOW CHART 9.1: Development of blood cells



Pronormoblast: 12–20 μm, large nucleus surrounded by a rim of deep basophilic cytoplasm and has a perinuclear halo. Nucleus is round and has several nucleoli.

Early normoblast: 10–16 μm, nucleus still large, chromatin coarser and deeply staining nucleoli disappear.

Intermediate normoblast: 8–14 μm, nucleus smaller, hemoglobinization commences, cytoplasm takes an acidophilic tint, chromatin becomes coarser and very deeply staining.

Late normoblast: 8–10 μm, cytoplasm is acidophilic, nucleus becomes much smaller, later it becomes pyknotic and is eccentrically placed, ultimately it is lost by extrusion.

Reticulocyte: Flat, non-nucleated, disc shaped, slightly larger than mature RBC. It shows diffuse pale basophilia, which appears in the form of a reticulum with supravital stains (brilliant cresyl blue or new methylene blue). In 1–2 days, it loses its basophilia and becomes a mature erythrocyte.

Control of erythropoiesis: Erythropoietin (formed in kidneys) is released in response to lowered tissue oxygen tension.

Erythropoietin is a glycoprotein and stimulates primitive cell differentiation to pronormoblasts. It affects the rate of multiplication and maturation. It acts up to early normoblast stage and also affects the rate of hemoglobinization.

Erythropoietin levels are reduced in:
- Acute starvation
- Hypophysectomy
- Transfusion-induced polycythemia.

Erythropoietin levels are increased in:
- All anemias except those of renal origin
- Aplastic anemia
- Polycythemia.

Leukopoiesis (Fig. 9.14)

The Myeloid Series

Specific granules are developed at the myelocyte stage, which determine the nature of the mature cell.

Development of a Mature Neutrophil

Maturation involves:
1. Development of specific granules
2. Loss of basophilia of the cytoplasm
3. Nuclear ripening till the segmented stage
4. Ability to be motile and to phagocytose (Mitotic division occurs till the myelocyte stage only).

Myeloblast: 15–20 μm has a large round or oval nucleus, evenly stained chromatin in strands or granules with reticular appearance, 1–6 nucleoli. The cell is peroxidase negative.

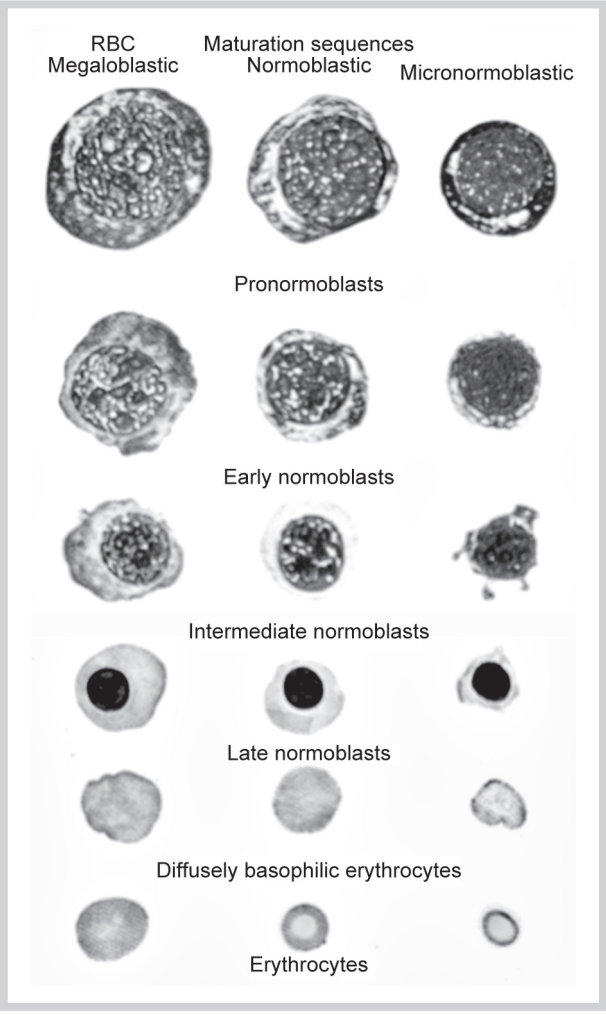


FIG. 9.13: Erythropoiesis

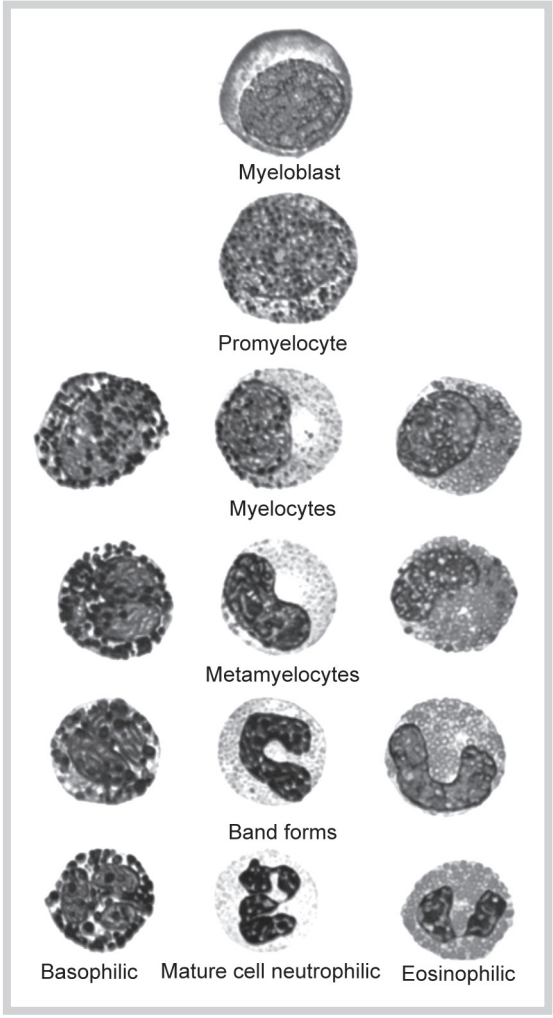


FIG. 9.14: Leukopoiesis

Promyelocyte: It is like myeloblast except that it contains azurophilic granules, which are peroxidase positive. Nuclear chromatin becomes condenser and nucleoli are less well defined.

Myelocyte: Specific neutrophilic granules appear, nucleus shows no nucleoli. N:C ratio reduces, cytoplasm is pale pink, chromatin thicker and deeply stained.

Metamyelocyte: Nucleus is smaller and indented, cytoplasm is pink with neutrophilic granules (purplish).

Band or stab form: Cell becomes still smaller, nucleus has a deep indentation, chromatin is coarsely clumped. Cytoplasm is pink with purplish granules.

Segmented neutrophil: 12–14 μm in size, nucleus shows 2–5 lobes, chromatin in dark purple clumps, cytoplasm has numerous, fine, evenly distributed purplish granules. In female at least 6 neutrophils or 500 should show drumsticks.

The mature eosinophil: 16 mm in size, granules are acidophilic and larger. Nucleus is bilobed and is not masked by granules.

The mature basophil: It usually has a bilobed nucleus, but the nucleus is masked by about 10 large basophilic granules.

Lymphocytic Series (Fig. 9.15)

Lymphoblast: 15–20 μm in size, resembles myeloblast, cytoplasm is agranular and moderately basophilic. Nuclear chromatin gives fine reticular appearance with up to 2 nucleoli. It is peroxidase negative.

Large lymphocyte: 12–16 μm in size, has abundant pale sky blue cytoplasm with a few purplish red granules seen in about 33% of the cells.

Small lymphocyte: 9–12 μm in size, has scanty cytoplasm. Nucleus is usually round and shows heavily clumped chromatin.

Monocytic Series (Fig. 9.14)

Monoblast: It resembles myeloblast.

Promonocyte: Up to 20 μm in size, has a large convoluted nucleus, chromatin is seen in skein like strands. Cytoplasm is dull gray-blue and may contain a few azurophilic granules.

Monocyte: 15–20 μm in size, has abundant dull gray-blue cytoplasm with a ground glass appearance and may show vacuolation and fine azurophilic granules. It has a kidney-shaped nucleus.

Thrombopoiesis (Fig. 9.14)

Megakaryoblast: 20–30 μm in size, has a large, oval or kidney-shaped nucleus with several nucleoli. It possesses relatively small amount of agranular cytoplasm.

Promegakaryocyte: 30 μm in diameter, cytoplasm is intensely basophilic with fine azurophilic granules. Nucleus may show mild lobulation and chromatin appears denser.

Megakaryocyte: 30–90 μm in diameter, it contains a single multilobulated or indented nucleus. Nuclear lobes may vary from 4–16 in numbers. Cytoplasm is bulky, light blue with fine azurophilic granulation. The margin is irregular and may show fragmentation or budding, precursor of circulating platelets.

Mature platelet: 1-4 μm. It is formed by fragmentation of megakaryocytic pseudopods. In circulation, they acquire a discoid shape. Cytoplasm stains light blue and contains purple reddish granules, which may be clumped centrally.

Control of platelet production: Perhaps by a humoral factor called thrombopoietin, acts by a feedback mechanism.

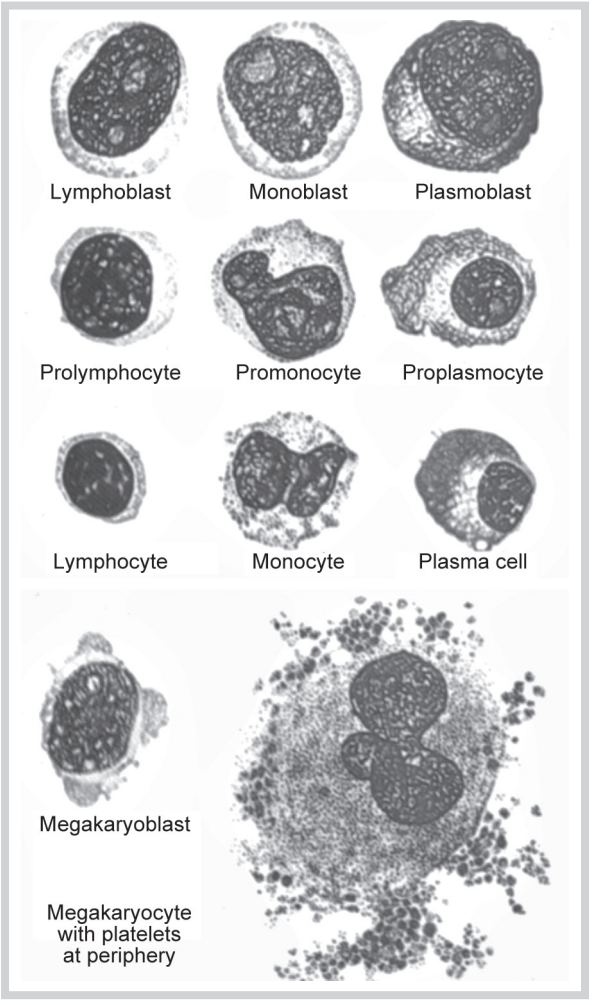


FIG. 9.15: Lymphocytic series

Examination of a Blood Film

Method

1. *Mount:* Cover the slide using a neutral mounting medium.
2. *Low power field examination:* Look for:
   - Quality of film
   - Number, distribution and staining of WBCs
   - RBCs examination, select an area where they just touch each other without overlapping, i.e. between tail and body of the film.
3. *High power field examination:* Assess RBC
   - Size
   - Shape
   - Hemoglobin concentration.
4. *Oil immersion examination:* Assess atypical cells and note fine details, e.g. inclusion bodies.

Always Note

RBCs

Size: Normocytes, microcytes, macrocytes, anisocytosis (variation in size).

Shape: Abnormal shape oval, pencil, tear, pear, oat and sickle-shaped cells, fragmented cells, target cells,

spherocytes, crenated cells, burr cells, acanthocytes, stomatocytes (poikilocytes).

Hemoglobin: Normochromic, hypochromic.

Immature forms: Polychromatic, stippled or nucleated red cells.

Inclusion bodies: Howell-Jolly bodies, Cabot rings, Pappenheimer bodies, malarial parasites, etc.

Arrangement: Autoagglutination, excess rouleaux formation.

WBCs

Number: Normal, increased, decreased.

Abnormal or immature forms: Immature forms, hypersegmented macropolycytes, abnormal forms.

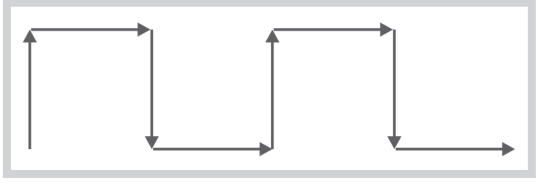
Platelets

Number: Normal, increased, decreased.

Form: Abnormalities of size and shape, present in groups or lying scattered.

Differential Leukocyte Count (DLC)

For differential leucocyte counts, choose an area where the morphology of the cells is clearly visible. Ensure that there is no tailing of the WBCs or else a false DLC may be obtained. Do differential count by moving the slide as shown in order to include central and peripheral areas of the smear.



While doing DLC, look for vacuolation, toxic granulation, size and maturity of the WBCs. Count at least 100 cells and give percentage of the cells seen. Counting becomes easier if 100 squares are made on a paper and the letters P for neutrophil, L for lymphocyte, M for monocyte, E for eosinophil and B for basophil can be entered in each square. Another easier way is using Laboratory cell counter (Fig. 9.16).

Bone Marrow Examination

Bone marrow can be obtained by using Salah's, Klima's or Jamshidi's marrow aspiration needles. Not more than 0.2 mL of bone marrow should be taken out at one time. If blood or dry taps have occurred on 2 different occasions, a trephine biopsy should be performed.

FIG. 9.16: Nine-unit laboratory cell counter

For aspiration of bone marrow, Klima's needle is better as the guard has no chances of getting slipped and hence dangers of puncturing substernal structures are less.

Before entering the site of puncture, take all aseptic precautions, i.e. cleaning with spirit, iodine and spirit again. The various sites for obtaining bone marrow are:

In Adults

- Sternal aspiration
- Anterior iliac crest
- Vertebral spinous processes
- Posterior superior iliac spine.

In Children

- Tibia, superior medial surface of the tibia, inferior to the medial condyle and medial to the tibial tuberosity
- Posterior iliac crest
- Calcaneum.

Marrow Film Preparation

Delay in marrow film preparation should not be there. Get rid of blood by using a capillary pipette leaving the grayish marrow particles behind. Make a smear as has been described for peripheral blood. The smear should be 3 to 5 cm long and should not be more than 2 cm wide. The particles should be dragged behind but not squashed. A trail of cells is left behind. Criteria of a good preparation—presence of both particles and free marrow cells in the smear.

Imprints

Another way is imprint method. The marrow particle is picked and transferred immediately to a slide and made to stick to it by a gentle smearing motion. The slide is air dried rapidly by waving.

Crush Preparation

Marrow particles in a small drop of aspirate are placed on a slide near one end. Another slide is carefully placed over the first. Slight pressure is exerted to crush the particles, and the slides are separated by pulling them apart in a direction parallel to their surfaces. Dry the smears immediately. The appearance of fat (irregular holes) on the smear implies that marrow has been obtained.

1. Good fixation is essential.
2. Any of the previously mentioned stains can be used for staining bone marrow films.

Examination

The examiner should be informed of the clinical picture before he or she examines the marrow films. An impression can be formed by examining various fields on the different stained slides prepared. Start with 10X through 40X, to oil immersion, now actually make a differential count of large number of cells (300 to 1000) and calculate the percentage of each type of cell.

The procedure given below is recommended for studying the marrow films:

1. Naked eye inspection of the slides to select the smear containing particles.
2. With 10X objective, survey the particles whether they are normoplastic, hypoplastic or hyperplastic.
3. Select a cellular area (usually in the tail portion of the film around particles), study the cytologic details by high power—40X and oil immersion—100X objectives.

Note the undermentioned points: Cellularity: Normo/hypo/hyperplastic marrow particles. Cellularity is better defined by studying histologic sections of aspirated particles, though crude estimates can be given on films. The normal cellularity of the marrow varies with age being more in infants and least in elderly individuals.

Next, look for *reaction of erythropoiesis,* whether it is normoblastic, megaloblastic or micronormoblastic. Look for maturity of leukopoietic cells. The M:E ratio is based on a count of 500 to 1000 marrow cells. In the normal adult, the reaction is normoblastic, leukopoietic maturity is normal and *ME ratio* is about 3 or 4:1. The ME ratio at birth is 1.85:1; during the first 2 weeks, it reaches its peak of 11:1. It then gradually drops to the (years 1 to 20) average of 3:1.

Look for megakaryocytes, their number, size, nuclear lobulations, functioning capacity.

Megakaryocytes with budding or cloud-like appearance at the periphery are functioning ones.

In addition, if suspected, look for metastatic cells, increased number of normal or abnormal (myeloma) plasma cells, cells containing unduly large amounts of lipid, carbohydrate, etc.—storage disease. One may also see hemoparasites in the marrow especially LD bodies of Kala-azar.

Bone Marrow Aspiration Analysis

Normal Values

Red marrow contains connective tissue, fat cells, and hematopoietic cells. Yellow marrow contains connective tissue and fat cells. Interpretation of cell count and histopathology by a hematologist, pathologist, or oncologist is required.

Response to staining: Iron stain for hemosiderin: 2+.
Periodic Acid-Schiff (PAS) glycogen reactions: Negative.

Sudan black B (SBB) granulocyte: Negative.

Differential cell count

	Adult (%)	*Child (%)*	*Infant (%)*
Basophils	0.1	0.06	0.07
Eosinophils	3.1	3.6	2.6
Hemocytoblasts	0.1–1.0		
Lymphocytes (all stages)	2.7–24	16	49
Megakaryocytes	0.03–0.5	0.1	0.05
Plasmacytes	0.1–1.5	0.4	0.02
Promyelocytes	0.5–8.0	1.4	0.76
Reticulum cells	0.1–2.0		
Undifferentiated cells	0.0–0.1		
Neutrophils, total	56.5	57.1	32.4
Metamyelocytes	9.6–24.6	23.3	11.3
Neutrophilic	10–32		
Eosinophilic	0.3–3.7		
Basophilic	0–0.3		
Monocytes (all stages)	0–2.7		
Myeloblasts	0.1–5.0	1.2	0.62
Myelocytes	4.2–15	18.4	2.5
Neutrophilic	5.0–20		
Eosinophilic	0.1–3.0		
Basophilic	0–0.5		
Segmented granulocyte	6.0–12.0	12.9	3.6
Neutrophilic	7.0–30		

Contd...

Contd...

	Adult (%)	*Child (%)*	*Infant (%)*
Eosinophilic	0.2–4.0		
Basophilic	0–0.7		
Band cells	9.5–15.3	0	14.1
Neutrophilic	10–35		
Eosinophilic	0.2–2.0		
Basophilic	0.3		
Erythroid series			
Normoblasts, total	25.6	23.1	8.0
Pronormoblasts	0.2–4.0	0.5	0.1
Basophilic normoblasts	1.5–5.8	1.7	0.34
Polychromatophilic normoblasts	5.0–26.4	18.2	6.9
Orthochromic normoblasts	3.6–21	2.7	0.54
Promegaloblasts	0		
Basophilic megaloblasts	0		
Polychromatic megaloblasts	0		
Orthochromic megaloblasts	0		

M:E ratio: (Myeloid:Erythroid is the ratio of WBCs to nucleated RBCs.)

Adult	6:1–2:1
Birth	1.85:1
2 weeks	11:1

Usage: Helps to distinguish primary and metastatic tumors. Assists in the identification, classification, and staging of neoplasias. Aids evaluation of the progress and/or response to the treatment of neoplasias. Assists in the definitive diagnosis of blood disorders. Culture of an aspirated sample can aid in the identification of infections such as histoplasmosis or tuberculosis. Histologic examination aids in the diagnosis of carcinoma, granulomas, lymphoma, or myelofibrosis. Iron stain showing decreased hemosiderin levels may indicate iron deficiency and SBB stain differentiates acute granulocytic leukemia from acute lymphocytic leukemia.

Increased eosinophils: Bone marrow carcinoma, eosinophilic leukemia, lymphadenoma, myeloid leukemia and pernicious anemia (relapse).

Increased lymphocytes: Aplastic anemia, hypoplasia of the bone marrow, infectious lymphocytosis or mononucleosis, lymphatic leukemoid reactions, lymphocytic leukemia (B-cell and T-cell), lymphoma, macroglobulinemia, myelofibrosis and viral infections.

Increased megakaryocytes: Acute hemorrhage, aging, chronic myeloid leukemia, hypersplenism, idiopathic thrombocytopenia, infection, megakaryocytic myelosis, myelofibrosis, pneumonia, polycythemia vera and thrombocytopenia.

Increased plasma cells: Agranulocytosis, amyloidosis, aplastic anemia, carcinomatosis, collagen disease, hepatic cirrhosis, Hodgkin's disease, hypersensitivity reactions, infection, irradiation, macroglobulinemia, malignant tumor, multiple myeloma, rheumatic fever (acute), rheumatoid arthritis, serum sickness, syphilis and ulcerative colitis.

Increased granulocyte: Hypoplasia of the bone marrow, infections, myelocytic leukemia, myelocytic leukemoid reaction and myeloproliferative syndrome.

Increased normoblasts: Anemia (iron deficiency, hemolytic, megaloblastic), blood loss (chronic), erythema, erythroid-type myeloproliferative disorders, hypoplasia of the bone marrow and polycythemia vera.

Increased M:E ratio above 7:1 Decreased hematopoiesis, erythroid hypoplasia, infection, leukemoid reactions, and myeloid leukemia.

Increased diffuse bone marrow hyperplasia: Myeloproliferative syndromes and pancytopenia reactions.

Decreased megakaryocytes: Anemia (aplastic, pernicious), bone marrow hyperplasia (with carcinomatous or leukemic deposits), cirrhosis, irradiation (excessive), and thrombocytopenia purpura. Drugs include benzene, chlorothiazides, and cytotoxic drugs.

Decreased granulocytes: Agranulocytosis, hyperplasia of the bone marrow, and ionizing radiation.

Decreased normoblasts: Anemia (aplastic, hypoplastic), folic acid or vitamin B_{12} deficiency.

Decreased M:E ratio below 2:1 Agranulocytosis, anemia (iron deficiency, normoblastic, pernicious, posthemolytic, posthemorrhagic), erythroid activity (increased), hepatic disease, myeloid formation (decreased), polycythemia vera, sprue, and steatorrhea.

Decreased diffuse bone marrow hypoplasia: Aging, cellular infiltrations, dengue fever, myelofibrosis, myelosclerosis, myelotoxic agents, osteoporosis, rubella, and viral infections.

Description: Bone marrow is the soft, organic, sponge-like material contained in the medullary cavities, long bones, some haversian canals, and within the spaces between trabeculae of cancellous bone. It is composed of red and

yellow marrow, with the chief function being production of erythrocytes, leukocytes, and platelets. Only the rusty, red marrow produces blood cells. The yellow marrow is formed of connective tissue and fat cells, which are inactive. During infancy and childhood, bone marrow is primarily red marrow, and in the adult, 50% is red marrow. The bone marrow aspiration procedure obtains a sample of bone marrow by needle. A stained blood smear of the sample is evaluated for bone marrow morphology and examination of blood cell erythropoiesis, cellularity, differential cell count, bone marrow iron stores, and M:E ratios.

Indications for Bone Marrow Aspiration

Absolute Indications

- Megaloblastic macrocytic anemia
- Aleukemic or subleukemic leukemia.

Diagnostic Importance

- Multiple myeloma
- Aplastic anemia
- Gaucher's disease.

Confirmatory Importance

- Leukemias of all types
- Hemolytic anemia
- Idiopathic thrombocytopenic purpura
- Idiopathic granulocytopenia
- Leishmaniasis
- Disseminated lupus erythematosus (LE cells)
- Metastatic disease
- Myeloproliferative disorders
- Lipid storage
- Sideroblastic anemia
- Iron deficiency anemia
- Lymphoma (staging).

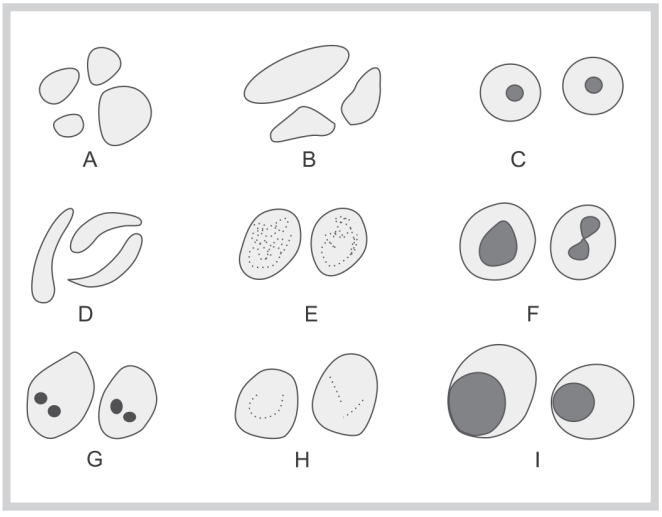
Therapeutic Importance

Bone marrow may be obtained from one person for transplantation into another.

In many cases, one may just obtain blood (*blood tap*) or nothing at all (*dry tap*). Under these conditions, a *bone marrow biopsy* has to be performed. It can be obtained with *Jamshidi's needle* or with *Sacker-Nordins bone biopsy trephine.*

Bone biopsy may be needed in:

- Malignant lymphoma
- Metastatic carcinoma
- Sarcoidosis
- Tuberculosis
- Brucellosis.



FIGS 9.17A TO I: Morphological alterations in RBCs: (A) Anisocytosis, (B) Poikilocytosis, (C) Target cells, (D) Sickle cells, (E) Basophilia, (F) Cabot's rings, (G) Howell-Jolly bodies, (H) Reticulocytes (large basophilic), (I) Normoblasts

MORPHOLOGICAL TYPES OF RED BLOOD CELLS (FIG. 9.17)

Usually anemias are described on two grounds:

- The average cell volume (MCV)
- The average hemoglobin concentration (MCHC).

Three main types of anemias are recognized:

1. The *normocytic anemias*, in which MCV is within normal range (76–96 fl). Most normocytic anemias are also normochromic (i.e. MCHC is between 30–35 g%) but in some, mild hypochromia may occur. For size of an RBC, compare its size with that of a small lymphocyte. As for hemoglobinization normally only a small area of central pallor is seen (central 1/3 rd).
2. The hypochromic, microcytic anemias, in which the MCV is reduced (less than 76 fl) and MCHC is also reduced (less than 30 g%).
3. The macrocytic anemias in which the MCV is increased (greater than 96 fl). Most macrocytic anemias are normochromic; but in some, a mild hypochromia may occur.

RBC Morphology

Normal Values

Microscopic interpretation is required.

Color	Uniformly normochromic
Size	6–8 μ only slight size variation
Shape	Round, biconcave disc
Stained appearance	Mature erythrocytes stain uniformly and contain a normal concentration of hemoglobin with an area of central pallor
Nucleus	Absent
Nuclear remnants	Absent
Cellular inclusions	Absent
Acanthocytes	Absent
Crescent bodies	Absent
Drepanocytes	Absent
Echinocytes	Absent
Leptocytes	Absent
Poikilocytes	Absent
Schizocytes	Absent
Spherocytes	Absent
Stomatocyte	Absent
Cabot rings	Absent
Heinz bodies	Absent
Siderocytes	Absent

Classification of Variation from Normal

Abnormal RBCs/HPF	*Score*	*Interpretation*
3–6	I+	Slight
7–10	2+	Moderate
11–20	3+	Marked
>20	4+	Very marked

Usage

Detection of blood dyscrasias; and differentiation of anemias, leukemia, and thalassemia (Table 9.2).

Reticulocyte Count

Reticulocytes: These are immature red cells which still contain the remains of ribonuclear protein. Their number in peripheral blood increases following increased erythroid activity in the bone marrow. This may occur when there is reduction in number of red cells in the peripheral blood, e.g. by hemorrhage or abnormal hemolysis. The reticulocyte count is of value in pernicious/megaloblastic anemias, for improvement is indicated by a rise in reticulocytes in the peripheral blood.

Normal Values

Comprises 1–2% of the total RBC count.

		SI Units
Adult females	0.5–2.5%	$0.005–0.025 \times 10^{-3}$
Adults males	0.5–1.5%	$0.005–0.015 \times 10^{-3}$
Cord blood	3.0–7.0%	$0.030–0.070 \times 10^{-3}$
Newborn	1.1–4.5%	$0.011–0.045 \times 10^{-3}$
Neonates	0.1–1.5%	$1.010\text{-}0.015 \times 10^{-3}$
Infants	0.5–3.1%	$0.005–0.031 \times 10^{-3}$
Children >6 months	0.5–4.0%	$0.005–0.040 \times 10^{-3}$

Staining

Ribonuclear protein is a living material, which requires special staining, using a supravital stain such as brilliant cresyl blue. Methyl alcohol destroys ribonuclear protein; and hence, it cannot be seen in Romanowsky stained preparation. In Romanowsky stained films, they appear as larger cells showing polychromasia.

Stain

1% Brilliant cresyl blue
Brilliant cresyl blue 1 g
Sodium chloride 0.7 g
Sodium citrate 0.6 g
Distilled water 100 mL.
Store in dark bottle under refrigeration, filter before use (new methylene blue can also be used instead of BCB).

Method

- Place I volume of filtered stain in small test tube
- Place I volume of capillary or venous blood and mix
- Incubate at room temperature or at 37°C for 10–30 minutes
- Remix the tube contents and spread 1 drop of the stained blood on a slide making a thin film.

When dry, examine with the oil immersion lens. Count systematically at least 500 cells, including in this the number of reticulocytes. Reticulocytes appear larger than mature cells and contain irregular dark purple granules or fine threads of ribonuclear material, calculate the percentage of retikulocytes.

Interpretation: Reticulocyte counts are low in ineffective erythropoiesis, e.g. myelosclerosis, aplastic anemia, megaloblastic anemia, thalassemia, erythroleukemia and sideroblastic anemia. Reticulocytosis occurs after blood loss or effective therapy for certain kinds of anemia, e.g. therapy of iron deficiency or megaloblastic macrocytic anemia. Reticulocytosis also occurs in hemolytic anemias.

TABLE 9.2: Various RBC abnormalities and their possible causes

Description of abnormalities of RBC color	*Possible causes of abnormal RBC color*
Anisochromia is demonstrated by variable staining intensities indicating unequal hemoglobin content due to multiple populations of red blood cells (RBCs)	*Anisochromatism:* Iron-deficiency anemia treated with transfused blood
Hyperchromia is demonstrated by the presence of cells having a smaller than normal area of central pallor, causing the cells to take on excessive staining and demonstrate higher than normal pigmentation. Increased amounts of these cells are called hyperchromatism	*Hyperchromatism:* Dehydration, increased bone marrow iron stores, inflammation (chronic), and in the presence of spherocytes that have increased cell wall thickness
Hypochromia is demonstrated by the presence of cells having a larger than normal area of central pallor, causing the cells to stain weakly and appear to have less than normal pigmentation. Increased amounts of these cells are called hypochromatism	*Hypochromatism:* Anemia (iron deficiency) and decreased hemoglobin concentration
Polychromatophils are cells that are stainable with many types of stains, such as stains with both an acid and base component. They are demonstrated by a bluish-pink tinge caused by the presence of both hemoglobin stained by acid and cytoplasmic ribonucleic acid (RNA) stained by the basic component. Both the larger-than-normal cell size and the presence of cytoplasmic RNA indicate that polychromatophils are reticulocytes (newly made RBCs). Increased amounts of polychromatophils is called polychromatosis and occurs in accelerated RBC production	*Polychromatosis:* Hemorrhage, hemolysis, reticulocytosis, and therapy for iron deficiency anemia or pernicious anemia
Description of abnormalities of RBC shape	*Possible causes of abnormal RBC shape*
Acanthocytes are cells with irregular, thorny, spiculated membrane surface projections containing bulbous, rounded ends. They result from an irreversible defect in the lipid content of the RBC membrane. The presence of acanthocytes is called acanthocytosis	*Acanthocytosis*: Abetalipoproteinemia (most common cause), alcoholic cirrhosis, hemolytic anemia (induced by pyruvate kinase deficiency), hepatic disease, postsplenectomy, and retinitis pigmentosa. Drugs include heparin calcium and heparin sodium.
Crescent bodies (achromocytes) are cells with a faint quarter-moon shape caused by RBC rupture	*Achromocytosis*: Any condition that increases the fragility of RBCs (i.e. sickle cell anemia, reduced oxygen supply)
Drepanocytes/sickle cells are cells formed in the shape of a sickle with a point at one end. The presence of these cells is called drepanocytosis	*Drepanocytosis:* Anemia (hemolytic, sickle cell) and hemoglobin SC disease
Echinocytes/burr cells/crenated: RBCs have a cell surface with 10–30 uniformly distributed, blunt spicules. Echinocytes may be commonly due to pH changes due to faulty drying during smear preparation, but certain physiologic conditions, including a reversible defect in the lipid content of the RBC membrane, have been associated with their presence. The presence of these cells is called echinocytosis	*Echinocytosis*: Bile acid abnormalities, blood loss (acute), burns (extensive), carcinoma of the stomach, disseminated intravascular coagulation (DIC), gastric ulcers (bleeding), increased free fatty acids, microangiopathic hemolytic anemia, pyruvate kinase deficiency, renal failure, thrombotic thrombocytopenic purpura, and uremia. Drugs include barbiturates, heparin calcium, heparin sodium, and salicylates
Elliptocytes/ovalocytes have a cigar shape, which distinguishes them from the more oval shape of the ovalocytes. They are normal constituents of mature RBCs. Higher than normal amounts of these cells are called elliptocytosis	*Elliptocytosis:* Anemias (iron deficiency, pernicious, sickle cell), hereditary elliptocytosis, leukemia, megaloblastic hematopoiesis, and thalassemia
Leptocytes/target cells have an increased ratio of surface to volume, often due to a shape that looks like a cup, bell, or hat. They have a colorless center and are thinner and lighter staining than normal RBCs due to abnormally low amounts of hemoglobin. When stained, the depth of the "cup" collapses, causing a bulls-eye appearance. The presence of leptocytes is termed leptocytosis	*Leptocytosis:* Anemia (iron deficiency, sickle cell), cellular dehydration, cirrhosis, hemoglobin C disease, hemoglobin SC disease, hepatitis, jaundice (obstructive), postsplenectomy and thalassemia

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Description of abnormalities of RBC shape	*Possible causes of abnormal RBC shape*
Poikilocytes occur in varying shapes, ranging from slightly irregular to dumbbell-like, pear-shaped or teardrop-shaped. Defective bone marrow production causes poikilocytosis, a general term used to describe the presence of cells demonstrating variation from the normal shape of the RBC	*Poikilocytosis:* Anemia (iron deficiency, hemolytic, megaloblastic. pernicious), myelofibrosis, myeloid metaplasia, and thalassemia
Schizocytosis/schistocytes are RBCs with adhesions of spiral and triangular RBC fragments due to hemolysis, hemoglobinopathies, or erythrocytic mechanical damage from fibrin strands. The presence of these cells is called schizocytosis	*Schizocytosis or Schistocytosis:* Anemia (acute hemolytic, microangiopathic hemolytic), burns (severe), disseminated intravascular coagulation (DIC), prosthetic heart valves, pyruvate kinase deficiency, renal graft rejection, uremic-hemolytic syndrome, valve prosthesis and valvular stenosis
Spherocytes are cells that are globe-like rather than biconcave, with an abnormally small dimple. They are thicker than normal, with many fine-needle-like projections. Spherocytes lack an area of central pallor (due to an increased mean corpuscular hemoglobin concentration) and have a smaller surface area relative to their size. Spherocytes are caused by mechanical fibrin strand damage to circulating RBCs. The presence of spherocytes is called spherocytosis	*Spherocytosis:* ABO hemolytic disease of the newborn, accelerated reticuloendothelial RBC destruction, anemia (hemolytic), following blood transfusion, hereditary spherocytosis, and thermal injury of the cell membrane
Stomatocytes are cup-shaped RBCs with an abnormal area of central pallor that may be oval or rectangular elongated, or slit-like. These cells are produced by antibodies or hydrocytosis. The presence of these cells is called stomatocytosis	*Stomatocytosis:* Alcoholism, cirrhosis, erythrocyte sodium pump defect, hepatic disease (obstructive), hereditary spherocytosis, hereditary, stomatocytosis, and Rh null cells
Description of abnormalities of RBC size	*Possible causes of abnormalities of RBC size*
Anisocytosis is a general term that describes any variation in the size of the RBC	*Anisocytosis:* Anemias (iron deficiency, pernicious), folic acid deficiency, following blood transfusion of normal cells into an abnormal RBC population, leukemia, newborns, and reticulocytosis
Macrocytes are large erythrocytes having a diameter > 8 µ, a mean corpuscular volume > 96 µ, and higher than normal hemoglobin content. They are usually increased due to stress erythropoiesis. Increased amounts of macrocytes are called macrocytosis	*Macrocytosis:* Anemia (hemolytic, pernicious), folic acid deficiency, following hemorrhagic states, hepatic disease, hyperthyroidism, idiopathic steatorrhea, newborns, reticulocytosis, and thalassemia
Microcytes have an RBC diameter <6 µ, a mean corpuscular volume <76 µ, and mean corpuscular hemoglobin concentration <27%. Increased amounts of microcytes are called microcytosis	*Microcytosis:* Anemia (from chronic hemorrhage, iron deficiency), hemoglobinopathies, hereditary spherocytosis, and thalassemia.
Description of abnormalities of RBC content of structure	*Possible causes of abnormal RBC content of structure*
Agglutination: Clumping together of RBCs is an immune mechanism caused by antibody formation	*Agglutination:* Invading antigen(s)
Basophilic stippling is demonstrated by the presence of minute basophilic granules that cause a bluish/purple color when reticulocytes are stained. They are caused by ribosomal aggregation that occurs as smears are prepared. Small amounts of basophilic stippling normally occur as the smears are dried. Increased amounts occur in conditions in which RNA has aggregated in the cells	*Increased basophilic stippling:* Alcoholism, anemia (megaloblastic, sickle cell), heavy metal intoxication (bismuth, lead, mercury, and silver), hemorrhage (gastrointestinal), leukemia, and thalassemia
Cabot's rings are cells containing mitotic spindle remnants appearing as fine, threadlike filaments of bluish purple color in the shape of a single ring or a double ring (figure-eight shape)	*Presence of Cabot's rings:* Anemia (severe, pernicious), lead poisoning, myelofibrosis, and myeloid metaplasia

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Description of abnormalities of RBC content of structure	*Possible causes of abnormal RBC content of structure*
Heinz bodies are denatured particles of hemoglobin attached to the RBC membrane that appear when stained with cresyl blue or new methylene blue. Heinz bodies usually indicate abnormal erythrocyte stability due to hemolytic conditions or hemoglobinopathies	*Presence of'Heinz bodies:* Alpha-thalassemia, anemia (hemolytic), glucose-6-phosphate dehydrogenase deficiency, hemoglobinopathies, methemoglobinemia, and post-splenectomy. Drugs include analgesics, antipyretics, chlorates, phenacetin, phenothiazines, phenylacetamide, phenylhydrazine phenylamine, primaquine phosphate, resorcinol, and sulfapyridine
Howell-Jolly bodies are nuclear fragments contained in red cells that stain purple or violet. They are normally present in immature RBCs and in mature erythrocytes before they pass through the splenic circulation. In conditions causing increased RBC production, erythrocytes contain higher than normal amounts of these bodies	*Presence of Howell-Jolly bodies:* Anemia (hemolytic, megaloblastic), leukemia, splenic absence congenital or surgical removal), and splenic atrophy
Platelets on RBCs appear as a halo that resists staining and can be easily confused with RBC inclusion bodies	
Rouleaux formation is demonstrated by a cellular configuration in stacks or rolls. Increased rouleaux formation may be caused by increased fibrinogen or globulins in the blood. Rouleaux formation is decreased by the presence of abnormally shaped RBCs, which inhibits adherence of the cells in a stacked shape. Rouleaux formation may also result from a delay in slide preparation	*Increased rouleaux formation:* Hyperfibrinogenemia, macroglobulinemia, and multiple myeloma *Decreased rouleaux formation:* Hereditary spherocytosis
Siderocytes/Pappenheimer bodies are cells with mitochondrial concentrations of ferritin (nonhemoglobin iron) deposits. These cells stain as purple-bluish granules only in the presence of iron stains such as Prussian-blue reactions. Pappenheimer bodies are noniron basophilic granules contained in the iron-protein matrix and stain positive for iron in the presence of noniron stains. Ferritin is normally absent in RBCs. During hemoglobin formation, in the premature infant and newborn, siderocyte free-iron granules commonly occur in developing normoblasts and reticulocytes. The presence of siderocytes is called siderocytosis	*Siderocytosis/Pappenheimer bodies:* Anemia (chronic hemolytic, congenital spherocytic, dyserythropoietic, megaloblastic, pernicious, refractory, sideroblastic), burns (severe), hemochromatosis, infection, lead poisoning, postsplenectomy, and thalassemia

Red blood cell abnormalities seen on stained smear (in brief)

Descriptive term	*Observation*	*Importance*
Macrocytosis	Cell diameter > 8 µm MCV > 96 fl	Megaloblastic anemias, severe liver disease Hypothyroidism
Microcytosis	Cell diameter < 6 µm MCV < 76 fl MCHC < 27	Iron deficiency anemia Anemia of chronic disease Thalassemia
Hypochromia	Increased zone of central pallor	Reduced Hb content
Polychromatophilia	Presence of red cells not fully hemogolobinized	Reticulocytosis
Poikilocytosis	Variability of cell shape	Sickle cell disease Microangiopathic hemolysis Leukemias Extramedullary hematopoiesis Marrow stress of any cause

Contd...

Contd...

Descriptive term	*Observation*	*Importance*
Anisocytosis	Variability in cell size	Reticulocytosis Transfusing normal blood into microcytic or macrocytic cell population
Leptocytosis	Hypochromic cells with small central zone Hb (target cells)	Thalassemias Iron deficiency anemia, obstructive jaundice
Spherocytosis	Cells without central pallor, loss of biconcave shape MCHC high	Loss of membrane relative to cell volume. Hereditary spherocytosis. Accelerated RBC destruction by reticuloendothelial system
Schistocytosis	Presence of cell fragments in circulation	Increased intravascular mechanical trauma Microangiopathic hemolysis
Acanthocytosis	Irregularly spiculated surface	Irreversibly abnormal membrane lipid content Liver disease Abetalipoproteinemia
Echinocytosis	Regularly spiculated cell surface	Reversible abnormalities of membrane lipid content High plasma free fatty acids, bile acid abnormalities Effects of barbiturates, salicylates, etc.
Stomatocytosis	Elongated, slit-like zone of central pallor	Hereditary defect in membrane sodium metabolism Severe liver disease
Elliptocytosis	Oval cells	Hereditary anomally, usually harmless

Red Cell Fragility Test

Screening Test

One needs 0.45% sodium chloride solution and hemocytometer for counting red cells. Blood is drawn to the 0.5 mark in 2 red cell pipettes. The first is diluted to the 101 mark with Hayem's (RBC diluting fluid) and the second with 0.45% sodium chloride solution. Both pipettes are shaken for 2 minutes and counts made from both pipettes, the percentage of cells hemolyzed in the 0.45% saline solution is thus determined. Less than 30% of normal erythrocytes are hemolyzed by this technique. An abnormal increase in red cell fragility, as in congenital hemolytic icterus will cause hemolysis of more than 70% of the cells.

Quantitative Test

Principle: Tubes containing solution of varying concentration of saline buffered to pH 7.4 are used. Heparinized or defibrinated blood is added to each tube in a proportion of 1 to 100 and the degree of hemolysis in each is noted using a photoelectric colorimeter. The result may be reported as a graph or stating the concentration at which hemolysis begins and that at which it is complete.

Reagents

A stock solution of buffered sodium chloride (AR) osmotically equivalent to 10% NaCl, is made up as follows. NaCl 180 g, Na_2HPO_4 27.31 g and $NaH_2PO_4 2H_2O$ 4.86 g are dissolved in distilled water and the final volume adjusted to 2 liters. This solution will keep for months in a well-stoppered bottle. In preparing solutions for use it is convenient to make first a 1% solution from the 10% stock solution by dilution with distilled water. Dilutions equivalent to 0.85, 0.75, 0.65, 0.60, 0.55, 0.45, 0.40, 0.35, 0.30, 0.20 and 0.10% NaCl are convenient test concentrations. Intermediate concentrations such as 0.475 and 0.525% are useful in critical work.

If the test is performed very occasionally, smaller volumes of the solutions may be made up as given at the next page.

Method

Use: Heparinized or defibrinated blood, oxalated and citrated blood may change the tonicity which is not desirable.

Add 0.05 mL of blood to each tube containing 5 mL of the different concentrations of saline. Mix well and let the tubes stand at room temperature for 30 minutes.

Distilled water drop	18	17	16	15	14	13	12	11	10	9	8	7
1% NaCl	9	10	11	12	13	14	15	16	17	17	17	18
NaCl%	0.28	0.32	0.36	0.40	0.44	0.48	0.52	0.56	0.60	0.64	0.68	0.72

Remix and centrifuge for 5 minutes at 2000 rpm and measure the amount of hemolysis in each tube in a photoelectric colorimeter with green filter. The supernatant from 0.85% NaCl is used as the blank because there is no hemolysis in this concentration (normal) of saline. The supernatant from the 0.1% NaCl is used to estimate 100% lysis (the supernatant can easily be decanted into the cuvette of the colorimeter). The depth of color should be such that the reading on the colorimeter scale for complete lysis does not exceed 50 (optical density 0.5). If necessary, the supernatant may be diluted with an equal volume of 0.1% NaCl or the initial proportion of blood may be 1:200 instead of 1:100. With a good colorimeter, as little as 1% hemolysis may be detected.

The blood added should be exactly 0.05 mL. It can be done by using capillary automatic pipettes. Alternatively straight glass pipettes graduated till 0.05 mL may be used. Less time consuming and far less accurate method is to add one drop of blood to each tube.

Factors Affecting Osmotic Fragility Tests

In carrying out osmotic fragility tests by any method three variables capable of markedly affecting the results must be controlled, quite apart from the accuracy with which the saline solutions have been made up. These are; (i) the relative volumes of blood and saline, (ii) the final pH of the blood-saline suspension, and (iii) the temperature at which the tests are carried out.

Interpretation

Spherocytes, being already round are unable to swell very much and therefore, rupture even when a small amount of water has entered the cell. Hemolysis may thus commence even at 0.75% and may be complete at 0.4% (a feature of spherocytosis). On the other hand, target cells seen in thalassemias and iron deficiency anemia cells can swell a great deal before they rupture because they are relatively flat. Fragility is, therefore, said to be decreased.

Clinical Implications

A. *Increased fragility (> 0.5%) occurs in:*

1. Hereditary spherocytosis
2. Hemolytic jaundice
3. Autoimmune anemia (ABO and Rh) incompatibility
4. Chemical poisons
5. Burns.

B. *Decreased fragility (< 0.3%) occurs in:*

1. Obstructive jaundice
2. Thalassemia
3. Sickle cell anemia
4. Iron-deficiency anemia
5. Polycythemia vera
6. Liver disease
7. Splenectomy (following).

Decreased fragility indicates that red cells are excessively flat. Occurs in iron deficiency anemia, thalassemia, and sickle cell disease.

QUALITATIVE ASSESSMENT OF G_6PD DEFICIENCY

Methemoglobin Reduction Test

Reagents

1. Sodium nitrite 1.25 g in 100 mL distilled water.
2. Glucose 5 g in 100 mL distilled water.
3. Methylene blue 150 mg in 100 mL distilled water.

Method

Withdraw 6 mL of blood and add to 1.2 mL of ACD solution. Label three test tubes as A, B and C, add as follows:

a. To tube A, add:
   0.1 mL sodium nitrite solution
   0.1 mL glucose solution
   0.1 mL methylene blue solution
   2 mL blood.
b. To tube B, add:
   0.1 mL sodium nitrite solution
   0.1 mL glucose solution
   2 mL blood.
c. To tube C, add 2 mL of blood only.
   Mix well and keep the tubes A, B and C at 37°C for 3 hours. Mix again and aerate at 1, 2, and 3 hours (hourly intervals).

Take three test tubes each containing 10 mL of distilled water.

To one, add 0.1 mL of mixture of A.

To second, add 0.1 mL of mixture of B.

To third, add 0.1 mL of contents from C.

Wait for 10 minutes.

Test tube with distilled water and contents from C should always be red.

Test tube with distilled water and solution from B should always be brown.

Interpretation

Test tube with distilled water and solution from A.

- If this is red—there is no G_6 PD deficiency
- If brown like B—full expression of deficiency of G_6PD
- If between red and brown—intermittent expression of G_6PD deficiency.

Aging red cells are especially susceptible to oxidative challenge by drugs, systemic infection, metabolic acidosis and other stress. Oxidative stress induces rapid intravascular destruction of susceptible cells, leading to hemoglobinemia, hemoglobinuria and a sudden drop of hematocrit.

Young cells have higher G_6PD content than the older ones, regardless of the genetic variant that is present. If the enzyme has defective activity, older cells are preferentially destroyed during a mild to moderate hemolytic phase. Reticulocytes released to replace lost cells have high enzyme levels. False negative test results often occur if blood is examined just after a hemolytic episode, because the non-hemolyzed remaining cells are, by definition, those with adequate enzyme levels. Newly generated reticulocytes have still higher levels, and this can affect the results for 3 to 10 days after the episode.

Drugs that hemolyze G_6PD deficient cells are those that either act as direct oxidants themselves or produce peroxide activity. Primaquine, an antimalarial drug is notable in this respect. Many sulfa drugs, quinine derivatives, nitrofurans and antipyretic-analgesic drugs can induce hemolysis in G_6PD deficient patients. Susceptibility seems to vary among different individuals. The presence of coexisting fever, metabolic disease, or hepatic or renal failure increases likelihood that symptoms will emerge.

Commercially Available Kit for G_6PD Assessment (Qualitative)

(Courtesy: Tulip Group of Companies)♥

Summary

Glucose-6-Phosphate-Dehydrogenase (G_6PD) deficiency is one of the most common human enzyme deficiency in the world. During G_6PD deficiency, the red cells are unable to regenerate reduced nicotine adenine dinucleotide phosphate (NADPH), a reaction that is normally catalyzed by the G_6PD enzyme.

Since the X chromosome carries the gene for G_6PD enzyme, this deficiency mostly affects the males. The two major conditions associated with G_6PD deficiency are hemolytic anemias and neonatal jaundice, which may result in neurological complications and death. Screening and detection of G_6PD deficiency helps in reducing such episodes, through appropriate selection of treatment, patient counseling and abstinence from disease precipitating drugs such as antimalarials and other agents.

Reagents

G-SIX test is a ready to use, three-component reagent system of the detection of G_6PD deficiency in human blood using the WHO recommended methemoglobin reduction method. The test system contains three vials *P, *T and *N predispensed with appropriate reagents along with Quantitation graph paper.

Each batch of the reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Storage and Stability

a. Ideally, the product should be stored at 2–8°C. It may also be stored between 20–25°C in a cool dark place away from light and moisture.
b. The shelf-life of the reagent system is as per the expiry date mentioned on the G-SIX carton.

Principle

The G-SIX test is based on the principle of reduction of methemoglobin by G_6PD activity of the red cells under test. The rate of reduction is proportional to the G_6PD activity of the red cells under test. During the test procedure, the test sample is processed in triplicate so as to simultaneously also derive positive and normal reference controls. During screening method, the color of the test sample is compared visually to the reference controls in order to arrive at the diagnostic conclusion. Quantitation of the percentage of G_6PD deficiency can also be done spectrophotometrically.

Note

Laboratory reagent for professional use only. Not for medicinal use.

Sample Collection and Preparation

1. The test requires minimum 3 mL of fresh whole blood sample collected in EDTA or Heparin only. The samples must be used within one hour of collection, since the G_6PD enzyme actively decreases on storage at 2–8°C.

2. Blood samples may be collected in ACD and can be stored up to 7 days at 2–8°C before performing the test.
3. No special preparation of the patient is required prior to sample collection by approved techniques.
4. If the hematocrit of the sample is less than 30%, enough plasma should be removed from the sample to bring back the PCV to 40 ± 5%.

Additional Material Required

1. 5 mL capacity clean and clear glass test tubes of same diameter and height.
2. Incubator at 37°C.
3. 0.05 mL, 1 mL, 5 mL clean precision pipettes, spectrophotometer with 490 nm filter or colorimeter with blue green filter.
4. Timer.
5. Test tube stand.
6. Distilled/Deionized water.

Screening Test Procedure

1. Open the pack of the reagent vials *P (for test reference),*T (for test reference) and *N (for normal reference). Mark patients ID on the three vials. Use immediately upon opening.
2. Add 1 mL of the blood sample under test to each of the vials *P, *T and *N and mix well by gentle inversion.
3. Recap the vials tightly using the screw cap (the plug may be discarded) and place them vertically in an incubator which has already been stabilized at 37°C.
4. Incubate undisturbed, at 37°C, for 3 hours.
5. Meanwhile set up three 5 mL test tubes on a test tube stand and dispense 5 mL distilled/deionized water into each of these tubes.
6. Label these reference tubes as PR, TR and NR respectively and mark patient ID on each tube, (if more number of samples are being run simultaneously set up equivalent number of such distilled/deionised water tube sets).
7. Remove the vials after 3 hours incubation and mix gently.
8. Uncap the incubated test vials*P,*T and *N and dispense exactly 50 µL (0.05 mL) of the well-mixed incubated samples using different pipettes into the corresponding appropriately labeled distilled water reference tubes PR, TR and NR.
9. Mix evenly by gentle inversion.
10. Observe and compare the color of tube TR with PR and NR against light to interpret the results.
11. The test results must be interpreted within 3 hours of the preparation of tubes PR, TR and NR for screening test and within 30 minutes for the quantitative procedure.

Quantitative Procedure

1. Set the spectrophotometer filter on 490 nm.
2. Dispense/aspirate required amount of NR as obtained in point no. 9 of screening procedure into the cuvette. NR serves as blank.
3. Similarly read the OD of PR and place the corresponding value on the G-SIX quantitation graph paper, which equates to 100% deficiency on the Y-axis.
4. Make a straight line joining the blank value (0.00) and the OD of PR.
5. Read the OD of TR and place it on the graph paper.
6. Find out the %G_6PD deficiency, corresponding to the OD value of TR on the Y-axis of the graph paper.

Interpretation of Results

Screening Test

Normal sample:	Tube TR has a clear red color, matching with the normal reference tube NR.
G_6PD deficient sample (full expression):	The test tube TR has a brown color matching with the positive reference tube PR.
G_6PD deficient sample (intermediate females):	The tube TR has intermediate color as compared to positive reference tube PR and negative reference tube NR depending on the degree of expression of the deficiency trait.

Quantitative Test

Class of deficiency	*% of G_6PD deficiency*	*Clinical relevance*
CLASS I	Complete	Chronic, congenital nonspherocytic, anemia without drugs/oxidative stress
CLASS II	90% or more	Acute hemolytic crisis induced by oxidative drugs
CLASS III	40–90%	Oxidative drugs/infection induces self-limiting hemolysis without previous hematologic disorder
CLASS IV	Less than 40%	Associated with milder clinical conditions, depending on the variant involved
% G_6PD deficiency of up to 20% as obtained by G-SIX test, corresponds to the normal range of 4.5 – 13.5 U/g Hb activity.		

Remarks

1. Do not expose the reagents during storage or during test to direct sunlight. Before performing the test, if the reagent vials show any moisture or condensation on the inner walls; they must be discarded, use another set for conducting the test.

2. The reagent vials should be used immediately after opening.
3. Young red cells have a higher G_6PD content than the older ones, regardless of the genetic variant that is present. If the enzymes have defective activity, older cells are preferentially destroyed during mild-to-moderate hemolytic phase. Since reticulocytes released to replace lost cells have high enzyme levels, false negative results may occur if blood is tested immediately after a hemolytic episode.
4. The blood should be tested within an hour of collection as recommended. Delay in testing may give rise to false positives.
5. It is extremely important that the 5 mL test tubes used for postincubation sample dilution are free from acids or alkalies as this may interfere with end color stability.
6. Transfer of correct samples to the correctly labeled reference tubes PR, TR and NR is extremely vital for achieving correct results.
7. Vitamin C supplements or a large dietary intake of vitamin C may interfere with the reaction.
8. The positive reference PR must be a brown color. The negative reference must have a cherry pink to cherry red color. These colors must be achieved to validate test run and correct transfer of incubated samples to correct and corresponding reference tubes.
9. If the positive and negative reference tubes (PR and TR) have a different color than expected the test must be re-run. It must be noted however, that the test reference will show varying colors from red to brown depending upon the degree of G_6PD deficiency in the sample.

Clinical Implications

1. A decreased level is associated with G_6PD deficiency, which is a sex-linked disorder. Affected males inherit the abnormal gene from their mothers who are usually asymptomatic carriers. In some cases of this disorder, there is lifelong hemolysis; but more commonly, the condition is asymptomatic and results only in susceptibility to acute hemolytic episodes that may be triggered by drugs such as primaquine, sulfonamides, and antipyretics, by ingestion of fava beans, or by viral or bacterial infections.
2. The major types of G_6PD deficiency are:
   a. Type A, found in blacks.
   b. Mediterranean type, found in both
      i. Caucasians and Orientals such as Greeks,
      ii. Sardinians and Sephardic Jews.
   c. Rare, congenital non-spherocytic anemia.
   d. Nonimmunologic hemolytic disease of the newborn.
3. G_6PD levels are increased in:
   a. Pernicious anemia.
   b. Werlhof's disease.
   c. Hepatic coma.
   d. Hyperthyroidism.
   e. Myocardial infarction.
   f. Chronic blood loss.
   g. Other megaloblastic anemias.

Quantitative Estimation of G_6PD

(Courtesy: Tulip Group of Companies)

Summary

Glucose-6-Phosphate-Dehydrogenase (G_6PDH) deficiency is one of the most common human enzyme deficiencies in the world. During G_6PD deficiency, the red cells are unable to regenerate reduced Nicotinamide adenine dinucleotide phosphate (NADPH), a reaction that is normally catalyzed by the G_6PD enzyme. Since the X chromosome carries the gene for G_6PD enzyme, this deficiency mostly affects the males.

The two major conditions associated with G_6PD deficiency are hemolytic anemias and neonatal jaundice, which may result in neurological complications and death. Screening and detection of G_6PD deficiency helps in reducing such episodes, through appropriate selection of treatment, patient counseling and abstinence from disease precipitating drugs such as antimalarials and other agents.

Principle

G_6PDH in the RBCs is released by a lysing agent present in the reagent. The G_6PDH released catalyzes the oxidation of glucose 6 phosphate with the reduction of NADP to NADPH. The rate of reduction of NADP to NADPH is measured as an increase in absorbance, which is proportional to the G_6PDH activity in the sample.

$$\text{G-6-P} + \text{NADP} \xrightarrow[\text{NADPH} + \text{H}]{\text{G-6 - PDH}} \text{Gluconate-6 - P} +$$

Normal Reference Values

G_6PDH activity	:	4.6 to 13.5 at 30°C/
(U/g Hb.)		6.4 to 18.7 at 37°C
($U/10^{12}$ RBCs)	:	146 to 376 at 30°C/
		202 to 522 at 37°c

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*5 × 1 tests*	*5 × 5 tests*
L1 : G_6PDH Reagent	5 × 1 mL	5 × 5.5 mL
L2 : Starter Reagent	10 mL	50 mL

Storage/Stability

Contents are stable at 2-8°C till the expiry date mentioned on the labels.

Reagent Preparation

Reconstitute G_6PDH reagent (L1) with distilled water as per the volume mentioned on the label. This working reagent is stable for 6 hours at RT and at least 3 days when stored at 2–8°C.

The Starter Reagent (L2) is ready to use.

Sample Material

Fresh whole blood sample collected in EDTA, Heparin or ACD. Red cell G_6PDH in whole blood is reported to be stable for 7 days at 2–8°C, but is unstable in hemolysates. Freezing is not recommended.

Procedure

Wavelength/filter : 340 nm
Temperature : 30/37°C
Light path : 1 cm.

Addition	*S Sequence (mL)*
G_6PD working reagent (L1)	1.0
Whole blood	0.01
Mix well and incubate for 5–10 minutes at RT and add substrate reagent	2.0

Mix well and incubate for 5 minutes at 30/37°C and read the initial absorbance A0 and repeat the absorbance reading after every 1, 2 and 3 minutes. Calculate the mean absorbance change per minute (DA/min).

If the G_6PDH activity is very low, the absorbance change per minute will also be very low. In such cases read the initial absorbance A1 and read another absorbance A2 exactly 5 minutes later. Calculate the mean absorbance change per minute (ΔA/minutes)

$$\Delta A/\text{min} = \frac{A2A1}{5}$$

Calculations

$$G_6\text{PDH Activity (U/10}^{12}\text{ RBC)} = \Delta A \times \frac{47780}{\text{RBC Count (/mm}^3\text{)}}$$

$$G_6\text{PDH Activity (U/gHb)} = \Delta A \times \frac{\times 4778}{\text{Hb (g/dL)}}$$

Temperature Conversion Factors

Assay Temperature	*Desired Reporting Temperature*		
	25°C	*30°C*	*37°C*
25°C	**1.00**	**1.32**	**1.82**
30°C	**0.76**	**1.00**	**1.39**
37°C	**0.55**	**0.72**	**1.00**

Notes

Since the activity of G_6PDH is reported in Hb concentration or RBC count the same should be determined before performing the assay. RBCs are well preserved when collected in ACD and such samples give an accurate count, for samples collected in Heparin counts become unreliable after 2 days and in such cases results are best reported in Hb concentration.

Copper and sulfate ions inhibit the G_6PDH activity; hence use of good quality deionized or distilled water for reconstitution of L1 and properly cleaned glassware is essential.

Young red cells have a higher G_6PD content then the older ones, regardless of the genetic variant that is present. If the enzymes have defective activity, older cells are preferentially destroyed during mild to moderate hemolytic phase. Since reticulocytes released to replace lost cells have high enzyme levels, falsely elevated results may occur if blood is tested immediately after a hemolytic episode.

Normally the activity contributed by WBC, platelets or serum is very small. In cases of severe anemia, leukocytosis, or very low G_6PDH levels, the use of a sample after removing the Buffy Coat is recommended.

EXAMINATION OF FETAL HEMOGLOBIN

Qualitative Method

Peripheral blood film staining method (Acid elution technique).

Always use freshly prepared smears

- Fix in 80% ethanol for 10 minutes
- Take 37.7 mL of citric acid solution prepared (21 g of citric acid by dissolving, in 100 cc of distilled water) in a jar kept at 37°C
- To this, add 12.2 mL of $Na_2H\ PO_4$ solution (prepared by dissolving 57.6 g of $Na_2H\ PO_4.12\ H_2O$ (in 1000 cc of distilled water)
- In this mixture keep the slide for 30 minutes
- Wash with distilled water
- Stain with 1% eosin solution for 5 minutes.

Interpretation

Normal red cells will appear as ghost cells. Fetal hemoglobin containing cells will appear as bright red cells.

Quantitative Method

STEP A

Preparation of hemolysate (Fig. 9.18):

1. Take 8 cc of EDTA blood, centrifuge it for 15 minutes, remove the plasma.
2. To the RBCs sediment, add normal saline and fill the centrifuge tube to 4/5th of it. Mix thoroughly and centrifuge for 20 minutes. Remove the saline and repeat the said process at least twice more.
3. To the packed RBCs, add equal quantity of distilled water and half the quantity of toluene. Mix them thoroughly and keep in the deep freezer for one hour.
4. Remove from the freezer chest. Thaw it and centrifuge it for 30 minutes. Now there will be 3 zones in the tube.
5. Pass a pipette to the hemolysate zone through the side of the test tube without disturbing the upper two zones, suck up the hemolysate.
6. Check the hemoglobin of the hemolysate and adjust to 8–10 g%. This hemolysate can be stored at –20°C for 6 months and can be used for quantitative (alkali denaturation method) estimation of fetal hemoglobin or for hemoglobin electrophoresis.

STEP B

Quantitative alkali denaturation method for estimation of fetal hemoglobin

1. Take 9.5 mL of Drabkin's solution in a test tube, add 0.5 mL of hemolysate. Cyanmethemoglobin (Hi CN) is formed. Mix well.

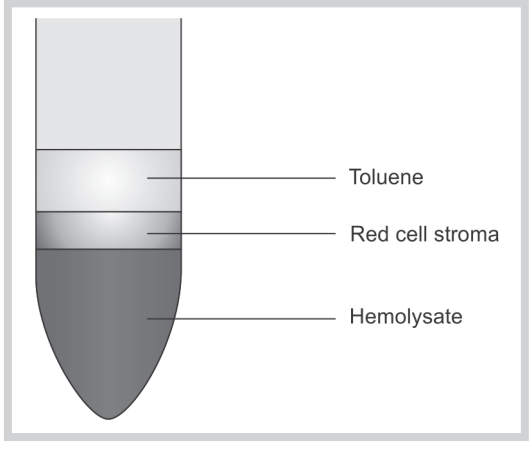


FIG. 9.18: Preparation of hemolysate

2. Transfer 2.8 mL of HiCN solution in a tube and keep at 20°C. To this, add 0.2 mL of 1.2 NNaOH solution. Mix rapidly—incubate for 2 minutes at 20°C. Add 2 cc of saturated solution of ammonium sulfate. Mix again and let stand for 5-10 minutes. Filter through Whatman filter paper number 3.
3. Prepare the total hemoglobin by adding 0.4 mL of the original HiCN solution to 6.5 mL of distilled water.
4. Using Drabkin's solution as blank at 540 nm, read the total hemoglobin and filtrate.
5. The optical density should fall between 0.05 and 0.5. If it is beyond 0.5, dilute the hemolysate with distilled water and repeat the said procedure.
6. Calculate as hemoglobin F (percentage of HbF).

$$\frac{\text{OD of total 10}}{\text{OD of total hemoglobin}}$$

HbF and HbS concentration as percentage of total Hb concentration in various disorders is given below:

Disorder	*HbF%*	*HbS%*
Normal	< 1% (in adults)	not present
Sickle cell trait (AS)	Normal	30–40%
Sickle cell anemia (SS)	1–20%	75–95%
HbS β thalassemia		
[Sβ$^+$ (some β chains present)]	2–10%	60–85%
[Sβ° (no β chains present)]	5–30%	76–90%
HbS-C (SC)	1–5%	50–55%
HbS-D (SD)	1–5%	95% (S+D)
β Thalassemia major	10–98%	—
α Thalassemia	Reduced	—

Normal Values

Adults	0–2% of total hemoglobin
Children	
Newborn	< 60–90% of total hemoglobin
1-5 months	< 75% of total hemoglobin
6-12 months	< 5% of total hemoglobin
1-20 years	< 2 % of total hemoglobin.

Tests for Sickling

Sickle cells are abnormal forms resulting from the presence of abnormal hemoglobin, which in the deoxidized state undergoes a hydrophobic bond dependent polymerization. Rigid threads or rods of S hemoglobin result, with consequent production of the rigid sickle cells. Sickling occurs in 10% of blacks, but only a few of these have anemia.

Methods

Moist Cover Slip Preparation

Cover a small drop of fresh blood on a slide with a coverslip, seal with petrolatum, paraffin or nail polish, keep at warm room temperature and examine for sickling every 12 hours up to 72 hours. To find percentage of circulating sickling forms, draw 4 mL of blood into a syringe containing 1 mL of 10% formalin, examine wet preparation.

Daland and Da Silva Method

Acceleration of removal of oxygen from hemoglobin by use of reducing substances.

Reagents

Sodium bisulfite or ascorbic acid, 2 g, in 100 mL water (to be made fresh each time).

Method

Use capillary blood, oxalated or defibrinated. Mix 2 drops of either reagent with one drop of blood on a glass slide. Cover, and seal coverslip with petrolatum or nail polish. If no sickling appears in 4 hours, the test is negative. Positive test: 10% or more red cells show sickled forms.

Dithionite Tube Test

The red cells are lysed, hemoglobin deoxygenated, and the β-globin chains are displaced to provide the molecular steric fit characteristic of sickling of S and no S hemoglobins. The test solution becomes turbid if sickling hemoglobins are present.

Reagents

Stock solution

$KH_2 PO_4$	160.48 g
$K_2H PO_4$	281.18 g
Distilled water to 1 liter.	

Working solution

Stock buffer	800 mL
$Na_2S_2O_4$	60 g
Saponin (5%) solution	20 mL
Distilled water to	1000 mL

Method

Add 20 μL (0.02 mL) of well mixed whole blood EDTA anticoagulated) to 2 mL dithionite working solution in a 12 × 75 mm test tube. Mix by inverting and allow to remain at room temperature for 56 minutes. Examine for opacity by holding the tube 2.5–3 cm from a sheet of black newsprint.

Interpretation

Turbidity (newsprint not visible) indicates the presence of sickling hemoglobin. Clear solution indicates a negative test. A positive test (turbidity) indicates the presence of any of the hemoglobins S (SS or AS), C (Harlem), C (Georgetown), Barts and perhaps Alexandra. All positive tests should be followed by hemoglobin electrophoresis. Hemoglobin S can be separated from other hemoglobins by the urea-dithionite test.

Urea-dithionite Test

Reagents

To 50 mL of working solution (above: from previous test), add 6 g of urea. Mix until dissolved.

Method

Add 20 μL (0.02 mL) whole blood to 2 mL of urea dithionite solution in a 12 × 75 mm test tube. Mix by inverting tube and allow to remain at room temperature for 5 minutes. Read with tube 2.5–3 cm away from newsprint background.

Interpretation

Turbidity produced by the sickled liquid crystal system of hemoglobin S in dithionite is dispersed in the urea-dithionite by the urea, which breaks the hydrophobic bonds essential to sickling.

Hemoglobin	*Dithionite*	*Urea-dithionite*
SS, AS and C (Harlem)	Turbid	Clear
Nonsickling C (Georgetown), Barts, Alexandra	Turbid	Turbid

Refer to Table given under HbF Estimation for Percentage of HbS in Various Disorders

The presence of HbA guards, to a modest extent, against tactoid (polymerization) formation, HbF exerts a very strong protective effect. It is probable that the cells of patients with sickle cell disease undergo intravascular sickling and unsickling many times as they circulate. Repeated shape changes, however, stresses the cell membrane and causes the loss of small membrane fragments. This reduces the ratio of surface to volume, making the cell less flexible and less responsive to physical changes. Some cells gradually lose their mechanical and osmotic resistance and undergo intravascular dissolution, whereas others are removed early by reticuloendothelial system. Red cells in HbS disease experience a chronically shortened lifespan.

The likelihood of sickling increases with low oxygen tensions, lowered pH and increased body temperature. HbS protects against *Plasmodium falciparum* infection. Sickle cell anemia rarely becomes clinically apparent before 6 months of age because protective amounts of HbF remain in cell; for the same reason, it is difficult to screen newborns for HbS disease.

Commercially Available Kit

(Courtesy: Tulip Group of Companies)

Summary

Hemoglobin S (HbS) differs from the normal hemoglobin A (HbA) by a single amino acid mutation at position 6 of the beta chain, wherein glutamic acid is replaced by valine.

During low oxygen conditions, the RBC morphology may range from mild elongation to irreversible elongated tactoid. This elongated filamentous tactoid formation results in the typical "sickle" appearance of the RBC.

Individuals with sickle cell anemia (homozygous S/S) may have early mortality with vascular occlusions of multiple organ systems, severe hemolytic anemia and hypoxia.

Individuals with the sickle cell trait (heterozygous A/S) are usually asymptomatic. However, under certain conditions of reduced oxygen tension such as hypoxia during anesthesia, fight in poorly pressurized airplanes, severe pneumonia, they can experience a sickle cell crisis.

Reagents

a. *Sicklevue reaction tubes*: Prefilled with sodium dithionite.
b. *Phosphate buffer:* A concentrated, ready-to-use solution containing a red cell lysing agent.

Reagent Storage and Stability

Store the reagents at RT (15–30°C). *Do not refrigerate or freeze.*

Do not expose to light for excessive periods. Best stored as supplied in the kit.

The shelf-life of the reagents is as per the expiry date mentioned on the Sicklevue carton.

Principle

Sicklevue is based on the solubility difference between HbS and HbA in concentrated phosphate buffer solution. RBCs under test are lysed by a powerful hemolytic agent and the released hemoglobin is then reduced by sodium dithionite in a concentrated phosphate buffer.

In the presence of sodium dithionite, HbS precipitates causing turbidity of the reaction mixture. Under the same conditions, HbA, as well as most other hemoglobins, are soluble.

HbS when reduced in the phosphate buffer forms a turbid solution, which is easily visualized. Normal HbA and most other hemoglobins remain in solution resulting in a clear suspension.

Note

1. Reagent for laboratory use only.
2. Do not pipette by mouth.
3. Phosphate buffer does not contain preservatives. Aseptic conditions should be followed to avoid contamination. However, as a powerful hemolytic agent is included in the composition, avoid contact with skin or mucosa. Wash hands after use.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures.
5. Use reagent of same lot numbers. Do not interchange reagent of different lot numbers.

Sample Collection and Preparation

No special preparation of the patient is necessary prior to specimen collection by approved techniques. Collect whole blood in EDTA, heparin, sodium citrate or ACD anticoagulant. Though fresh blood samples are preferable, the sample can be stored at 2–8°C for up to 24 hours, in case of delay in testing.

Materials Provided with the Kit

Reagent Pack

Sicklevue reaction tubes, phosphate buffer and result reading card.

Additional Material Required

The 2 mL pipettes, micropipette (20 µL), test tube rack, stop watch, laboratory centrifuge.

Test Procedure

Bring all reagents and samples to room temperature before use.

Screening Method

1. Retrieve the required number of Sicklevue reaction tubes, as the number of samples to be tested.
2. Label the reaction tubes appropriately and set on a test tube rack.
3. Add 2 mL of the buffer to each of the reaction tubes, using the pipette. Alternately, fill up the tubes by pouring the buffer up to the 2 mL marking.
4. Mix well and allow to stand for 5 minutes at RT.
5. With the help of a micropipette, add 20 µL of whole blood sample.
6. Mix and allow to stand for 10 minutes.
7. To read the results, place the tubes into the slots of the Result Reading Card provided.
8. Read the turbidity in the tubes by holding the tubes against a dim illumination and viewing the horizontal

black lines printed on the viewing card, below the 2 mL marking, through the solution, in the Sicklevue tube.

Differentiation Method

To differentiate between sickle cell trait (Hb AS) and sickle cell anemia (Hb SS).

1. If positive results are obtained during the screening method, take a fresh Sicklevue reaction tube and repeat the test procedure as in Screening Method with 100 µL of whole blood sample.
2. Centrifuge the reaction tube at 1200 g for 5 minutes in a laboratory centrifuge.
3. Allow the centrifuge to stop without braking and carefully remove the reaction tubes without disturbing the contents.
4. Observe the pattern formed in the reaction tubes.

Interpretation of Results

Screening Method

a. A turbid solution (horizontal black lines on the Result Reading Card are barely visible or cannot be seen) indicates a positive test for sickle cell hemoglobinopathies.
b. A clear solution (horizontal black lines on the Result Reading Card are clearly visible) indicates a negative test result.

Differentiation Method

a. Hb AS-Hb S forms a red precipitate at the top and soluble Hb if present forms a red color solution below this precipitate.
b. Hb SS-A red precipitate at the top and solution is colorless. If substantial amount of Hb F is present, the solution may be pink.
c. All other Hb yield clear red solutions.

Remarks

1. All positive results should be confirmed on electrophoresis.
2. Severe anemia can cause false negative results. If the hemoglobin concentration is 8 g% or less the sample volume for testing should be doubled to 40 µL.
3. Blood samples from patients with multiple myeloma, cryoglobulinemia and other dysglobulinemias may give false positive results.
4. It is recommended that the performance of reagents should be verified with known positive and negative controls.
5. As with all tests, the result of the test should be correlated with clinical findings to arrive at the final diagnosis.

LABORATORY DIAGNOSIS OF DISORDERS RELATED TO RBCS

Laboratory Diagnosis of Iron Deficiency Anemia

Peripheral Blood

MCV < 76 fl.
MCH < 27 pg.
MCHC < 30 g%
RBC count—normal.

Peripheral Smear

1. Anisocytosis, microcytic red cells.
2. Poikilocytosis, pencil-shaped cells and target cells.
3. Hypochromia, ring or pessary cells.
4. Few polychromatophils.
5. Reticulocyte count is variable.
6. RBC osmotic fragility is slightly decreased.
7. Hematocrit low, plasma appears paler.
8. Radiochromium—^{51}Cr studies show reduced red cell span; however, TLC, DLC and platelets have normal lifespan.

Bone Marrow

1. Micronormoblastic erythroid hyperplasia.
2. Predominantly intermediate normoblasts are seen.
3. Cytoplasm is decreased and shows differential staining.
4. Cytoplasm matures so slowly that nucleus may be pyknotic, while cytoplasm is still polychromatic.
5. Bone marrow iron is reduced or absent. [Perl's reaction done on fixed bone marrow slide shows absent/reduced free iron (blue particles) and lack of siderotic granules in normoblasts].

Serum Biochemistry

1. Serum iron is reduced (15–16 µg%).
2. Total iron binding capacity is raised (up to 550 µg%).
3. Unsaturated iron-binding capacity is also raised.
4. Percentage saturation is reduced to about 10%.
5. Red cell protoporphyrin increased (no iron available to form hemoglobin).

Normal Values for Iron Metabolism

Serum iron (Fe): 60–170 µg%; Total iron-binding capacity (TIBC): 300-360 µg%; Saturation: 20–45%; Serum ferritin:12–300 µg/L.

Laboratory Findings in Iron Deficiency

Blood Count

- Microcytic, hypochromic red cells if Hb < 12 g% (men), < 10 g% (women)
- Degree of Hb reduced, RBC count diminished depends on severity. Leukopenia may occur

- Platelets increased in number with active bleeding
- Reticulocytes lower than expected for degree of anemia.

Bone Marrow
- Erythroid hyperplasia
- Micronormoblastic reaction
- Stainable iron is reduced.

Others
- Serum iron is reduced, iron-binding capacity is increased % saturation is diminished
- Serum ferritin < 10 ng%
- Free erythrocyte protoporphyrin increased
- RBC survival time slightly increased.

Causes of Iron Deficiency Anemia

Blood Loss

Uterine (menorrhagia, metrorrhagia) *chronic gastrointestinal blood loss, in:*
- Esophageal varices
- Hiatus hernia
- Peptic ulcer
- Chronic aspirin ingestion
- Carcinoma of:
  - Stomach
  - Colon
  - Cecum
  - Rectum
- Ulcerative colitis
- Hemorrhoids
- Diverticulosis
- Hookworm infestation (anemia with eosinophilia).

Other Causes of Chronic Blood Loss
- Hematuria
- Repeated epistaxis
- Hemoptysis.

Increased Requirements
- Prematurity (decreased iron stores)
- Growth (iron deficiency anemia is commonest in children 6–24 months of age).
- Females in reproductive age group:
  - Menstruation
  - Pregnancy
  - Lactation.

Impaired Absorption
- Achlorhydria (especially in middle-aged females) achlorhydria → iron deficiency anemia
- Gastrectomy (HCl not available)
- Gastroenterostomy (Inflamed anastomosis or intestinal hurry hence no time for absorption).

Inadequate Intake
- Improper feeding in infants and young children
- Poverty
- Dietary fads
- Anorexia (nervosa, of pregnancy or malignancies).

Laboratory Diagnosis of Megaloblastic Macrocytic Anemias

Peripheral Blood Findings in Vitamin B_{12} or Folic Acid Deficiency

1. Anemia with macro (ovalo) cytosis.
2. Anisopoikilocytosis.
3. RBCs may show:
   - Howell-Jolly bodies
   - Cabot's rings
   - Basophilic stippling.
4. MCV > 96 fl.
5. Moderate leukopenia due to neutropenia.
6. Hypersegmented neutrophils, i.e. more than 3 neutrophils having more than 5 nuclear lobes/100 neutrophils.
7. Macropolycytes (large neutrophils).
8. Mild, usually asymptomatic thrombocytopenia.

Bone Marrow

Dyserythropoiesis
1. Megaloblasts (larger normoblasts with large open sieve-like nucleus) a constant feature.
2. Late megaloblast
   - Has an eccentric, indented lobulated nucleus
   - May show Howell-Jolly bodies.
4. Dissociation of cytoplasmic-nuclear maturation (hemoglobinization occurs faster than the nuclear maturation).
5. Mitoses common, may be abnormal, i.e. tri or quadripolar.
6. Maturation arrest, promegaloblasts and early megaloblasts constitute 50% of the erythroblasts.

WBC Series
1. Large atypical cells.
2. Giant metamyelocytes.
3. Absolute number of granulocytes increases but is not evident because of simultaneous erythroid hyperplasia.

Megakaryocytes
1. Number is variable, occasionally diminished.
2. Have deep basophilic cytoplasm.
3. Hypersegmented nuclei.

Special Tests for Diagnosing Vitamin B_{12} Deficiency

1. Serum vitamin B_{12} assay
2. Increased urinary excretion of methylmalonic acid
3. Radioactive vitamin B_{12} absorption test.

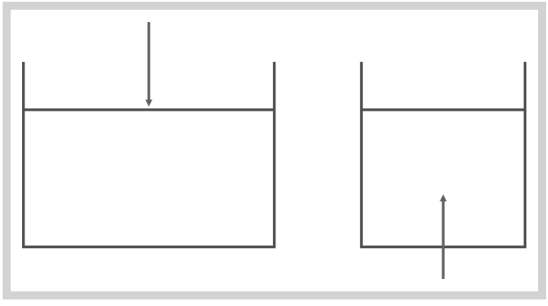
Serum Vitamin B_{12} Assay

- Microbiological
- Radioisotope assay.

Microbiological

Organisms used:

- *Euglena gracilis*
- *Lactobacillus leichmannii*

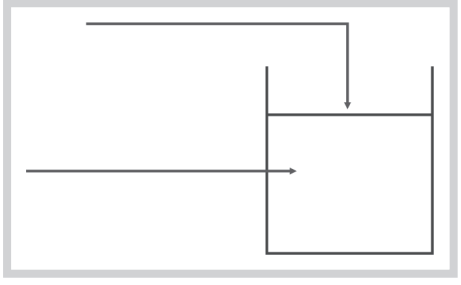


- Test serum
  Microorganism + all microorganism + necessary growth factors all necessary growth except vitamin B_{12} factors + known amount of vitamin B_{12}

  Both the tubes are inoculated and later the turbidity developed due to growth of the microorganism is measured and serum vitamin B_{12} level is deduced.
- Using *E. gracilis,* normal values are 160–925 ng/L (mean = 475 ng/L).
- A value < 100 ng/L implies frankly megaloblastic anemia.

Radioisotope Assay

Test serum + Vitamin B_{12} labeled with ^{57}Co



1. Vitamin B_{12} binding protein or intrinsic factor.
2. Separate free and bound form.
3. Compare with standards
   — Normal values = 200–300 ng/L.

Radioactive Vitamin B_{12} Absorption Test

Principle: Ability to absorb ^{57}Co-labeled vitamin B_{12} orally, if simultaneous administration of intrinsic factor improves absorption, it implies lack of intrinsic factor.

Absorption of radioactive vitamin B_{12} can be measured in 5 ways:

1. Radioactivity in feces.
2. Radioactivity in urine (Schilling test).
3. External counting over liver (chief storage organ).
4. Whole body counting.
5. Estimation of plasma radioactivity.

Schilling Test

1. Give 1 mg unlabeled vitamin B_{12} parenterally.
2. Give 1 μg labeled vitamin B_{12} orally.
3. Within 24 hours 1/3rd of absorbed radioactive vitamin B is flushed out in urine.
4. Normal excretion is > 10% of oral dose.
5. Pernicious anemia patients excrete < 5%.
6. If the test is normal no further testing necessary.
7. If it is abnormal—repeat the said procedure with simultaneous oral administration of intrinsic factor.
8. If excretion increases, it implies lack of intrinsic factor. If it does not, then there is some defect in absorption (Repeat test can be done 48 hours later).

Variant of Schilling Test

- Give orally
  Free ^{58}Co-labeled vitamin B, and
  ^{57}Co-labeled intrinsic factor bound vitamin B
- Estimate amounts in urine
- If ^{57}Co-labeled vitamin B is excreted more, there is deficiency of intrinsic factor.

Causes of Vitamin B_{12} Deficiency

1. *Reduced intake*—nutritional deficiency.
2. *Impaired absorption.*

   Gastric causes
   - Adult pernicious anemia
   - Congenital lack of IF
   - Total or partial gastrectomy.

   Intestinal causes
   - Chronic tropical sprue
   - Intestinal stagnant loop syndrome, e.g. jejunal diverticulosis, blind loops, strictures
   - Crohn's disease and ileal resection
   - Congenital selective malabsorption with proteinuria
- Fish tapeworm infestation
- Severe pancreatitis

- Celiac disease
- Therapy with metformin or phenformin.

Special Tests for Diagnosing Folate Deficiency

Serum Folate Assays

1. *Microbiological:* As for vitamin B_{12} except that *Lactobacillus casei* is used here. Normal values = 6–21 μg/L
2. *Radioisotope assay:* As for vitamin B_{12} except that Cow's milk is used here as the binding protein. Value < 4 μg/L implies megaloblastic, macrocytic anemia (Normal red cell folate 160–640 μg/L).

FIGLU Test

Folate is essential for conversion of histidine to glutamic acid. Formiminoglutamic acid (FIGLU) is an intermediate product. In folate deficiency, FIGLU is increased which appears in urine. This test, however, is not very specific.

Radioactive Folic Acid Test-like Schilling Test

Deoxyuridine (dU) suppression test (for both vitamin B_{12} and FA deficiency)

1. Short-term in vitro bone marrow cultures are used.
2. In normoblastic cultures, added deoxyuridine enters DNA thymine pathway and supresses the subsequent incorporation of subsequently added tritiated thymidine in DNA.
3. In vitamin B_{12} and folic acid deficiency, the added deoxyuridine causes less suppression, but the defect can be corrected by supplying the missing vitamin.

Causes of Folic Acid Deficiency

Nutritional

- Especially old age
- Poverty
- Scurvy
- Partial gastrectomy
- Goat's milk anemia.

Malabsorption

- Tropical sprue
- Celiac disease
- Partial gastrectomy
- Extensive jejunal resection
- Crohn's disease.

Increased Demand

Physiological

- Pregnancy
- Lactation
- Prematurity.

Pathological

1. Hematological diseases
   - Hemolytic anemias
   - Myeloproliferative disorders
   - Myelosclerosis
   - Sideroblastic anemia
   - Multiple myeloma.
2. Various carcinomas
3. Inflammatory diseases
   - Crohn's disease
   - Tuberculosis
   - Rheumatoid arthritis
   - Psoriasis
   - Exfoliative dermatitis.
4. Hyperthyroidism
5. Excess urinary loss
   - Active liver disease
   - Congestive heart failure.
6. Anticonvulsant drug therapy and oral contraceptives
7. Mixed
   - Liver disease
   - Alcoholism.

Laboratory Findings in Megaloblastic Anemias

Blood Counts

- Severe anemia (Hb may fall up to 3 g%)
- Macrocytosis (MCV 100–140 fl), with anisocytosis
- Ovalocytes, and macro-ovalocytes numerous
- WBCs, platelets often low in number
- Hypersegmented neutrophils > 3%
- Reticulocytes disproportionately low vis-a-vis degree of anemia.

Bone Marrow

- Marked erythroid hyperplasia
- Megaloblastic nuclear appearance in all 3 cell lines
- Storage iron normal or increased.

Blood Chemistry

- Bilirubin increased (indirect)
- Serum iron increased
- Increased LDH, with LDH-1 > LDH-2.

Other Studies

- Schilling test abnormal (pernicious anemia). Antibodies to gastric cells, intrinsic factor (pernicious anemia)
- Serum RBC levels of vitamin B reduced (vitamin B_{12} deficiency)
- Urine methylmalonate increased (vitamin B_{12} deficiency)
- Urine FIGLU—folic acid deficiency.

Causes of Bone Marrow Megaloblastosis

- Vitamin B_{12} deficiency
- Folic acid deficiency
- Folic acid antagonists
- Inhibitors of purine or pyrimidine synthesis
- Alcoholism

- Genetically determined enzyme defects:
  a. Lesch-Nyhan syndrome
  b. Orotic aciduria
- Cytotoxic drugs
- Liver disorders
- Myxedema
- Sideroblastic anemia
- Multiple myeloma
- Widespread neoplastic disease
- Metastatic deposits in bone marrow.

Laboratory Diagnosis of Hemolytic Anemias

Causes and Classification of Hemolytic Anemias

Intracorpuscular Defects

Hereditary or congenital

Membrane defects

Hereditary spherocytosis
Hereditary elliptocytosis
Hereditary stomatocytosis.

Hemoglobin defects

Hemoglobinopathies
Sickle cell disease
Hb CDE, etc.
Unstable hemoglobin disease.

Thalassemias

β Thalassemia major
HbH disease

Enzyme defects

Nonspherocytic congenital hemolytic anemia due to phosphokinase deficiency or other EM pathway enzyme defects. Due to G_6PD deficiency or other pentose phosphate pathway enzyme defects.

Drug-induced hemolytic anemia—Favism

Acquired

Paroxysmal nocturnal hemoglobinuria.

Extracorpuscular Defects

Acquired

Immune mechanism

- Autoimmune hemolytic anemia. Warm antibody type Cold antibody type
- Hemolytic disease of the newborn
- Incompatible blood transfusion
- Drug-induced hemolytic anemia
- Non-immune mechanism
- Mechanical hemolytic anemia
- Cardiac hemolytic anemia
- Microangiopathic hemolytic anemia
- March hemoglobinuria.

Miscellaneous

- Hemolytic anemia due to direct action of drugs/chemicals
- Hemolytic anemia due to infection (*Clostridium welchii*)
- Hemolytic anemia due to burns
- Lead poisoning.

Evidences of Hemolysis

Increased Breakdown of Hemoglobin

- Jaundice and hyperbilirubinemia
- Reduced plasma haptoglobin and hemopexin
- Increased plasma LDH
- Increased urinary urobilinogen
- Increased fecal urobilinogen
- Hemoglobinuria and hemoglobinemia
- Methemalbuminemia
- Hemosiderinuria

evidences of intravascular hemolysis

Compensatory Erythroid Hyperplasia

- Reticulocytosis, erythroblastemia
- Macrocytosis, polychromasia
- Erythroid hyperplasia of bone marrow (reversal of M:E ratio)
- Skeletal X-ray (Widening of marrow space)
- Radiological changes in skull and tubular bones (in congenital hemolytic anemias only).

Damage to Red Cells

- Spherocytosis
- Fragmentation of red cells
- Heinz bodies.

Demonstration of Shortened Lifespan of Red Cells

Normal plasma haptoglobin level = 1–1.5 g/L. Levels are assessed by rapid latex agglutination test. Levels under 1 g/L imply two to three times hemolysis or the half-life of red cells is 17 days or less. Normal plasma hemopexin level is = 0.5–1 g/L. Its concentration is measured by radial immunodiffusion technique. In most intravascular hemolysis, its levels are diminished.

Laboratory Diagnosis of Hereditary Spherocytosis

Blood Picture

- Anemia with spherocytosis (Hb 9–12 g%)
- Osmotic fragility, reticulocyte count (5–7%) and serum bilirubin (indirect) are raised
- Negative Coombs' test
- Autohemolysis after 48 hours at 37°C—10–50% (normal ≤ 4%)
- Glucose or ATP addition abolishes autohemolysis.

Peripheral Smear

- Spherocytes
- Polychromatophils.
- Platelets diminished in number if splenomegaly present
- MCV normal or reduced
- MCH normal
- MCHC often increased (34–40%)
- Reticulocyte count raised (5–20%).
- There can be pancytopenia in aplastic crisis.
- ^{51}Cr autologous red cell life reduced with excessive counting over spleen (the main pitting organ).

Chemistry

- Bilirubin slightly (indirect) increased
- Urine urobilinogen increased
- Haptoglobin reduced.

Laboratory Diagnosis of Hereditary Elliptocytosis

- In peripheral smear, ovalocytes are > 50% usually. Both MCV and MCH are normal
- Osmotic fragility may be raised in anemic patients.

Laboratory Diagnosis of Enzyme Deficiency Related Anemias

G_6PD Deficiency

Blood Picture

- Polychromasia
- Basophilic stippling
- Spherocytosis ±
- Heinz bodies, 1–2, after commencement of therapy. Besides methemoglobin reduction test already described, other tests which can be done are:
  1. Brilliant cresyl blue (BCB) reduction test.
  2. Heinz body test.
  3. Fluorescent spot test: NADPH autofluorescences in long wave ultraviolet light.
  4. Enzyme assays.

Laboratory Diagnosis of Autoimmune Hemolytic Anemia (AIHA)

Laboratory Diagnosis of Warm Antibody AIHA

Blood Picture

- Hemolytic anemia
- Positive direct antiglobin (Coombs') test
- Monocytosis in peripheral smear ± erythrophagocytosis
- Variable total leucocyte count TLC.
- Blood withdrawn often shows autoagglutination because RBCs, are heavily coated with immunoglobulins.
- Red cell and serum folate levels are diminished.

Immunology

- Autoantibodies demonstrated in vitro in most cases
- Antibody is found on red cell surface and in serum (surface antibodies are revealed by direct Coombs' test)
- IgA immunoglobulin deficiency.

Laboratory Diagnosis of Cold Antibody AIHA

This has two forms:

- Cold hemagglutinin disease (CHAD)
- Paroxysmal cold hemoglobinuria (PCH).

CHAD

Blood picture

- Anemia
- Red cell agglutination of peripheral (smear) blood (avoidable by raising temperature to 37°C)
- Reticulocytosis
- Hyperbilirubinemia
- Positive (direct) Coombs' test (because of C_3d on red cell surface as shown by specific antiglobulin sera).

PCH

- Donath Landsteiner antibody test, chill the blood, take back to 37°C, hemolysis occurs.
- Positive Coombs' (direct) test only during the attack.

Laboratory Diagnosis of Paroxysmal Nocturnal Hemoglobinuria

Blood Picture

- Anemia, macrocytosis, polychromasia
- Reticulocytosis, moderate leukopenia, mild thrombocytopenia
- HbF occasionally raised
- Hyperbilirubinemia
- Neutrophil alkaline phosphatase score is low, but normal in aplastic cases
- Hypercoagulability
- Direct Coombs' test ±.

Further Investigations

- Hemoglobinuria
- Hemosiderinuria
- Serological tests.
  - *Ham's serum test:* Patient's red cells will undergo lysis in compatible acidified serum at 37°C (serum may be patient's own). About 10–50% lysis implies a positive test with patient's own serum.
  - *Sucrose hemolysis test:* Isotonic solution of low ionic strength if causes hemolysis of more than 10% cells—indicates diagnosis of PNH.

Laboratory Diagnosis of Lead Poisoning

Blood Picture

- Basophilic stippling in peripheral smear and bone marrow
- Normocytic, normochromic or microcytic, hypochromic anemia
- Polychromasia, slight reticulocytosis and erythroblastemia
- Osmotic fragility of red cells is decreased
- TLC is usually normal.

Bone Marrow

- Stippling of erythroblasts
- Sideroblastosis.

Urine

- Increased excretion of coproporphyrin and ALA.

Laboratory Diagnosis of Hemoglobin Structure and Synthesis Disorders

Clinical finding
Ethnic origin of patient
} Suggest hemoglobinopathy

↓

Any family history
Preliminary blood studies
} Hemoglobin electrophoresis confirms

Special Tests

Tests Depending on Physiochemical Properties

- Sickle test, Hb solubility tests—HbS
- Intracellular Hb crystals—HbC
- HbH inclusions—Thalassemia
- Heinz bodies
- Heat instability test
- Isopropanol precipitation test—Unstable Hb
- Oxygen dissociation studies—High O_2 affinity Hb
- Alkali denaturation, acid elution test—HbF.

Hemoglobin Electrophoresis

The apparatus consists of two basic units:

1. The electrophoresis trough.
2. The voltage and amperage regulator. There are two troughs on either side containing the appropriate fluid.

The paper strips or agar gel covered slides are placed in the center of the instrument, and either end is connected to the respective trough with the help of wet filter paper strips—with one end dipping in the fluid and the other touching the gel or paper strip. This kind of connection is made on both the sides. The hemolysate is applied gently with the help of coverslip (broken) to get about 0.5–1 cm size. The positive and negative points are connected to the electrophoresis trough and then it is covered. Electrophoresis is allowed to continue for a specific time. The agar gel slides are dipped in amido black and left overnight for destaining in dilute acetic acid solution. The hemoglobin bands become clearly visible, the agar slide can be left for drying and a permanent record obtained. Having used the apparatus once, change the anode and cathode terminals for use next time. Most electrophoresis troughs are provided with water cooling systems, attach the inlet tube to a slow water stream from a tap and the outlet water to be drained into a waste sink.

Cellulose acetate electrophoresis done at pH = 8.6. Agar gel electrophoresis, using citrate buffer done at pH = 6.0. The mobilities of electrophoresis are presented in Figure 9.19.

(For further precise quantitations, electrophoresis can be done on starch gel or starch block)

Autoscanning and Computing Densitometer 205

Densitometer 205 is microprocessor based, designed to quantify electrophoretically separated bands (Fig. 9.19). The embedded software allows quantification of serum protein pattern by default, and any other multiband pattern (e.g. hemoglobin, lipoprotein, etc.) in an N-BAND mode. Postscan facilities like base line shifting, deletion of area, selection of minimas make the unit practically very useful and enhance its application in research-oriented studies.

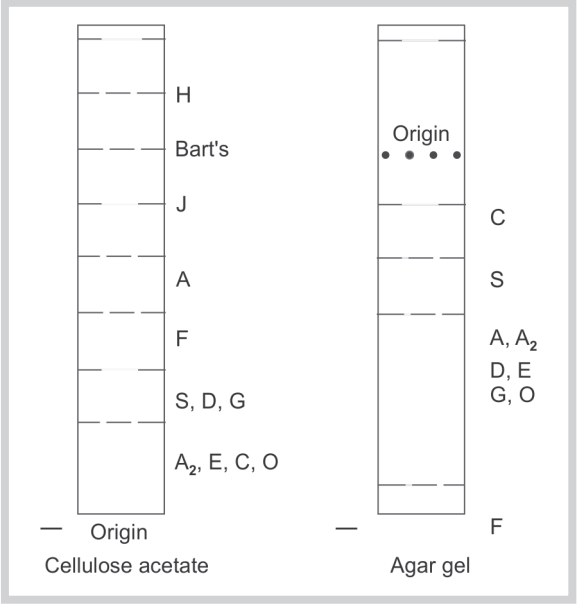


FIG. 9.19: Electrophoretic band separation

FIG. 9.20: Representative image of electrophoresis and densitometer apparatus

A special 'PC LINK 205' software (optional) links the Densitometer 205 to a PC. With this, densitograms can be conveniently stored/recalled/displayed/edited/compared on a CRT monitor using the keyboard of the PC (Fig. 9.20).

Features

- Quantification of five classical bands of serum protein by default
- N-Band (2–15) to quantify any pattern
- Accepts any multiband pattern on a transparent dry media
- Postscan EDIT facilitie
- Patient identification with date and time
- Hard copy of densitogram and/or computed values
- Optional 'PC-LINK 205' software.

Minimum System Configuration

- Systronics Densitometer 205
- 80 column dot-matrix printer (EPSON compatible).

System Configuration with 'PC-:LINK 205' Software

- Systronics Densitometer 205
- 80 column dot-matrix printer (EPSON compatible)
- Systronics PC-LINK 205 software
- PC (286, 386, 486 or Pentium or higher) with VGA/EGA color monitor.

Technical Specifications

Optical

Wavelength range:	400–700 nm
Filters:	520 nm, 600 nm (interference) and white light; another up to 5 filters optional
Light source:	6 V, 6 W tungsten lamp
Slit size (projected):	0.5 mm × 7 mm
Detector:	Silicon photodiode
Density range:	0 to 2 OD

Mechanical

Scanning length:	120 mm, programmable in steps of 0.1 mm
Scanning speed:	40 mm per minute
Carriage movement:	X-axis: automatic Y-axis: manual.
Scanning resolution:	10 readings per mm
Maximum pattern size:	L-120 mm, W-50 mm, H-5 mm

Electronics

Microprocessor:	8085 (Intel)
Display:	1. Segment LED display for set-up/programing interface. 2. 80-column dot-matrix printer for display of: a. Densitogram (with graduated X and Y axes to facilitate editing) b. Computed values c. Patient ID No. d. Date and time.

Computing and Editing

Input of date and program keys:	11 numerical and 13 functional soft-touch
Computations:	Proportional fraction in % and values with reference to total for: 1. Five classic bands serum proteins (by default). 2. N-band (2 to 15, selectable).
Editing:	1. Baseline shifting 2. Deletion of area. 3. Selection of minimas (rejection or introduction).

Printed Records

1. 5-band Serum: Protein mode
   a. Proportional fraction % of albumin, alpha-1, alpha-2, beta, gamma
   b. Protein values of albumin, alpha-1, alpha-23, beta, gamma with reference to total protein value
   c. Albumin/globulin ratio

   OR

   N-band mode:
   a. Proportional fraction % of N-bands
   b. Values with reference to total
2. Patient ID No: Up to 999.
3. Date and time: D/M/Y/and Hr/min.

Power

230 V ± 10%, 50 Hz.

Optional Accessories

i. RS 232 interface to communicate with external computer
ii. 'PC-LINK 205', software.

Laboratory Diagnosis of Sickle Cells Trait

1. Sickling tests (positive).
2. Solubility tests for HbS (positive).
3. Hb electrophoresis.

Laboratory Diagnosis of Sickle Cell Anemia

Blood Picture

1. Anemia—moderate to marked; normocytic, normochromic (MCV and MCH are normal).
   - Anemia occurs because of reduced RBC lifespan to about 8 days and in part due to ineffective erythropoiesis
   - Hemolytic and aplastic crises further reduce Hb concentration
   - Irreversibly sickled cells (ISC) have a life of 2 days
   - Osmotic fragility is decreased
2. ESR is decreased, sickling prevents rouleaux formation.
3. There is moderate to marked anisopoikilocytosis.
4. Serum and red cell folate values are decreased.
5. There is evidence of intravascular hemolysis.
6. Sickle and solubility tests are positive.
7. There is irregular distribution of HbF (acid elution test).
8. Hemoglobin electrophoresis shows increased HbS.

Laboratory Diagnosis of Unstable Hemoglobinopathy

Blood Picture

1. Mild to severe anemia:
   - Hemolytic anemias
   - Peripheral blood
   - Anisopoikilocytosis
   - Punctate basophilia, polychromasia
   - Reticulocytosis.
2. MCH is reduced
3. Red cell life is 20–30 days.

Special Tests

1. *Demonstration of Heinz bodies:* Preformed Heinz bodies cannot be shown by supravital staining except in 50% of splenectomized patients. Sterile incubation of affected red cells at 37°C for 24 hours leads to Heinz body formation.
2. *Heat instability test:* Incubate a fresh hemolysate with phosphate or tris buffer at 50°C for 1 hour—precipitation implies presence of unstable Hb.
3. *Isopropanolol precipitation test:* Incubate fresh hemolysate with isopropranolol tris buffer at 37°C for 1 hour, precipitation denotes presence of unstable Hb.
4. *Hemoglobin electrophoresis:* Abnormal Hb band (at pH = 8.6) in 50% cases.

THALASSEMIAS (REDUCED SYNTHESIS RATE)

Thalassemias are of two types:

- α, affecting mainly α chain
- β, affecting mainly β chain.

In β Thalassemia

There is reduced β chain production hence reduced HbA leading to microcytic, hypochromic anemia. Total Hb is maintained by increase in γ and δ chains so HbA_2 and HbF increase. Because of lack of β chains, α chains accumulate in cells forming aggregates → causing ineffective erythropoiesis.

In β Thalassemia

Level of HbA, A_2 and F are equally depressed leading on to microcytic, hypochromic anemia. In the absence of normal chains, β or γ chains increase and form HbH (β_4) or Hb Bart's (γ_4).

Laboratory Diagnosis of β Thalassemia Minor

Blood Picture

Hemoglobin is normal, MCV and MCH. MCHC is normal.

Peripheral Smear

- Microcytic, hypochromic cells; target cells
- Basophilic stippling, reticulocytosis (up to 6%)
- Osmotic fragility is decreased.
- Evidence of hemolysis
- HbA_2 increased, slight increase in HbF.

Laboratory Diagnosis of β Thalassemia Major

Blood Picture

(Resembles iron deficiency anemia)

- Marked microcytosis with hypochromia
- Moderate degree of anisopoikilocytosis, teardrop cells
- Target cells are prominent
- All; MCV, MCH and MCHC are diminished
- Normoblasts (intermediate/late) in peripheral smear.
- Granular inclusions in cytoplasm, represent α-chain aggregates which can be shown by methyl violet
- Polychromasia and moderate degree of punctate basophilia
- Reticulocytosis (10%)
- Leukocytosis (5,000–40,000) with shift to the left (immature forms seen)
- Platelet count normal but decreases with splenomegaly
- Osmotic fragility decreased, the curve has a tail because of a few very fragile cells
- Evidences of intravascular hemolysis.

Bone Marrow

- Erythroid hyperplasia
- Micronormoblastic reaction
- Increase in early and intermediate normoblasts
- Methyl violet positive inclusion bodies and PAS positive glycogen (PAS-periodic acid Schiff's reagent)
- Sideroblastosis, often ring sideroblasts.

Hb Pattern on Electrophoresis

- HbF (10–98%) estimated also by acid elution and alkali denaturation tests
- HbA (very little or absent)
- HbA_2 (variable).

Laboratory Diagnosis of HbH Disease

Blood Picture

- Marked microcytosis with hypochromia
- Target cells, fragmented cells and normoblasts in peripheral blood
- Reticulocytosis, basophilic stippling
- Numerous HbH inclusions.

Hb Pattern (Electrophoresis)

- HbH = 5–25%
- Remainder is HbA, A_2 and F.

Demonstration of HbH Inclusions

With a redox dye, e.g. brilliant cresyl blue (BCB) or new methylene blue (NMB); HbH being relatively unstable, precipitates and the red cells appear pitted with numerous inclusions (golfball appearance).

Hemoglobin Electrophoresis

At pH 8.6, both HbH and Hb Bart's are fast moving, they migrate in front of HbA towards anode.

Hb Bart's—Hydrops Fetalis

Blood Picture

- Marked anisopoikilocytosis with hypochromia
- Polychromasia
- Target cells and normoblasts.

Hb pattern

- Hb Bart's 80–90%
- Some HbH and Hb Portland
- No HbA, A_2 or F.

Laboratory Diagnosis of Aplastic Anemia

Blood Picture

- RBC morphology is usually normal (polychromasia, normoblasts and stippling usually not seen)
- Sometimes macrocytosis, mild anisopoikilocytosis
- Hb and PCV are diminished to about 7 g% and 20% respectively
- Red cell osmotic fragility is normal
- Leukopenia—neutropenia, polymorphs are qualitatively and quantitatively abnormal, show coarse granules
  - Absolute lymphocytopenia but relative lymphocytosis
  - Neutrophil alkaline phosphatase count is increased

- Thrombocytopenia—bleeding time is increased
- Clot retraction time is increased
- Coagulation parameters are abnormal
- Serum iron is increased
- Radio-iron bone marrow uptake is reduced
- Red cell life may be decreased
- Plasma erythropoietin level is raised.

Bone marrow may be:
- Aplastic
- Hypoplastic
- Normal or
- Patchily hypercellular.

Aspiration
- Blood tap or dry tap
- Is hypoplastic (there is both myeloid and erythroid hypoplasia)
- Fat cells are increased
- Plasma cells and reticulum cells are prominent
- Megakaryocytes are diminished in number
- WBCs may show maturation arrest
- Developing granulocytes are abnormal and may show:
  - Abnormal granulation or.
  - Vacuolization.
- Bone marrow iron may be normal or increased.

Hence, the diagnosis is based upon:
- Pancytopenia
- Rapid ESR
- No immature cells in peripheral smear
- Bone marrow is aplastic/hypoplastic (if 2 consecutive aspirations have been unsuccessful do a bone marrow trephine biopsy).

Classification and Causes of Aplastic Anemia

1. *Primary*
   - Idiopathic (cause unknown).
2. *Secondary*
   - Effects of chemical/physical agents on bone marrow.
3. *Miscellaneous*
   - Familial hypoplastic anemia (Fanconi's syndrome)
   - Aplastic anemia associated with
     - Infective hepatitis (1–2%) (usually post hepatitis A)
     - Pancreatic insufficiency
     - Paroxysmal nocturnal hemoglobinuria (PNH).
4. *Pure red cell aplasia*
   - Congenital (Diamond Blackfan type)
   - Acquired
     - With thymoma
     - Without thymoma. (In both primary and secondary aplastic anemia clinical and hematological features are similar)
     - *Primary type is less common.*

Secondary type occurs due to:
- Chemicals
  - Pancreatic
  - Industry
  - Domestic
  - Drugs.
- Physical
- Ionizing radiation.

Drugs

Especially those which have an amino group near a benzene ring.

Bone marrow depression depends upon:
- Dosage and period of treatment
- Idiosyncrasy, susceptibility and hypersensitivity to the drug.

Drugs which regularly cause aplastic anemia:
- Anticancer drugs.

Drugs which occasionally cause bone marrow depression:
(Either because of idiosyncrasy or hypersensitivity)
- Antiepileptics
  - Phenylhydantoin
  - Mesantoin
  - Paradione.
- Antibacterials
  - Chloramphenicol
  - Sulfas
  - Streptomycin
  - Chlortetracycline
  - INH.
- Tranquillizers
  - Chlordiazepoxide
  - Chlorpromazine.
- Antidiabetics
  - Tolbutamide
  - Chlorpropamide.
- *Antirheumatics*
  - Oxyphenylbutazone
  - Phenylbutazone
  - Indomethacin
  - Gold salts
  - Aspirin
  - Colchicine. (Commonest drugs—phenyl and oxyphenylbutazone and chloramphenicol).

Chemicals
- Benzene
- Lindane
- TNT
- DDT.

Physical Agents
- Ionizing radiation is most dangerous (e.g. X-ray, γ rays, neutrons).
- Particles have a limited range and hence cause effects only on entering the body.

Pancytopenia

Reduction in number of the three blood cells types RBCs, WBCs and platelets.

Blood Picture

Hb < 13.5 g% in males
< 11.5 g% in females
TLC < 4,000 cells/cu mm,
Platelet count < 1.5 lakh/cu mm.

Causes

- Subleukemic leukemia: Refractory anemia with medullary myeloblastosis
- Aplastic anemia
- Pancytopenia with hyperplastic bone marrow
- Bone marrow infiltration or replacement
- Hypersplenism
- Megaloblastic macrocytic anemia
- Systemic lupus erythematosus
- Disseminated tuberculosis.

NORMAL WHITE CELL VALUES AND PHYSIOLOGICAL VARIATIONS

Normal total leukocyte count = 4,000–11,000 cells/cu mm.

Total leukocyte count (TLC) undergoes minor physiological and diurnal variations. It increases slightly in the afternoon 'afternoon tide'. Various stimuli that may increase the count are:
- Food intake
- Physical exercise
- Emotion
- Pregnancy and following parturition.

Pathological Variations in White Cell Counts

Neutrophilia

Infections
- Pyogenic bacteria
  - Staphylococcal
  - Streptococcal
  - Pneumococcal
  - Meningococcal
  - Gonococcal
- Nonpyogenic
  - Acute rheumatic fever
  - Diphtheria
  - Scarlet fever
  - Acute poliomyelitis
  - Cholera
- Herpes zoster
- Mycobacterial
- Fungal
- Spirochetal
- Parasitic.

Metabolic Disorders—due to Varied Causes Leading to
- Acute yellow atrophy of liver
- Uremia
- Diabetes
- Acidosis
- Gout
- Eclampsia.

Neoplasms
- Myeloproliferative disorders
- Myeloid leukemia
- Lymphomas
- Polycythemia vera
- Myelosclerosis
- Other malignancies
  - Carcinomas (metastatic or otherwise)
  - Sarcomas.

Conditions Causing Cell Necrosis or Destruction
- Acute hemolysis (especially intravascular, type)
- Infarctions
- Drug intoxication
  - Nephrotoxins
  - Hepatotoxins.

Various Drugs/Chemicals Implicated are
- Phenacetin
- Digitalis
- Quinine
- Organic arsenicals
- Lead
- Mercury
- Carbon monoxide.

Trauma and Hemorrhage
- Hemorrhage
  - Acute hemorrhage (especially internal hemorrhage)

- Trauma
  - Operative
  - Fractures
  - Crush injuries
  - Burns.

Cardiac Disorders
- Paroxysmal tachycardia.

Collagen Diseases
- Polyarteritis nodosa
- Acute phases of:
  - Rheumatoid arthritis
  - Dermatomyositis.

Miscellaneous
- Serum sickness
- Acute anoxia
- Spider venom poisoning
- Histiocytosis-X.

Eosinophilia

Allergic States
- Asthma
- Hay fever
- Exfoliative dermatitis
- Erythema multiforme
- Urticaria
- Food sensitivity
- Angioneurotic edema
- Serum sickness
- Drug allergy.

Parasitic Diseases

Intestinal
- Hookworm
- Roundworm
- Tapeworm.

Tissue form
- Toxocara
- Trichina
- Strongyloides
- *Echinococcus*
- Filariasis
- Malaria.

Skin disorders
- Pemphigus
- Dermatitis herpetiformis
- Psoriasis
- Scabies
- Prurigo.

Drug Administration
- Liver extracts
- Penicillin
- Streptomycin
- Chlorpromazine.

Neoplasms
- Myeloproliferative
  - Eosinophilic leukemia
  - Chronic myeloid leukemia
  - Polycythemia.
- Others
  - Hodgkin's lymphoma
  - Multiple myeloma
  - Metastatic and necrotic tumors
  - Occult abdominal tumor.

Miscellaneous
- Familial eosinophilia
- Eosinophilic granulomatosis (visceral larva migrans)
- Eosinophilic syndrome
- Scarlet fever
- Polyarteritis nodosa
- Tropical eosinophilia
- Pernicious anemia
- Postsplenectomy
- Post-transfusion mononucleosis
- Idiopathic neutropenia.

Lymphocytosis

Acute Infections
- Infectious mononucleosis
- Infectious lymphocytosis
- Pertussis
- Mumps
- Chickenpox
- Rubella
- Infective hepatitis
- Convalescent stage of many acute infections
- Toxoplasmosis
- Influenza.

Chronic Infections
- Brucellosis
- Tuberculosis
- Syphilis (secondary).

Endocrine Disorders
- Thyrotoxicosis
- Adrenocortical insufficiency
- Hypopituitarism
- Myasthenia gravis.

Neoplasms
- Non-Hodgkin's lymphomas
- Chronic lymphatic leukemia
- Lymphosarcoma
- Multiple myeloma.

Monocytosis

Infections
- Bacterial
  - Brucellosis
  - Tuberculosis
  - Subacute bacterial endocarditis
  - Typhoid fever
  - Recovery stage of an acute infection.
- Rickettsial
  - Rocky mountain spotted fever
  - Typhus.
- Protozoan
  - Malaria
  - Kala-azar
  - Trypanosomiasis
  - Oriental sore.
- Viral
  - Infectious mononucleosis.

Neoplasms
- Monocytic leukemia
- Hodgkin's and other lymphomas
- Myeloproliferative disorders
- Multiple myeloma
- Carcinomatosis.

Collagen Diseases
- Rheumatoid arthritis
- SLE.

Miscellaneous
- Chronic ulcerative colitis
- Regional enteritis
- Sarcoidosis
- Lipid storage disease
- Hemolytic anemia
- Hypochromic anemia
- Recovery from agranulocytosis.

Basophilia

- Chronic myeloid leukemia
- Myelosclerosis
- Polycythemia vera
- Hypersensitivity states
- Myxedema
- Iron deficiency anemia (some cases)
- Hemolytic and toxic anemias of long standing
- Preleukemia (some cases).

Morphologic forms of Lymphocytes

Virocyte

(Also called stress lymphocytes, Downey type cells, or atypical lymphocytes)

1. These are small atypical cells that appear in viral diseases such as mononucleosis, viral hepatitis, viral pneumonia, and viral upper respiratory tract infections.
2. These may also be found in numerous nonviral conditions:
   a. Fungoid and protozoid nonviral conditions
   b. Autoimmune states
   c. Allergic reactions
   d. After transfusions and tissue graft.
3. When seen in stress response, these are called stress lymphocytes.
4. May be found in apparently healthy children.
5. Up to 10% of all lymphocytes, can be considered normal.

Transformed Lymphocytes

1. *Examples*
   a. Lymphocyte cells that may be seen in macroglobulinemia.
   b. Turk cells and Reider cells that are seen in acute lymphatic leukemia.
   c. Vacuolated lymphocytes that are seen in lipidosis.
2. Culturing of lymphocytes in laboratory:
   a. Stimulates small lymphocytes to transform into large atypical cells which produce immunoglobulin.
   b. Transformation response is impaired in culturing of lymphocytes from patients with:
      - Hodgkin's disease
      - Lymphatic leukemia
      - Lymphocytosis
      - Agammaglobulinemia.
   c. Transformation response increased in sarcoidosis.
3. Other uses of transformation test are to determine histocompatibility of recipient and donor for tissue grafts:
   a. Lymphocytes from donor not related to recipient stimulate the production of up to 3% of transformed lymphocytes in recipient.
   b. Lymphocytes from sibling react less strongly.
   c. No reaction occurs on cultures from fraternal twins.

Arneth Count

Neutrophils can be divided into five main groups according to the number of lobes in their nuclei.

Group 1 One lobe, even if it shows indentation and thinning out at one or more places.
Group 2 Two lobes connected by one thin filament.
Group 3 Three lobes connected by two thin filaments.
Group 4 Four lobes connected by three thin filaments.
Group 5 Five or more lobes connected by four or more thin filaments.

Using the above-mentioned classification, count 100 neutrophils and the number in each group is to be expressed as percentage. Usual normal values:

Group	1	2	3	4	5
Number	5	35	42	16	2

Interpretation: In acute infections, there is rapid turnover of neutrophils and in the process younger neutrophils with lesser number of lobes are released into circulation, thus increasing the number of cells in groups 1 and 2 (shift to the left).

In macrocytic megaloblastic anemias, the neutrophil production rate is slow, hence cells with hypersegmented nuclei are released into circulation (shift to the right).

However, Arneth count is now no longer in use.

Arneth Index

The percentage of cells in groups 1, 2 and ½ of 3 is about 60 (normal range 51–65). Only 2–5% fall in group 1.

Neutropenia and Agranulocytosis

Discussed elsewhere.

Lymphopenia

- Severe pancytopenia
- Congestive heart failure
- Adrenocorticosteroid therapy (transient)

Eosinopenia

Drug/Hormone Therapy

- Adrenocortical steroids
- Adrenaline
- Ephedrine
- Insulin.

Response to Stress

- Acute infections
- Traumatic shock
- Surgical operations
- Severe exercise
- Burns
- Acute emotional stress
- Exposure to cold.

Endocrine Diseases

- Cushing's disease
- Acromegaly
- Pheochromocytoma.

Miscellaneous

- Aplastic anemia
- Discoid lupuserythematosus.

Basophilopenia

- neutrophil leukocytosis or leukemoid reaction associated with:
  - Infection
  - Neoplasia
  - Tissue necrosis
  - Acute anemia
  - Allergic conditions
  - Hyperthyroidism
  - Myocardial infarction
  - Cushing's syndrome
  - Following prolonged corticosteroid therapy.

Leukemoid Reactions

Excessive leukocytic response to a stimulus and/or immature cell spilling over in peripheral blood.

Neutrophilic

- Hemolytic crises
- Hemorrhage
- Hodgkin's disease
- Infections
  - Tuberculosis
  - Other bacterial infections
  - Congenital syphilis
- Burns
- Eclampsia
- Mustard gas poisoning
- Vascular thrombosis and infarction
- Marrow replacement and myeloid metaplasia.

Lymphocytic

- Infectious lymphocytosis
- Infectious monocytosis

- Pertussis
- Varicella
- Tuberculosis.

Eosinophilic

- Visceral larva migrans.

Bone Marrow Plasmacytosis

Acute Infections

- Rubella
- Rubeola
- Varicella
- Infective hepatitis
- Scarlet fever.

Chronic Infections

- Tuberculosis
- Syphilis
- Fungal.

Allergic States

- Serum sickness
- Drug reactions.

Collagen—Vascular Disorders

- Acute rheumatic fever
- Rheumatoid arthritis
- Systemic lupus erythematosus.

Neoplasms

- Disseminated carcinoma
- Hodgkin's disease
- Multiple myeloma.

Others

- Cirrhosis of liver.

WHITE BLOOD CELLS

Neutropenia and Agranulocytosis

Neutropenia is the reduction in number of circulating neutrophils below 2500 cells/cu mm.

Blood Picture of Drug-induced Neutropenia

- Neutropenia with no anemia or thrombocytopenia
- In some cases, there may be lymphopenia and monocytopenia also
- Neutrophils may show toxic and degenerative changes
- ESR is usually raised.

Bone Marrow

- Absence of granulocytic precursors with normal erythropoiesis and a normal number of megakaryocytes (sometimes depleted)
- Toxic granulation in developing granulocytes
- Granulocytic hyperplasia implies recovery.

Causes of Neutropenia

Drugs

1. *Drugs that cause aplastic anemia.*
2. *Drugs that induce selective neutropenia:*
   Antipyretic analgesics—Amidopyrine.
   Antithyroid drugs—Thiouracil, methimazole, carbimazole.
   Antihistamines—Promethazine, chlorpheniramine, mepyramine, etc.
   Tranquillizers and antidepressants —Chlorpromazine, meprobamate, imipramine, amitri-ptiline, etc.
   Antibacterials—Tetracycline, streptomycin, ristocetin, salazopyrin, sodium methicillin, etc.
   Anticoagulants—Phenindione, dicoumarol.
   Antituberculars—Isoniazid, PAS, thiacetazone.
   Antimalarials—Primaquine, amodiaquin.
   Miscellaneous—Procainamide, penicillamine, metronidazole, etc.

Other Causes of Neutropenia

1. *Chronic idiopathic neutropenia (agranulocytosis)* neutrophil count = 500–2000 cells/cu mm— absolute or relative lymphocytosis.
2. Infections
   - Acute viral
     - Rubeola
     - Hepatitis.
   - Bacterial
     - Typhoid
     - Brucellosis
     - Rickettsial
     - Protozoan—malaria
     - All grave infections
   - Bacteremia
   - Miliary tuberculosis.
3. *Marrow aplasia:* All causes of aplastic anemia.
4. *Due to known cause or myelophthisis*
   - Leukemia
   - Neoplasia.
5. *Nutritional deficit*
   - Folic acid or Vitamin B_{12} deficiency causes megaloblastic or macrocytic anemia also.

6. *Hypersplenism:* Congestive or infiltrative.
7. *Miscellaneous*
   - SLE
   - Anaphylaxis
   - Antileukocyte antibodies
   - Immunodeficiencies
   - Pancreatic exocrine deficiency
   - Cyclic neutropenia (familial/sporadic).

Symptomatic neutropenia occurs usually in aplastic anemia, drug-induced neutropenia, hypersplenism and idiopathic neutropenia, and acute leukemia.

Laboratory Diagnosis Of Infectious Mononucleosis

Blood Picture

- There is both absolute and relative lymphocytosis, large numbers of them atypical
- Hemoglobin value and platelet counts are normal
- Initial leukopenia due to reduction in number of neutrophils, the neutrophil alkaline phosphatase count is often low
- Lymphocytosis is maximum at about the tenth day
- Lymphocytosis (atypical) as described by Downey and Mckinley
  - Type I—Monocytoid lymphocytes
  - Type II—Plasmacytoid lymphocytes
  - Type III—Blastoid lymphocytes
- ESR is raised in 50% cases
- Wasserman reaction may be positive in 3–10% cases
- ELISA test is available
- Latex and particle agglutination tests are also available.

Paul Bunnel Test for Heterophile Antibody

This is based upon the presence of antisheep red cell hemagglutinins in unusually high titers in the sera of these patients. It is positive in about 80–90% of cases. It remains positive for a variable period of time. In addition to the said antibodies, at least 2 other types of agglutinin for sheep red cells occur in human serum. They are:

1. An antibody present in low titers in normal persons and in malignant lymphomas. This antibody is absorbed by guinea pig kidney but not by ox cells.
2. An antibody occurring following the injection of horse serum and in serum sickness. This is absorbed both by guinea pig kidney and ox cells. This antibody of infectious mononucleosis is not absorbed by guinea pig kidney but is absorbed by ox cells.

Lupus Erythematosus (LE) Cell/Phenomenon

Method

1. Draw 5–10 mL of venous blood. Place in a 50 mL flask containing 20–30 glass beads 3–5 mm in diameter or clear metal paper clips. Swirl or shake gently for 10–15 minutes to defibrinate the blood.
2. Let stand 15 minutes (preferably at 37°C).
3. Transfer blood and a few beads to a test tube or container and mix on a rotator or by inverting for 30 minutes.
4. Let stand at room temperature (preferably at 37°C) for 1 hour.
5. Centrifuge at 2000–3000 rpm for 5–10 minutes.
6. Transfer the buffy coat to Wintrobe's hematocrit tube and centrifuge again for 5–10 minutes.
7. Transfer the buffy coat and an equal volume of plasma to a small tube, mix well, and prepare smears. Dry rapidly and stain with Giemsa's or Leishman's stain.
8. Examine smears for clumps of platelets and neutrophilic leukocytes where the typical LE cell is most likely to be found. Look for neutrophils containing ingested homogeneous blue to magenta colored bodies (LE cell) or a group of neutrophils encircling (garlanding) such a body (LE phenomenon).

It should be differentiated from 'tart cell' in which though neutrophil may show an inclusion that is not homogeneous and is of the same color and appearance of their nuclei.

Interpretation: Positive LE cells in blood are found in:

- Systemic lupus erythematosus (70–80%)
- Rheumatoid arthritis (10%)
- Occasionally other collagen disorders
- Malaria
- Drug induced, e.g. hydralazine and procainamide.

Classification of Acute Myelomonocytic Leukemias

FAB Classification

M1. *Myeloblastic leukemia without maturation:* Non-granular blasts with occasional Auer rods or azurophilic granules, 3% or more are myeloperoxidase positive; no maturational changes.

M2. `*Myeloblastic leukemia with maturation:* Maturation to promyelocytic stage: 50% of marrow cells are blasts or promyelocytes, later stages variably present, often with bilobed nuclei, Pelger-Huet anomaly or decreased granulation.

M3. *Hypergranular promyelocytic leukemia:* Predominant cell is heavily granulated promyelocytes; bundles of Auer rods common in cytoplasm or free on smear.
M4. *Myelomonocytic leukemia:* Promonocytes and monocytes comprise 20% of nucleated cells in marrow, blood or both; myeloblasts plus promyelocytes are 20% of marrow cells; monocytic cells have strong nonspecific esterase reaction inhibited by fluoride; esterase activity in myelocytic cells persists after fluoride exposure.
M5. *Monocytic leukemia:* Granulocytic cells less than 10% differentiated and poorly differentiated subtypes depend on degree of maturation; esterase reaction inhibited by sodium fluoride.
M6. *Erythroleukemia:* Marrow has 50% erythropoietic forms, often with bizarre morphology or megaloblastic changes (show PAS+ve granules), myeloblasts and promyelocytes 30% or more; abnormal megakaryocytes present.

Laboratory Diagnosis of Leukemias

Cytochemical Methods for Staining Leukocytes

Neutrophil Alkaline Phosphatase (Kaplow's Method)

Principle: The enzyme, located in the neutrophil specific granules, is exposed to the substrate (a naphthol phosphate) in the presence of a diazonium salt (fast blue or fast violet) at an alkaline pH 9.5. The substrate is hydrolyzed by the enzyme, releasing a phosphate and an aryl naphthol amide. The latter is immediately coupled to the diazonium salt, forming an insoluble azo dye.

Reagents

Fixative: 10% formalin in absolute methanol. To 10 mL 37% formaldehyde, add 90 mL absolute methanol. Store at 10 to 20°C.

Buffer stock: 0.2 M propanediol. Dissolve 21 g of 2-amino-2 methyl-1, 3-propanediol in distilled water and dilute to 1000 mL. Store at 4°C.

Working: 0.05 M propanediol pH 9.4 to 9.6. Add 70 mL 0.1 N HCI to 250 mL of stock buffer and dilute to 1000 mL with distilled water. Store at 4°C.

Substrate mixture: Dissolve 5 mg of naphthol ASBI phosphate or naphthol AS-MX phosphate or naphthol AS phosphate in 0.2 to 0.3 mL dimethyl formamide in a dry flask and add 60 mL of 0.05 M propanediol buffer and 40 mg of fast blue salt RR, BB, or BBN (or fast red violet LB). Shake well, filter into a Coplin jar and use immediately.

Counterstain: Mayer's hematoxylin. Add 1 g hematoxylin to 500 mL distilled water. Heat just to boiling and add another 500 mL distilled water. Add 0.2 g sodium iodate and 50 g of aluminum potassium sulfate. Shake well, filter and store in brown bottle at room temperature.

Procedure

Use freshly made blood films. If venous blood is used, heparin should be the anticoagulant, as the enzyme activity diminishes rapidly in EDTA.

Fix air dried blood films in 10% formal methanol for exactly 30 seconds at 0 to –10°C.

Wash in gently running tap water for 30 to 60 seconds.

Air dry slides, then place them in substrate mixture for exactly 10 minutes. Wash in gently running tap water again for 30 to 60 seconds.

Counterstain for 6 to 8 minutes in filtered Mayer's hematoxylin.

Wash in running tap water for 2 minutes. Air dry. Positive controls are run with each batch of slides. Women in last trimester of pregnancy are good controls, because their scores are high normal or somewhat increased.

Scoring procedure: Examine 100 mature neutrophils in the thin part of the film, where red cells barely touch each other and score each as follows:

Unstained cells	0
Cells stained faintly diffusely, or a few discrete granules	1
Cells with moderate number of granules	2
Cells with granules filling the cell	3
Cells staining deeply, almost obscuring the nucleus	4

Adding the scores for 100 cells can give a possible range of 0 to 400. The normal range with this method is 20 to 100.

The NAP scores are raised in:

- Bacterial infections
- Myocardial infarction
- Trauma
- Diabetic acidosis
- Polycythemia vera
- Myelosclerosis
- Following corticosteroid therapy
- During pregnancy
- Use of oral contraceptives.

The NAP scores are lowered in:

- Chronic myeloid leukemia } always
- Paroxysmal nocturnal hemoglobinuria } always
- Idiopathic thrombocytopenic purpura } sometimes
- Infectious mononucleosis } sometimes
- Pernicious anemia relapse } sometimes
- Collagen disorders and refractory anemias } sometimes
- Hypophosphatemia. } sometimes

FAB classification of lymphoblastic leukemias			
Observation	*L1*	*L2*	*L3*
Consistency of appearance	Homogeneous	Heterogeneous	Homogeneous
Cell size	Uniformly small	Large, but variable	Uniformly large
Nuclear shape	Regular, little clefting	Irregular, clefted, indented	Regular, rounded
Nucleoli	None or inconspicuous	One or more large	One or more, prominent
Amount of cytoplasm	Scant	Variable, often abundant	Abundant
Other findings	T d T* usually	T d T usually	May have B cell
		Myeloperoxidase negative	markings
(T d T—Terminal deoxynucleotidyl transferase)			

Peroxidase (Myeloperoxidase, Kaplow's Method)

Principle: In the presence of hydrogen peroxide, peroxidase in leukocyte granules oxidizes benzidine from a colorless form to blue or brown derivative, which is localized at the site of the enzyme.

Reagents

Fixative: Mix 10 mL of 37% formaldehyde with 90 mL of absolute ethanol:

Incubation mixture

Ethanol 30% (v/v) in water	100 mL
Benzidine dihydrochloride	0.3 g
Zn SO_4. $7H_2O$, 0.132M (3.8% w/v)	1.0 mL
Sodium acetate ($Na_2C_2H_3O_2$. $3H_2O$)	1.0 g
3% hydrogen peroxide	0.7 mL
Sodium hydroxide, 1.0 N	1.5 mL
Safranin O	0.2 g

Reagents are mixed in the stated order. A precipitate forms after adding zinc sulfate but dissolves after other reagents are added. The pH is not critical between 5.8 and 6.5. The mixture is filtered and may be kept in a closed container and reused for a period of several months.

Procedure

Freshly made films or imprints are used. Peroxidase is unstable in the light, but unfixed films are satisfactory for as long as 3 weeks if kept in the dark. Heparin/oxalate/EDTA can be used as an anticoagulant.

Place slides in fixative for 60 seconds at room temperature. Wash in gently running tap water.

Place slides in incubation mixture for 30 seconds at room temperature. Wash in gently running tap water for 30 to 60 seconds.

Allow slides to dry, and examine under the microscope.

The slides may be counterstained with Wright's stain or with 1% aqueous cresyl violet if greater nuclear detail is wanted.

Interpretation

Peroxidase activity is indicated by blue granules in the cytoplasm. The nucleus and background cytoplasm stain red.

In neutrophilic series peroxidase becomes positive in late myeloblasts and on till mature neutrophil. In eosinophils specific granules contain peroxidase. Basophils, lymphocytes and erythroid cells do not stain. Monocytes stain less intensely than do neutrophils, and the granules are smaller.

Peroxidase reaction is used in differentiating acute myeloblastic leukemia (+ve) from acute lymphoblastic leukemia (–ve). It parallels Sudan Black B reaction; Auer rods are positive with both.

Peroxidase activity may be absent in some toxic neutrophils in infection.

Periodic Acid-Schiff (PAS) Reaction

Principle: Periodic acid (HIO_4) is an oxidizing agent that converts hydroxy groups on adjacent carbon atoms to aldehydes. The resulting dialdehydes are combined with Schiff's reagent to give a red colored product. A positive reaction is, therefore, seen with polysaccharides, mucopolysaccharides, and glycoproteins.

Reagents

- *Fixative:* Mix 10 mL of 37% formaldehyde with 90 mL of absolute ethanol
- Periodic acid, 5 g, is dissolved in 500 mL of distilled water. Stored in dark bottle and is good for 3 months
- *Schiff's reagent:* Dissolve 5 g of basic fuchsin in 500 mL of hot distilled water and filter after it has cooled. Saturate with sulfur dioxide gas by bubbling for 1 hour. Extract the

solution with 2 g of activated charcoal for a few seconds in a hood and immediately filter through Whatman No. 1 filter paper into a dark bottle. The solution is kept for 2 to 3 months.
- Harris hematoxylin.

Procedure
- Place air-dried blood and marrow films or imprints in fixative for 10 minutes. Wash briefly with tap water
- Control slides are exposed to digestion with saliva (diastase) for 30 minutes. Place slides in periodic acid for 10 minutes. Wash briefly with tap water and blot dry
- Immerse slides in Schiff's reagent for 30 minutes
- Rinse slides in several changes of sulfur dioxide water for 20 to 30 minutes
- Wash for 5 to 10 minutes in tap water and counterstain with Harris hematoxylin for 10 minutes.

Interpretation
In blood cells a positive PAS reaction usually indicates presence of glycogen. This is shown by digestion with diastase and consequent loss of staining.

Neutrophils react at all stages of development, the most strongly in the mature stage (similarly for eosinophils). The glycogen is not in the granules but in the background cytoplasm. Myeloblasts contain a few small PAS-positive granules. Monocytes have a faint staining reaction in the form of fine granules. Lymphocytes may contain a few small or large granules. Normoblasts are normally PAS negative.

In erythroleukemia and in thalassemia some of the erythroid precursors are PAS positive. In acute lymphoblastic leukemias, the lymphoblasts often contain large coarse clumps of PAS positive material (block positivity).

Sudan Black B Stain (Sheehan and Storey)

Principle: Sudan black B stains phospholipids and other lipids. It appears to stain both azurophilic and specific granules in neutrophils, whereas the peroxidase is found only in azurophilic granules. In early forms, late myeloblasts and early promyelocytes, the Sudan black B reaction is therefore, parallel to the peroxidase in its utility in separating acute lymphoblastic from acute myeloblastic leukemia.

Reagents
Stock solution of stain: Dissolve 0.3 g of Sudan black B powder in 100 mL ethyl alcohol.

Buffer solution: Dissolve 16 g crystalline phenol in 30 mL ethyl alcohol. Add this to a solution of 0.3 g hydrated disodium hydrogen phosphate (Na_2HPO_4. $12H_2O$) dissolved in 100 mL distilled water.

Working Stain Solution
Add 40 mL buffer solution to 60 mL stock stain solution. Filter using suction. This is stable for approximately 2 months.

Procedure
- Fix air dried films in formalin vapor for 10 minutes. Slides need not be freshly made.
- Wash slides in running tap water for 10 minutes
- Place slides in working stain solution (in Coplin jar) for 60 minutes
- Wash slides with 70% ethyl alcohol for 2 to 3 minutes to remove excess dye
- Wash slides in tap water for 2 minutes
- Allow slides to dry. Counterstain slides with Wright's stain or hematoxylin.

Interpretation
Cytoplasmic granules stain faintly in neutrophil precursors and strongly in mature neutrophils with a brown black color. Eosinophilic granules are brown but often show a central pallor. Monocytes have scattered fine brown-black granules. Lymphocytes and lymphoblasts are negative, but at least some myeloblasts contain Sudan black positive granules.

The peroxidase and Sudan black B reactions show roughly similar patterns in the various cell types. These techniques are most useful in distinguishing myeloblasts from lymphoblasts when large numbers of primitive blast forms are present in acute leukemias.

Nonspecific Esterase (Yam et al)

Alpha-naphthol acetate esterase.

Reagents
Fixative: Buffered formalin and acetone.

Formaldehyde, 37%; 25 mL, $Na_2 HPO_4$, 20 mg, KH_2PO_4 100 mg; distilled water, 30 mL; acetone 45 mL.
Buffer: Sorensen's phosphate buffer (M/15. pH = 7.6)

Incubation mixture: Add in the following manner:
- Buffer 44.5 mL.
- Hexazotized pararosaniline 3.0 mL
- Alpha-naphthol acetate 50 g dissolved in 2.5 mL ethylene glycol monomethyl ether
- Filter mixture through Seitz filter
- Harris hematoxylin.

Procedure
- Place air dried blood or marrow films in fixative for 30 seconds at 4°C. Wash in running tap water
- Place slides in incubation mixture for 60 minutes. Wash in running tap water
- Counterstain with Harris hematoxylin for 10 minutes.

Interpretation

Alpha-naphthol acetate esterase activity is found in monocytes but not in neutrophils or neutrophil precursors, other granulocytes or lymphocytes. It may be found, however, in activated or atypical lymphocytes, in imprints of active lymphoid tissue, and probably in the poorly differentiated lymphocytes of some lymphomas.

Acute Leukemias: Laboratory Diagnosis

Routine Hematologic Investigations

- A normocytic, normochromic anemia
- The white cell count may be decreased, normal or increased up to 2 lakh/cu mm
- Thrombocytopenia occurs in most cases, often extremely low in AML

Blood Film Examination

Shows variable numbers of blast cells. In AML the blasts may show Auer rods and other abnormal cells may be present, e.g. promyelocytes, myelocytes, agranular neutrophils, pseudopelger cells, myelomonocytic cells. In erythroleukemia, many normoblasts may be seen but these may be seen in smaller numbers in the other forms. The differentiating features of various blast cells have been discussed elsewhere. In leukemias one may see typical and atypical blasts.

Bone marrow is hypercellular with a marked proliferation of leukemic blast cells, which typically amount to over 75% of the marrow cell total. In ALL marrow may be difficult to aspirate due to increased reticulin fiber.

Differentiation of ALL from AML

In most cases, the clinical features and morphology on routine stains separate ALL from AML. In ALL blasts, show no differentiation whereas in AML some evidence of differentiation, to granulocytes is often seen in the blasts of their progeny. Special cytochemical staining techniques just described are needed when cells are undifferentiated.

Cytochemistry	ALL	AML
Peroxidase	—	+ (including Auer rods)
Sundan black B	—	+
Nonspecific	—	+ (in monoesterase cytic types)
PAS	+	(coarse) + (fine)
Acid phosphatase (T cell ALL)	—	

Other Investigations

Tests for disseminated intravascular coagulation (DIC) are positive in promyelocytic leukemia. Lumbar puncture shows raised spinal fluid pressure, it contains leukemic cells in patients with meningeal leukemia.

Chronic Myeloid Leukemia

Laboratory Investigations

Diagnostic Features

- Leukocytes usually > 50,000/cu mm and sometimes > 5 lakh/cu mm
- A complete spectrum of myeloid cells in the peripheral blood. The levels of neutrophils and myelocytes exceed those of blast cells and promyelocytes.

Additional Features

- Philadelphia chromosome on cytogenetic analysis of blood or bone marrow
- Bone marrow is hypercellular with granulopoietic predominance (especially myelocytes)
- Neutrophil alkaline phosphatase score invariably low
- Increased circulating basophils
- Normocytic, normochromic anemia
- Platelet count is usually increased but may be normal or decreased
- Serum vitamin B_{12} and vitamin B_{12} binding capacity are increased
- CML is said to be undergoing blast transformation when percentage of myelocytes and promyelocytes exceeds 20% in peripheral smear or 30% in bone marrow.

Chronic Lymphocytic Leukemia (CLL)

Laboratory Findings

- *Leukocytosis:* The absolute lymphocyte count is above 5000/cu mm and in the majority of patients it is 30–3000 × 10^9/L. Between 70 and 99% of white cells on blood film appear as mature lymphocytes. Smudge or smear cells are also present
- Normocytic, normochromic anemia in later stages
- Thrombocytopenia occurs in many patients.

Bone marrow aspiration: Shows lymphocytic replacement of normal marrow elements. Lymphocytes comprise 25–95% of all the cells.

Reduced concentration of serum immunoglobins: They are found in most cases, particularly with advanced disease.

Leukemia can be differentiated from leukemoid reaction: By taking into consideration, the clinical picture, neutrophil alkaline phosphatase score (high or normal in leukemoid reaction but low in leukemia). In addition, cytochemical stains may be used for differentiation.

Paraproteinemias

Causes

- Multiple myeloma
- Macroglobulinemia
- Malignant lymphoma
- Chronic lymphocytic lymphoma
- Benign monoclonal gammopathy
- Chronic cold hemagglutinin disease
- Rarely with carcinomas.

Multiple Myeloma

Multiple myeloma (can be solitary myeloma called plasmacytoma in extramedullary sites) is a neoplastic proliferation of plasma cells. Characterized by lytic bone lesions, plasma cell (myeloma cell) accumulation in the bone marrow and the presence of monoclonal protein in serum and in about half the cases in urine also.

Laboratory Diagnosis

1. In about 98% cases, monoclonal protein occurs in serum and/or urine. Incidence of serum paraprotein
   IgG 66%
   Ig A 33%
   Ig M or
   Ig D 1%.
   The normal immunoglobulins are depressed. The Bence-Jones proteins found in urine are free light chains, either kappa or lambda of the same type as serum paraprotein.
2. The bone marrow shows more than 10% plasma, cells and often with abnormal 'myeloma cells'.
3. 60% of patients have osteolytic areas 20% show generalized bone rarefaction or osteoporosis, which may cause pathological fractures. 20% show no such lesions. Most often two of the three diagnostic features stated above are present.

Other Laboratory Findings

1. Normochromic, normocytic anemia is usual. Marked rouleaux formation is seen. Neutropenia and thrombocytopenia seen in advanced cases. There may be myeloma cell spillover in the peripheral blood or there may be (very rare) plasma cell leukemia. Leukoerythroblastic changes (immature cells of both myeloid and erythroid series in the peripheral blood) are occasionally found.
2. About half the cases have raised serum calcium levels and elevated serum alkaline phosphatase.
3. ESR is markedly raised.
4. Renal damage leads to raised blood urea and serum creatinine levels.
5. Serum albumin is low.

The monoclonal peak (M peak, spike) is found by immunological and electrophoretic techniques. Immunoglobulins IgG, IgM and IgA can be measured quantitatively by using immunoturbidimetric or nephelometric kits available for estimation of the immunoglobulins IgG, IgM and IgA separately.

Polycythemia Vera

Laboratory Diagnosis

1. Raised red cell count, hematocrit and hemoglobin.
2. Anisocytosis and poikilocytosis in late stages.
3. Neutrophilic leukocytosis (50% cases), in some cases basophilia.
4. Raised platelet count (50% cases).
5. Reticulocyte count raised.
6. Raised neutrophil alkaline phosphatase score.
7. Increased vitamin B_{12} binding capacity.
8. Bone marrow is hypercellular (generalized hypercellularity), with prominent megakaryocytes. Storage iron diminished Reticulin diminished (late stages).
9. Serum uric acid may be raised, serum iron— histamine levels (blood and urine)—arterial oxygen saturation normal (92%).

Myelosclerosis

Laboratory Diagnosis

1. Anemia is usual.
2. At the onset, white cell and platelet counts are frequently high but later leukopenia and thrombocytopenia are common.
3. A leukoerythroblastic blood picture is seen. The red cells characteristically show 'tear drop' poikilocytes.
4. Bone marrow is usually unobtainable by aspiration. A trephine biopsy may show a hypercellular marrow with an increase in reticulin pattern. Increased megakaryocytes are frequently seen. In some cases, there is increased bone formation.
5. Low serum folate, raised serum vitamin B_{12}, and raised vitamin B_{12} binding capacity, increased neutrophil alkaline phosphatase.
6. High serum urate, LDH and hydroxybutyrate dehydrogenase levels reflect the increased but largely ineffective turnover of hemopoietic cells.

7. Extramedullary hemopoiesis may be documented by radioiron studies, by liver biopsy or splenic aspiration.

Hodgkin's Disease

This is a neoplastic disorder of lymphoreticular tissue and four morphologic types are known, viz.

1. Lymphocyte predominance (5–13%).
2. Nodular sclerosis (40–50%).
3. Mixed cellularity (35–40%).
4. Lymphocyte depletion (5–10%).

Laboratory Findings in Hodgkin's Disease

Early in Course

- Mild normocytic, normochromic anemia; from depressed erythropoiesis
- Moderate leukocytosis, with eosinophilia up to 10%
- Normal or increased platelet count
- Increased ESR
- Decreased serum iron and iron-binding capacity; normal or ↓ marrow iron
- Decreased cell-mediated immunity, antibody activity normal.

Later in Disease

- Lymphopenia
- More severe anemia
- Coombs' positive hemolysis (relatively rare)
- Thrombocytopenia
- Mild hypoalbuminemia, hyperglobulinemia
- Hypercalcemia
- Hyperuricemia
- Low serum zinc, high serum copper.

QUALITY CONTROL IN HEMATOLOGY

Quality control in medical laboratories encompasses a set of procedures, which ensure that reliable and timely test results are received by the users of laboratory service. Reliability implies both precision and accuracy.

There are four components of quality assurance program:

- Internal quality control (IQC)
- External quality assurance (EQA)
- Standardization
- Proficiency surveillance.

Internal Quality Control

Since now most of the laboratories are dependent on automated machine, it has become extremely important to maintain good internal quality control, which is done by:

Testing Control Sample

The best-known method is testing a control sample along side the routine specimen in each batch of test. Control material is either obtained commercially or prepared individually, but its stability and homogeneity should be ensured.

Control Chart (Levy-Jennings or L-J Chart)

In this process when a batch of samples is dispensed (after being run along a control sample), the mean and standard deviation of each diameter is obtained and linear graphs are ruled, showing the +2 standard deviation (SD) limits. Statistically, not more than 1 in 20 samples should fall outside these limits if the system is in control.

Cusum Analysis

Cumulative Sum (Cusum) Charting was introduced in 1960s. Deviation from the largest is plotted in a cumulative manner so that each point represents the sum of all the deviations to date from the mean or target value. This method of plotting exaggerates trends in data and makes shifts of the mean much more obvious than by other plots. The rules for using the Cusum system for quality control are less well defined than for the L-J system.

Duplicate Tests

A well-known method for checking precision in clinical analysis is duplicate testing. In this process, a few of the specimens that were measured in an earlier batch, are rechecked with the next batch control.

Inbuilt Quality Control

This includes:

- These of cumulative reports of a single patient
- Clinical correlation: If a physician can not interpret a report on clinical grounds, a repeat test with a fresh specimen is indicated
- Red cell indices: If reports are giving erroneous rise or fall in the red cells indices, this usually points to an error in analysis
- Blood film examination ultimately helps in double checking the analysis done by the instrument.

External Quality Assessment

The college of American Pathologist first introduced "proficiency testing" survey program in 1960. In the late 60s, the British Committee for Standards in Hematology, finally developed the National External Quality Assessment

Scheme (NEQAS) for Hematology. Such methods are used by various laboratories all over the world to keep up with international standards.

Standardization

Modern diagnostic systems depends on a calibration procedure for accurate performance. Calibrators or testing standards are commercially prepared products, made by a direct comparison with a primary international standard. They are used for accuracy and interlaboratory harmonization of test results. The calibrator has an assigned value as close to the true value as can be established.

The WHO (World Health Organization) provides a wide range of biologically important international reference standard material. Some examples of these which are available for use in hematology are:

a. Hemoglobin preparation
b. Hemoglobin A_2 and F
c. Thromboplastin
d. Blood type sera
e. Various coagulation factors.

Proficiency Surveillance

This is concerned with the pre-analytical parts of the process that require control, if tests are to be reliable and effective. This involves following a standard guideline at various steps of a laboratory analysis.

The steps are:

1. Standard of blood collection tube
2. Phlebotomy technique
3. Identification of sample with special reference to hazardous specimens
4. Maintenance of transportation standards
5. Data processing of results
6. Establishing normal reference values, assessment of the significance of results and taking decisions for further tests.

Technical Proficiency has always been the corner stone of the laboratory, but in recent years with the advent of sophisticated instruments and automation, quality control has assumed an even more important role in good laboratory practice. It is the duty of the laboratory staff to ensure that the tests, which are carried out, are appropriate and to provide reliable analytical results.

CHAPTER 10

Clinical Hematology: Bleeding Disorders

PLATELETS, COAGULATION AND BLEEDING DISORDERS: LABORATORY INVESTIGATIONS

Platelet Count—Dealt in Depth Elsewhere

Capillary Fragility Test of Hess (Rumpel-Leede Sign, Tourniquet Test)

1. Inflate sphygmomanometer cuff around arm at 80 mm of Hg pressure for 5 minutes.
2. Look for petechiae in an area 5 cm in diameter just below the elbow.
3. Under normal circumstances the number of petechiae should be less than 5, more than 5 indicate a positive test.

A positive test may be found in reduced capillary resistance (or increased capillary fragility) as in non-thrombocytopenic purpura and scurvy. It may also be positive in thrombocytopenia when the platelet count is below approximately 70,000 mm^3 of blood.

Clinical Implications

1. Increased petechiae formation occurs most commonly in thrombocytopenia and less commonly in: (i) thrombasthenia, (ii) vascular purpura, (iii) senile purpura, and (iv) scurvy.
2. The number and size of petechiae are roughly proportional to the bleeding tendency and possibly to the degree of thrombocytopenia. However, the test can be positive because of capillary fragility in the presence of normal platelet count.
3. Results will be normal in coagulation disorders and vascular disorders.

Laboratory Diagnosis of Vascular Bleeding Disorders

Hess's test is positive in these

Causes and classification

1. *Hereditary*
   - Hereditary hemorrhagic telangiectasia.
2. *Acquired*
   - Simple easy bruising
   - Senile purpura
   - Purpura of infections
   - Henoch-Schonlein syndrome
   - Scurvy
   - Steroid purpura.

Interfering Factors

1. *Menstruation:* Capillary fragility is normally increased before menstruation.
2. *Infectious disease:* Capillary fragility is increased in measles and influenza.
3. *Age:* Women over 40 years with decreasing estrogen levels may have a positive test that is not indicative of a coagulation disorder.
4. *Readministration:* Repetition of test on same arm within 1 week of the first test may lead to error.
5. *Variation:* Results may vary because of differences in texture, thickness, and temperature of the skin.

LABORATORY DIAGNOSIS OF PLATELET DISORDERS

Idiopathic Thrombocytopenic Purpura (ITP)

1. Platelet count is usually 10-50 × 10^9/L.

2. The blood film shows reduced numbers of platelets, those present are often large.
3. The bone marrow usually shows increased number of megakaryocytes.
4. Sensitive tests can demonstrate antiplatelet IgG, either alone or with complement, on the platelet surface or in the serum in most patients.
5. Autologous platelet survival studies with ^{51}Cr or $DF^{32}P$-labeled platelets may be used to show reduced platelet survival. In severe cases, the mean platelet survival may be reduced to one hour.
6. Hess's test may be positive in some cases.

Drug Induced Immune Thrombocytopenia

1. Thrombocytopenia. Platelet count is often $<14 \times 10^9$/L.
2. Bone marrow may show normal or increased numbers of megakaryocytes.
3. Drug dependent antibodies against platelets may be demonstrated in sera of some patients.
   Drugs usually incriminated are:
   - Quinine
   - Quinidine
   - Sulfonamides
   - PAS
   - Rifampicin
   - Stibophen
   - Digitoxin, etc.

Disseminated Intravascular Coagulation (DIC)

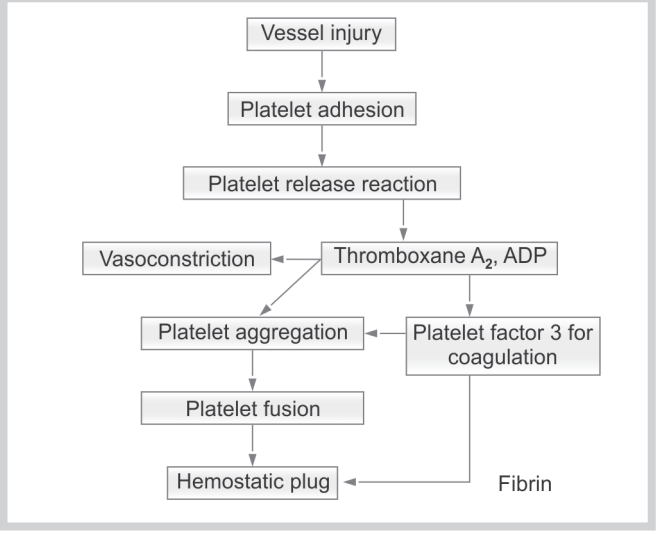
1. In acute cases blood may not clot due to gross fibrinogen deficiency.
2. Platelet count is low.
3. Fibrinogen screening tests, titers or assays indicate deficiency.
4. Thrombin time is prolonged.
5. High levels of serum fibrin/fibrinogen degradation products are found in serum and urine.
6. Prothrombin time and partial thromboplastin time are prolonged.
7. Factor V and factor VIII activity is diminished.
8. Due to microthrombi causing mechanical hemolytic anemia, RBCs may show crenation and poikilocytosis.

Causes of DIC

1. DIC may be caused by entry of procoagulant material into circulation, for example,
   - Amniotic fluid embolism
   - Premature placental separation
   - Widespread mucin secreting adenocarcinoma
   - Severe falciparum malaria
   - Hemolytic transfusion reaction
   - Promyelocytic leukemia
   - Some snake bites.
2. DIC may also be initiated by extensive endothelial damage and collagen exposure, for example
   - Endotoxemia
   - Gram-negative and meningococcal septicemia
   - Septic abortion
   - Certain viral infections (purpura fulminans)
   - Severe burns
   - Hypothermia.
3. Massive intravascular platelet aggregation can also precipitate DIC as occurs in some:
   - Bacterial and viral infections
   - Immune complexes may have a direct effect on platelets.

Functional Platelet Disorders

Platelet reactions in the hemostatic process.



Laboratory Diagnosis

1. Platelet count normal.
2. Prolonged bleeding time.
3. Abnormal platelet aggregation studies with ADP, adrenaline, collagen and ristocetin.
4. Abnormal adhesion studies and nucleotide pool measurement.
5. Factor VIII clotting assay (for von-Willebrand's disease).

Abnormal platelet function should be suspected in cases where bleeding is prolonged despite a normal platelet count. Various causes included in this are as follows:

Hereditary Disorders

1. *Platelet storage pool disease:* There is defective release

of ADP and 5HT due to an intrinsic deficiency in the number of dense granules.
2. *Thrombasthenia (Glanzmann's disease):* There is failure of primary platelet aggregation.
3. *Bernard-Soulier syndrome:* Platelets are larger than normal, lack surface glycoprotein and fail to make phospholipid available or to adhere to vessel walls.
4. von Willebrand's disease: There is defective platelet adhesion as well as coagulation factor VIII deficiency.

Acquired Disorders

1. *Aspirin therapy:* It may lead to abnormal bleeding time although purpura is rare. Aspirin leads to impaired thromboxane-A_2 synthesis. So, there is failure of the release action aggregation with ADP and adrenaline.
2. *Hyperglobulinemia:* Interferes with platelet adherence, release and aggregation.
3. *Myeloproliferative disorders:* Intrinsic abnormalities of platelet function may occur in patients with essential thrombocythemia and other myeloproliferative disorders.

Bleeding Time

The duration of bleeding from a standard puncture wound of the skin is a measure of the function of platelets as well as the integrity of the vessel wall.

Duke's Method

Requirements

- Stop watch
- Lancet
- Filter paper
- Glass slide
- Alcohol sponges.

Method

1. Clean the lobe of the ear or tip of a finger with alcohol and let dry.
2. For ear—glass slide is placed behind the ear lobe and held firmly in place. This provides a firm site for incision.
3. Pierce the lobe of the ear by a firm stroke against the glass slide (or pierce the finger-tip). Discard the glass slide if ear lobe has been incised. Start the stop watch when the stab was made.
4. Bleeding of the wound should be allowed to proceed without pressure and the blood is allowed to drop on the filter paper. The paper should be moved so that each drop will fall on a fresh area. When bleeding slows, the wound is touched gently with a fresh area of the filter paper at 30 second intervals. When blood no longer stains the filter paper, the watch is stopped and the time recorded.

Normal Values

The normal range is up to 6 minutes. Between 6 and 10 minutes, the results are borderline. Over 10 minutes is definitely abnormal.

Precautions

1. In children, heel should be used.
2. In suspected cases of a bleeding disorder, the bleeding may not be controlled easily from the ear lobe hence, fingertip puncture wounds are better.
3. The area to be punctured should not be congested.
4. The size and depth of the wound may vary if one does not have a standardized technique.
5. If bleeding persists for more than 15 minutes it should be stopped by placing a dry gauge sponges over the site and applying finger pressure (the filter paper used to collect the drops of blood can be dried and saved as a record of the procedure).

Ivys's Method

(Preferred because of greater ease of standardization).

Method

1. Cleanse the inner aspect of the forearm with spirit and let dry.
2. Place a blood pressure cuff on the upper arm, inflate at 40 mm Hg, and maintain the same throughout the test.
3. Select an area on the forearm—Volar aspect which is devoid of superficial veins. Stretch the skin laterally between the thumb and forefinger and hold in a taut position.
4. Take a cork, through which a no. 11 surgical blade has been inserted with the tip extending 3 mm beyond the cork surface (both cork and blade should have been sterilized before), the blade should be withdrawn from the cork and autoclaved before being used again.
5. Hold the cork with the thumb and forefinger of the free hand, and with the heel of the hand resting on the patient's arm, quickly make two skin punctures (actually they are small incisions) in the selected area. It is important that the surface of the cork meet the skin to ensure a 3 mm deep incision. Holding the skin taut prevents the test area from being depressed when the blade enters the skin.
6. Timing is begun as soon as the incisions are made and bleeding starts.
7. Using the edge of a piece of a filter paper to collect the blood, gently touch paper to the drop of blood, which

forms over the wound every 30 seconds. Do not rub or remove the clot. Do not touch the skin. Any disruption of formed fibrin or clot will prolong the bleeding time.

8. The bleeding time is reported when no blood stain is seen on the filter paper after a gentle touch. It is reported in intervals of 30 seconds. One can measure both wounds and average them, or take the reading of the last one to stop bleeding.

Normal Values

Normal values are 1 to 6 minutes. More than 6 minutes should be taken as abnormal.

Interpretation

1. Results of duplicate tests performed on the same individual should agree within 2 to 3 minutes at most.
2. Bleeding time is prolonged:
   - When platelet count < 100,000/mm^3
   - In patients on aspirin therapy.
   - In acquired fibrinogen disorders.

(If the platelets are young even in a thrombocytopenia patient, the bleeding time may not be raised as young platelets have enhanced hemostatic capabilities).

When platelet counts are low, one can calculate the expected bleeding time with the following formula:

$$\text{Bleeding time} = 30.5 \times \frac{\text{Platelet count/cu mm}}{3850}$$

A bleeding time longer than that calculated from platelet numbers alone, suggests defective platelet function in addition to reduced number. It is also possible to detect above-normal hemostatic capacity in cases in which active young platelets comprise the entire population of circulating platelets.

Clinical Implications

1. Bleeding time is prolonged when the level of platelets is decreased or when the platelets are qualitatively abnormal, as in
   a. Thrombocytopenia
   b. Platelet dysfunction syndromes
   c. Decrease or abnormality in plasma factors such as von Willebrand's factor and fibrinogen
   d. Abnormalities in walls of the small blood vessels—vascular defects
   e. Severe liver disease
   f. Leukemia
   g. Aplastic anemia
   h. DIC disease.
2. Bleeding time can be either normal or prolonged in von Willebrand's disease. It will definitely be prolonged if aspirin is administered prior to testing.
3. A single prolonged bleeding time does not prove the existence of hemorrhagic disease because a larger vessel may have been punctured. The puncture should be done twice (on the contralateral side) and the average of the bleeding times can be taken.

Interfering Factors

1. The normal range may vary when the puncture is not of standard depth and width.
2. Touching the incision during the test will break off any fibrin particles and prolong the bleeding time.
3. Heavy alcohol consumption (as in alcoholics) may cause bleeding time to be increased.
4. Prolonged bleeding time will result from the ingestion of 10 g of aspirin up to 5 days before the test.
5. Other drugs that may cause the bleeding time to be increased include:
   - Dextran
   - Streptokinase—streptodornase
   - Mithramycin
   - Pantothenyl alcohol.

Patient Preparation

1. Explain the purpose and procedure of the test to patient.
2. Warn patient not to consume aspirin for 5 days prior to test.
3. Advise patient not to consume alcohol in any form.

Coagulation Time

Capillary Tube Method of Wright

Blood is collected in about a dozen capillary tubes from a finger prick made after aseptic precautions. The tubes are sealed with plasticine and immersed in water bath at 37°C. After 4 minutes, remove the first tube from the bath and expel the blood in it with one end immersed in a dish containing water. Repeat this every 30 seconds with the other tubes till the blood is expelled in a worm clot and note the time.

An alternative way of determining the end point is to break the capillary tubes every 30 seconds until a clot is seen between the two broken ends. By these methods, the normal clotting time is 5 to 10 minutes at 37°C and longer if performed at room temperature. This test should be avoided as tissue thromboplastin contaminates the oozing blood and hence, false reports may be obtained.

Lee and White's Method

Principle: Whole blood, when removed from the vascular system and exposed to a foreign surface, will form a solid

clot. Within limits, the time required for the formation of the solid clot is a measure of the coagulation system.

Requirements

1. Stop watch
2. Equipment for collection of blood
3. Clean, dry glass test tubes (10 × 75 mm)
4. Water bath (37°C).

Method

1. Make a clean venipuncture with as little trauma to (or time spent passing through) the connective tissue between skin and vein as possible. One may routinely or in selected cases use the two-syringe technique, whereby one rinses the needle of all interstitial tissue fluid by drawing back 1 cc. of blood after entering the vein. Then remove the first syringe from the needle and quickly place on a second clean and dry syringe and draw back blood for the test.
2. Timing is begun when the blood first enters the syringe. The second syringe in the 'Two-syringe' technique.
3. Draw 3–5 mL of blood and withdraw the syringe and needle. Disconnect the needle. Place approximately 1 mL of blood in each of three (10 × 75 mm) test tubes.
4. Place the tubes in a stand so that they remain upright and undisturbed, at room temperature for 10 minutes. If a 37°C water bath is available one may do the entire test at 37°C, and shorter clotting times will be found (if the test has been done at 37°C, do not wait for more than 5 minutes).
5. After 10 minutes (or 5 minutes) take the first of the tubes and gently tip it every 30 seconds to test for clotting. Do not tip it further than necessary to get the information.
6. When the first tube is clotted (can be inverted without blood running down the edge of the tube), record the time and start the tipping of the second tube every 30 seconds until it is also found to be clotted. Then do the same with the third tube (tipping is intended to allow one to ascertain when blood is clotted—not as a means of hastening clotting or of assuring mixing of the blood).
7. The time recorded for the clotting of the third tube is taken as the clotting time (the purpose of the first two tubes is to tell one when to start looking in the third tube, since the agitation of tipping does hasten the clotting).

Some choose to tip the tubes in rotation (at 37°C) every 15 seconds, or tip all tubes at once, and average the results of the three tubes.

Normal Values

Normal times depend on method used. Normal range at 37°C is usually 5 to 10 minutes. Normal times at room temperature will vary with the degree of temperature present and the method used. If one uses the method which waits 10 minutes before starting to tip, then normal values may go as high as 22 to 25 minutes, especially in the cool season. Values shorter than 10 minutes should be suspected and the test repeated using the two-syringe technique to rule out contamination by tissue fluid (in the heat of April, May or June warm tropical climate blood will clot before 10 minutes without having been contaminated by tissue fluids). If one uses the method which waits 5 minutes before tipping begins, normal results are between 8 to 18 minutes. Longer than 20 minutes is abnormal. If clotting occurs in less than 7 minutes, the test should be repeated using two-syringe technique.

Precautions and Errors

1. The venipuncture must be without trauma to avoid contamination with tissue thromboplastin.
2. If all three tubes are clotted at 10 minutes (or 5 minutes) when one starts to tip the first tube, the test is unsatisfactory and should be repeated. If blood was drawn by single syringe technique, the most likely explanation is contamination, of the blood by tissue thromboplastin. If a two-syringe technique is used, it suggests that the patient's blood is hypercoagulable.
3. Vigorous agitation of the tubes will significantly shorten the coagulation time. So tipping should really be very gentle just to see if the blood has clotted.

Clinical Implications

1. Severe deficiencies of any of the coagulation factors must be present before the coagulation time will be prolonged. Fibrinogen for example, needs to be decreased to 50 mg/100 mL or less before the coagulation time is affected, the normal range of fibrinogen is 200 to 400 mg/100 mL.
2. When prothrombin is diminished to a level of 30% of normal, there will be a small change in coagulation time.
3. Prolonged coagulation time will be noted in afibrinogenemia and marked hyperheparinemia.

Interfering Factors

1. *Quality of venipuncture:* The venipuncture must be carefully done because either tissue thromboplastin obtained as a contaminant when the venipuncture is done, or hemolyzed red blood cells suctioned when the blood is drawn, can cause a marked shortening of the coagulation time. The time required for a severe hemophiliac's blood to clot can be shortened from 1 hour to a normal value when a poor venipuncture is done.

2. *Type of test tube:* The coagulation time will be lengthened to 20 to 40 minutes if plastic or silicone coated test tubes are used.
3. *Drugs:* Increased coagulation time may be seen with:
   - Mithramycin
   - Tetracyclines
   - Anticoagulants
   - Azathioprine
   - Carbenicillin.

   Decreased coagulation time may be seen with:
   - Corticosteroids
   - Epinephrine.

Clot Retraction

Principle

When whole blood is allowed to clot spontaneously, the initial coagulum is composed of all elements of the blood. With time the coagulum reduces in mass and fluid serum is expressed from the clot. This is due to an action of platelets on the fibrin network.

Requirements

- Equipment for collecting blood
- Clean, dry plain glass graduated centrifuge tube
- Timer
- Water bath 37°C.

Method

1. 5 mL blood is obtained with a standard two-syringe technique and transferred to the centrifuge tube.
2. Incubate it at 37°C in vertical position.
3. Record degree of retraction after 1, 2, and 4 hours. It may be necessary to loosen the clot gently from the wall of the test tube if contraction is not apparent at the end of 1 hour. The degree and rate of retraction should be noted. Note also any digestion of clot or discoloration of serum.

Clot retraction is directly related to platelet count, hence, it is impaired in thrombocytopenia, but is normal in hemophilia. In the method just described, one can remove the clot by using a hooked long needle and the volume of serum left behind can be measured. The percentage of clot can be calculated from the initial 5 mL of blood taken. In normal individuals, the clot percentage is about 50% at the end of one hour of the original blood volume taken.

Interpretation

1. Patients with qualitative or quantitative platelet disorders have samples with scant serum and a soft, plump, poorly demarcated clot.
2. The clot is small and serum voluminous if the patient has a low hematocrit.
3. Patients with polycythemia have poor clot retraction because the large numbers of captured red cells separate fibrin stands and interfere with platelet contraction.
4. If fibrinogen levels are low, the initial clot is so fragile that the delicate strands rupture and red cells spill out into the serum when retraction begins.
5. Serum contamination by red cells is especially striking if fibrinolysis is abnormally brisk, as often happens with reduced fibrinogen levels. Sometimes in these cases, the incubated tube contains only cells and plasma with no fibrin clot at all.

Errors

1. When fibrinogen is reduced in amount, the clot may be very small and retraction may be interpreted as normal even though it is inadequate.
2. In the presence of active fibrinolytic activity, the clot may dissolve.
3. In normal blood the exuded serum will be clear and free of RBC's. The presence of significant number of RBC's in the serum suggests fibrinolytic activity.
4. With a low hematocrit value, the mass of the clot will be proportionately small and may give enormously high values.

Heparin Therapy

Protocols and Blood Coagulation Tests

1. Heparin combines in the blood with an alpha globulin (heparin cofactor) for a potent antithrombin.
2. The intravenous injection of heparin will give an immediate anticoagulant effect, so it is used when rapid effects are desired.
3. Because of heparin not remaining in the blood very long, the clotting time is measured before each injection.
4. The coagulation time is ordinarily maintained at two to two and one half times the normal limit.
5. To evaluate the effect of heparin, the blood is tested for coagulation time:
   - Before therapy is started for baseline
   - One hour before the next dose is administered
   - Dependent upon the status of patient during heparin therapy (signs of bleeding).
6. Protamine sulfate is the antidote for heparin overdose and hemorrhage.

QUALITY ASSURANCE FOR ROUTINE HEMOSTASIS LABORATORY

Introduction

Coagulation tests for the routine assay parameters in laboratories are fairly simple to perform and master.

The performance of basic tests require simple apparatus such as water bath, test tubes, pipettes and stop watch and/or automatic clot timer. Moreover, It is precisely for this reason that these techniques appear deceptively easy.

There are a number of pretest variables that affect the accuracy and precision of coagulation results. These may relate to collection techniques, processing of samples, selection and preparation of reagents. In order to achieve optimum and reproducible results the impact of variables needs to be understood and controlled so as to reduce variability and errors and improve accuracy and reproducibility. Various variables that affect the results are discussed, along with the basis that leads to such recommendation.

Preparation of Patients

Although no special preparation of patients is required prior to approve techniques, it is preferable that patients are not heavily exercised before blood collection. Fasting patients or patients on a light non-fatty meal are preferable.

- Patients who are on fasting or on a light non-fatty meal prior to blood collection provide samples with desirable lower opacity this improves the sensitivity of clot detection especially when photo-optical instruments are being used
- Turbid, icteric, lipemic or grossly hemolyzed samples generate erroneous results due to varying opacity.

Sample Collection Techniques (Phlebotomy)

1. Blood should be withdrawn without undue venous stasis and without frothing into a plastic syringe with a short needle of 19 to 20 SWG.
2. The venipuncture must be a 'clean' one and incase of difficulty with a new syringe and needle another vein should be tried. The tourniquet should not be placed too tightly or for extended lengths of time. Patting the venipuncture site should also be avoided.
3. Distribute blood into test tubes (preferably plastic) after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant by gentle inversion of the tube.
   - 'Clean' venipuncture is essential to avoid formation of microclots at the site of venipuncture and consumption of factors, which will lead to artificially prolonged results.
   - Usage of short bigger bore needle allows free flow of blood within the syringe and reducing blood contact with metal surface. With smaller bore longer needles, blood will remain in contact with metal surface for longer time. This will lead to initiation of clotting or partial consumption of factors being assayed leading to erroneous results during test procedures.
   - Frothing when distributing the blood into anticoagulant tube should be avoided because frothing induces microclot formation.

Sample Preparation

1. The anticoagulant of choice for most coagulation procedures is sodium citrate or preferably buffered sodium citrate.
2. Sodium citrate is an ideal anticoagulant since Factor V and Factor VII are more stable in citrate. These factors are more labile in sodium oxalate. Heparin neutralizes action of thrombin on fibrinogen.
3. The recommended molarity of sodium citrate for coagulation studies is 0.109M, which equates to 3.2% of tri-sodium citrate.
4. Use of buffered sodium citrate is preferred over plain sodium citrate solution.
   - After collection of blood in citrate during centrifugation for preparation of platelet poor plasma (PPP) or platelet free plasma (PFP), the pH of the solution shifts releasing of carbon dioxide (CO_2). This shift in pH affects the labile factor V leading to erroneous results during test. Use of appropriately formulated buffered citrate overcomes this phenomenon.
   - When samples are collected in 3.8% citrate (129 mm) the prothrombin time of samples especially with patients receiving oral anticoagulants give prolonged results. Also the ISI of thromboplastins is lowered. It is for this reason 3.2% citrate is recommended universally instead of 3.8% for increasing accuracy of test results.
5. The optimum ratio of citrate to blood is 1 part of anticoagulant to 9 parts of blood.
   - When the molarity of citrate is accurate the anticoagulant supplied in this amount and ratio is sufficient to bind all the available calcium in the collected sample so as to prevent clotting. A shift in this ratio leads to erroneous results as follows:
   - *More blood less citrate:* The chelating activity of citrate will not be sufficient to bind the calcium present in the sample. This will lead to formation

of clots, consumption of factors and subsequent prolongation of results during test
- *More citrate less blood*: Excess citrate remaining in the blood sample would consume the calcium from the reagents thereby giving prolonged results.

6. The optimum concentration of calcium chloride to be used for APTT test should be 0.02 M:
   - The concentration of 0.02 M $CaCl_2$ replaces the calcium necessary to activate the intrinsic coagulation cascade. This ultimately generates thrombin from prothrombin via the coagulation cascade.'
   - Appropriate volumes of $CaCl_2$ should be aspirated for the day's work. Prewarmed $CaCl_2$ should always be discarded at the end of the working day.
7. The standard ratio of blood to anticoagulant of 9:1 is for normal hematocrit or PCV:
   - For occasional patients with PCV less than 20% (e.g. microcytic hypochromic anemia) and greater than 50% (e.g. polycythemia vera) the anticoagulant to blood ratio must be readjusted using the following formula,
     $C = 1.85\ L \times 10^{-3}\ (100\text{-}H)\ V$
     Where,
     C = Volume of sodium citrate in mL
     V = Volume of whole blood–sodium citrate in mL
     H = Hematocrit in percentage
   - When the PCV is higher than 55% the patient blood contains so little plasma that excess unutilized anticoagulant remains and is available to bind reagent calcium to prolongation of test results.
   - On the other hand, if the PCV is less than 20 percent the patient blood contains excess of plasma but less of anticoagulant and the chelating activity of citrate will not be sufficient to bind the calcium present in sample. This will lead to formation of clots in vitro, consumption of factors and prolongation of results.

Sample Processing and Storage

- Containers for collection and processing of plasma should be ideally made out of plastic or siliconized glass tubes. They should be scrupulously clean and dry.
  - The containers should be ideally made out of plastic and not from glass as scratched glass surfaces can activate in vitro the coagulation mechanism within the sample due to contact with silica. While plastic tubes overcome this problem they should be free from leavening chemicals used by the plastic industry during molding. These chemicals usually have an inhibitory effect. Scrupulous washing and drying overcomes this problem.
  - All the containers used for collection, storage and test should be free from detergents, acids and alkalies. These chemicals have a varying effect on pH. Change in pH effects factor stability. Detergents inhibit reactive characteristics of the sample/reagent mixture.
  - Ideally the cleaning of glassware used in coagulation tests should be the responsibility of one individual and should be handled separately from routine laboratory glassware. Alternatively, disposable labware should be used.
- The specimen to be tested for coagulation studies must be used preferably immediately.
  - As most of coagulation factors are time as well as temperature labile it is of utmost importance that they should not be subjected to high temperatures and tests be performed as early as possible, preferably immediately.
  - If specimens are held at 22 to 24°C then they must be tested within 2 hours and if the specimens are held at 2 to 4°C then they must be tested within 3 hours.
  - Plasma samples held at 4 to 8°C for prolonged periods may undergo cold activation leading to erroneous results.
  - Samples obtained for factor assays and tests for fibrinolysis should be stored in crushed ice if a delay in testing is anticipated.
  - Citrated blood for platelet aggregation studies should remain in capped tubes at room temprature (20 to 25° C) before testing.
- The sample collected must be stored tightly capped.
  - If the tubes are not capped the samples will absorb atmospheric CO_2 leading to shift in pH to an unacceptable range. This hampers factor stability and accuracy of results.
  - Centrifugation speed and time are of absolute importance in coagulation studies. The PT test uses PPP while the APTT test uses PFP.
  - Excessive centrifugation may destroy clotting factors due to the heat generated during centrifugation.
  - Under centrifugation would lead to the presence of platelets in plasma sample, which could lead to activation of clotting mechanism in vitro which leads to erroneous results.
  - Normally, centrifugation for 15 minutes at approximately 1500 g yields PPP (platelet poor plasma) and centrifugation at approximately 2000 g for 15 minutes yields PFP (platelet free plasma). The 'g' is a function of length of rotor head and RPM. It is for this reason each laboratory must calibrate its own equipment to achieve satisfactory samples depending on test performed and kind of plasma sample required.

BUFFERED 3.2% CITRATE SOLUTION (PROFACT)

(Courtesy: Tulip Group of Companies)
Ready to use 3.2% buffered citrate solution for coagulation assays and ESR by Westergren method.

Summary

Accurate coagulation testing is dependent on numerous preanalytical variables, which may affect the results of routine coagulation assays. To improve the precision and accuracy of laboratory testing, it is important to identify these variables and control their potential effect on results.

Preanalytical variables pertinent to routine coagulation testing can be classified into three major categories: specimen collection, specimen processing and specimen storage and transport. 3.2% citrate is also the anticoagulant of choice for performing ESR by Westergren method.

Reagent

Laboratory reagent: Ready-to-use solution.

Profact is a unique ready-to-use 3.2% buffered trisodium citrate solution formulated for collection of blood for routine coagulation assays. Profact can be used for sample preparation in the following clot based assays such as PT, APTT, TT, quantitative estimation of fibrinogen, test for factor deficiency, test for lupus anticoagulants, protein C and protein S tests. Profact can also be used for collection of blood to perform ESR by Westergren method.

Principle

About 3.2% trisodium citrate is the anticoagulant of choice for coagulation studies. When anticoagulated blood is centrifuged for preparing PPP for routine coagulation assays, the centrifugation process leads to release of carbon dioxide (CO_2). The end result being shift in pH, which has an adverse impact on the results of clot-based assays. Profact incorporates 3.2% trisodium citrate in a unique protective solution, which arrests shift in pH due to the release of carbon dioxide during centrifugation. Also labile factor V and VIII are well preserved and the results of clotbased assays are more accurate.

Also Profact incorporating 3.2% citrate is the anti-coagulant of choice for ESR by Westergren method.

Storage and Stability

- Store the reagent at 2 to 8°C
- Stability of unopened vial: 12 months from the date of manufacturing.
- Stability of opened vial: 90 days from the date of opening, provided it is not contaminated.

Material Required But Not Provided

Sterile and clean 0.5/1 mL micropipettes, micropipette tips or glass blow out pipettes, ESR tube.

Sample Collection and Preparation

For Coagulation Assays

Though no special preparation of the patient is required prior to sample collection by approved techniques, it is preferable that patients are not heavily exercised before blood collection. Fasting or only light non-fatty meals prior to blood collection provides sample with a desirable low opacity.

Withdraw blood without undue venous or frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and if there is any difficulty, take a new syringe and try another vein. Transfer the blood into tubes containing Profact, after detaching the needle from the syringe. Do not delay mixing blood with Profact. Avoid foam formation during mixing. Mix exactly nine parts of freshly collected blood with one part of Profact. For occasional patients with hematocrit less than 20% or greater than 50%, this ratio must be readjusted to ensure valid results. Centrifuge immediately for 15 minutes at 1500 to 3000 rpm (approximately 1500 g) on a laboratory centrifuge and transfer the plasma into a clean test tube. It should be ensured that the plasma is free from platelets (PPP). Cap the test tubes to prevent deterioration of samples. Plasma must be tested preferably immediately. However, if the specimen is held at 22 to 24°C then they may be tested within 2 hours and if the specimen is held at 2 to 4°C then they may be tested within 3 hours. Also plasma samples obtained after collection with Profact may be stored at –20°C for 2 to 3 weeks before testing.

For ESR by Westergren Method

For performing the test, venous blood is mixed accurately in the proportion of 1 part of Profact and 4 parts of whole blood.

The sedimentation rate is reduced in stored blood, hence, the test should be carried out within 4 hours of collecting the blood, and a delay up to 6 hours is permissible provided that the blood is kept at 4°C.

Precautions

1. Take every possible aseptic precaution to minimize contamination while drawing the reagent.

2. Avoid dipping contaminated pipettes/micropipette tips in the reagent vial. Ideally pour the required quantity for the day's work into another sterile clean vial.
3. Recap and replace the reagent vial immediately back at 2 to 8°C.

Remarks

1. Since most of the routine coagulation assays use PPP, each laboratory must calibrate the necessary force and time required during centrifugation to yield PPP.
2. Incorrect mixture of blood and Profact is a potential source of error both in coagulation assays and ESR estimation.
3. If the reagent vial develops turbidity, do not use the reagent as this would lead to erroneous results.

Calibration of Instruments/Equipments

- Water baths or heating blocks calibrated and preset at 37 ± 0.5°C are an important requirement to achieve accuracy and reproducibility.
  - The whole process of the coagulation tests is based on a series of enzymatic reactions, which are dependent on pH, ionic strength and the temperature of the reaction process. A correct temperature at 37 ± 0.5°C is critical as most of the reagent systems are standardized at this temperature. Day-to-day shift in reaction temperature of equipment will introduce uncontrolled variation into test conditions. Therefore, temperature of all equipments must be calibrated daily and diligently to avoid erroneous results and ensure accuracy and reproducibility.
  - Sample/reagent dispensing mechanisms must be accurate and precise.
  - Well-calibrated dispensing mechanisms are required for all coagulation- based tests to accurately dispense samples as well as reagents. Any shift in ratio or individual volumes of the sample and/or reagent can lead to shortening or prolongation of results.
  - Straight 0.1 and 0.2 mL glass pipettes are usually satisfactory, provided they are scrupulously clean and dry.
  - Automatic micropipettes, which are able to deliver the required volumes, are replacing the glass pipettes, provided these pipettes are calibrated frequently. The use of clean disposable tips places this system at an advantage over the older mechanisms.

Storage of Reagents

- Usually reagent manufacturers recommend aspiration of adequate reagent for the days use in a thoroughly clean and dry tube instead of intermittent aspiration from reagent vials at the time of test.
  - Most coagulation reagents are extremely delicate reagents. For them to maintain their sensitivity and performance the reagent formulations must maintain reagent integrity over the usage period. Repeated intrusions into the reagent vial exponentially increases the chances of reagent contamination and destruction of reagent formulations and integrity. Undried and/or contaminated pipettes, tips, glassware are usually the main culprits. Such contaminated reagents perform suboptimally.
  - The reagent vials must be immediately stored back to the recommended storage temperatures after the aspiration of the day's requirement separately so that the remaining reagent remains at optimal temperature for future use. Keeping unused reagents at higher ambient temperatures during the day causes steady deterioration of the reagent due to thermal stress.
- The recommended storage temperature for reagents should be strictly complied to:
  - Most of the liquid stable or reconstituted reagents such as PT and APTT are colloidal suspensions of lipoproteins and/or phospholipids. Subjecting them to elevated temperatures through repeated freeze-thaw cycles stresses the colloidal system. Especially detrimental are the effects of freezing (below 2°C). After freezing the reagent colloidal suspension undergoes an irreversible change and precipitates out or present itself as a particulate mass. Such reagents give erroneous results.
- Bringing reagents/samples to room temperature should be a two-step process:
  - When enough reagents are aspirated out for the days testing as recommended the reagent and samples stored at 2 to 8°C should be first allowed to attain room temperature (25 to 30°C) and then they should be subsequently brought to the optimal test temperature of 37 ± 0.5°C.
  - When reagent samples from 2 to 8°C are directly brought to 37°C the required time of 3 to 5 minutes may not be sufficient for the reagent samples to attain a homogeneous temperature of 37°C within the recommended time. This affects the reaction kinetics leading to erroneous results.

End Point Reading

- Reading of endpoint of clot-based tests varies from user to user.
  - Usually when manual techniques are followed the definition of "end point" is important. Ideally, the end point tests should be read "as soon as the first fibrin strand is visible and the gel clot formation begins"
  - When some users use a fully formed gel clot as an end point, there is a variation of 1 to 3 seconds between the end points as recorded by the ideal method and user-based variation.
  - It is advisable to have well illuminated background for reading the clot-based end points. Since user variations based on proficiency continue to influence results, it is advisable not to change personnel involved in coagulation tests off and on.
- Manufacturer's instructions must be followed meticulously when instrument based or automatic clot detection systems are used.
  - Each clot detection system works on a different principle, such as electro mechanical, turbidimetric or photo-optical. Each system of clot detection has its requirements for optimum functioning. Special care must be taken while using optical instruments for clot detection since reagent-induced turbidity can influence the results dramatically. Usually low turbidity reagents are preferred for manual as well as instruments-based clot detection.

Drug/Clinical Conditions Influencing Patient Results

- Drugs/clinical conditions influence results of patients coagulation studies.

PT tests are influenced on administration of following drugs

PT may be shortened drugs
- Antihistamines
- Butabarbital
- Phenobarbital
- Caffeine
- Oral contraceptives
- Vitamin K

PT may be prolonged drugs
- Corticosteroids
- EDTA
- Asparaginase
- Clofibrate
- Erythromycin
- Ethanol
- Tetracycline
- Aspirin
- Anticoagulants such as warfarin and heparin

APTT tests are influenced on administration of following drugs

APTT may be shortened drugs
- Oral contraceptives
- Conjugated estrogen therapy

APTT may be prolonged drugs
- Diphenylhydantoin
- Heparin
- Warfarin
- Naloxone
- Radiographic agents

Thrombin time test is prolonged in the following clinical conditions
- Normal newborn infant
- Systemic lupus erythematosus
- Macroglobulinemia
- Presence of exogenous/endogenous circulating anticoagulants
- Hepatic diseases
- Toxemia of pregnancy
- Multiple myeloma

Mean Normal Prothrombin (MNPT) and International Normalized Ratio (INR)

- MNPT is a critical requirement in the derivation of INR.
- MNPT is a critical requirement in the derivation of INR. Ideally each laboratory must derive its own MNPT from 20 or more normal patients for a given PT reagent and Lot under use. This corrects within laboratory test variables that influence PT results.
- By definition INR represents the PT ratio which would have been obtained for a particular patient sample as if the WHO reference thromboplastin itself (ISI=1.0) had been used in the PT determination.

$$INR = [R]^{[ISI]}$$

$$INR = \left[\frac{\text{Patient PT in seconds}}{\text{Mean of the normal range}}\right]^{ISI}$$

A PT ratio is obtained by dividing the patient PT in seconds by the **"Mean of the normal range"** (MNPT). This ratio is then "normalized" by raising the results to the power of the ISI of the PT reagent used.

If "normal control plasmas" are used in place of patient plasma for arriving at the MNPT it can affect the evaluation of the patients level of anti-coagulation.

For example:

Reagent ISI=2.5	*Test Day 1*	*Test Day 2*	*Test Day 3*
Patient PT (sec)	16.0	16.0	16.0
Normal Control (10.4-12.3 sec)	11.5	10.4	12.3
INR Formula	$16.0^{2.5}$	$16.0^{2.5}$	$16.0^{2.5}$
$[R]^{*SI}$	11.5	10.4	12.3
Resulting INR	2.27	2.89	1.92

If the control time is greater than the mean normal range (MNPT), the PT ratio for any patient, PT will be smaller, potentially leading to over coagulation. If the control time is lesser than MNPT the ratio for any patient PT will be greater, leading to under coagulation.

On the other hand, MNPT for a particular laboratory using the same combination of methodology, reagent and instrument would remain constant.

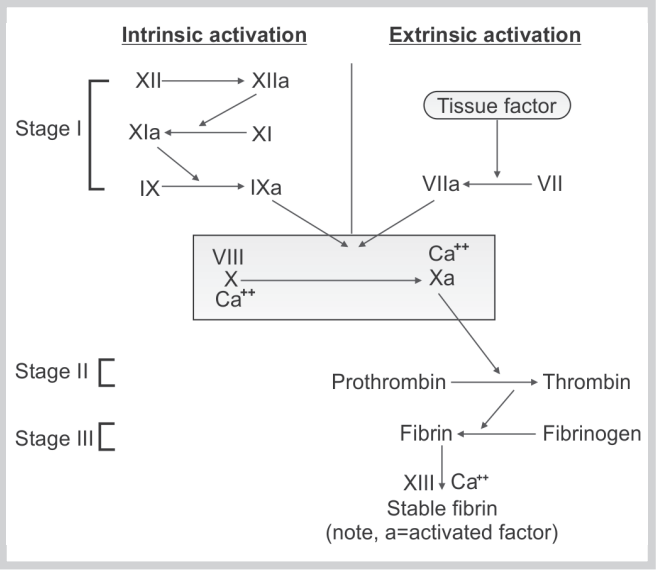
Quality Control Aspects

- Quality of water used for reconstituting lyophilized coagulation reagents must be good.
  - The water used for reconstitution of lyophilized coagulation reagents should be at least distilled twice and kept separately labeled for "coagulation studies". The reagents employed for coagulation studies are extremely delicate and inability to use good quality distilled water could lead to incorporation of metallic impurities in the reagent formulation as well as change in pH. Such changes can alter reaction kinetics and overall stability and performance of reagents.
- Quality assurance for coagulation-based reagents must be performed preferably on a daily basis.
  - Each laboratory should test coagulation reagents with normal and abnormal control plasma specimens at the beginning of each day's work to verify instruments, temperature calibration and also reagent performance.

If the control results fall within the stated limits, the test results are considered valid.

But if the results fall outside the stated control limits then the reagents, control and equipments are checked and the problem should be corrected.

Control results should be recorded and analyzed after regular intervals to ascertain the long-term validity of results.



Clotting mechanism—cascade system

PROTHROMBIN TIME (QUICK ONE-STAGE METHOD) LIQUIPLASTIN®

(*Courtesy:* Tulip Group of Companies)

Thromboplastin Reagent for Prothrombin Time (PT) Determination

Summary

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues.

Tissue thromboplastin, in the presence of calcium, is an activator, which initiates the extrinsic pathway of coagulation, which includes plasma coagulation factors VII, X, V, prothrombin and fibrinogen. During oral anticoagulant therapy, most of these factors are depressed, as also during the deficiencies of clotting factor activity which may be hereditary or acquired.

Prothrombin time determination is the preferred method for presurgical screening, determination of congenital deficiency of factors II, V, VII and X and for monitoring of patients on oral anticoagulant therapy and as a liver function test.

Reagent

Liquiplastin® is a liquid ready to use **calcium thromboplastin reagent,** which is derived from rabbit brain. Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Reagent Storage and Stability

(a) Store the reagent at 2 to 8°C. Do not freeze.
(b) The shelf-life of reagent is as per the expiry date mentioned on the reagent vial label. The uncontaminated reagent is stable for: 1 year at 2 to 8°C, 1 week at 18 to 25°C, 2 days at 37°C.

Principle

Tissue thromboplastin in the presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When Liquiplastin® reagent is added to normal anticoagulated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period. The time required for clot formation would be prolonged if there is a deficiency of factors/factor activity in the extrinsic pathway of the coagulation mechanism.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Liquiplastin® reagent is not from human source hence, contamination due to HBsAg and HIV is practically excluded.
3. Liquiplastin® reagent contains 0.01% Thimerosal as preservative.
4. It is very important that clean and dry micropipette tips be used to dispense the reagent.
5. Avoid exposure of the reagent to elevated temperatures and contamination. Immediately replace cap after use and store at recommended temperatures only.

Sample Collection and Preparation of PPP

Though no special preparation of the patient is required prior to sample collection by approved techniques, it is preferable that patients are not heavily exercised before blood collection. Fasting or only light non-fatty meals prior to blood collection provide samples with a desirable lower opacity. Withdraw blood without undue venous stasis or frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into anticoagulated tubes, after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant. Avoid foam formation during mixing. Mix exactly nine parts of freshly collected blood with one part of trisodium citrate (0.11 mol/L, 3.2%) or Profact available from Tulip; For occasional patients with hematocrit less than 20% or greater than 50%, this ratio must be readjusted to ensure valid results. Centrifuge immediately for 15 minutes at 1500-3000 rpm (approximately 1500 g) on a laboratory centrifuge and transfer the plasma into a dean test tube. It should be ensured that the plasma is free from platelets (PPP). Cap the test tubes to prevent deterioration of samples. Plasma must be tested preferably immediately. However if the specimen are held at 22 to 24°C then they may be tested within 2 hours and if the specimen is held at 2 to 4°C then they may be tested within 3 hours.

Additional Material Required for Manual and Calibration Curve Methods

12 × 75 mm test tubes (plastic tubes are preferred), 0.1 mL and 0.2 mL precision pipettes, Stop watch, Water bath or heating block at 37°C, fresh normal plasmas for establishing MNPT.

Test Procedure

Manual Method

1. Aspirate from the reagent vial enough reagent for immediate testing requirements in a thoroughly clean and dry test tube (plastic test tubes are preferred).
2. Bring this reagent to room temperature before prewarming at 37°C for testing purposes.
3. Recap the reagent vial and replace immediately to 2–8°C.
4. To a 12 × 75 mm tube add 0.1 mL of plasma and place the tube in a water bath for 3 to 5 minutes at 37°C.
5. To the tube forcibly add 0.2 mL of Liquiplastin® reagent (prewarmed at 37°C for at least 3 minutes) and simultaneously start a stopwatch. Shake the tube gently to mix contents.
6. Gently tilt the tube back and forth and stop the stopwatch as soon as the first fibrin strand is visible and the gel/clot formation begins. Record the time in 'seconds'.
7. Repeat steps 4-6 for a duplicate test on the same samples.
8. Find the average of the duplicate test values. This is the prothrombin time (PT). If a coagulation instrument is being used to perform the tests, the instrument manufacturer's instructions must be strictly adhered to.

Calculation of Results

Manual Method

The result may be reported directly in terms of the mean of the double determination of PT of the test plasma in 'seconds.'

or as a ratio'R':

$$R = \frac{\text{Mean of the patient plasma PT in seconds}}{\text{MNPT for the reagent}}$$

Or as international normalized ratio (INR), INR = $(R)^{ISI}$ 'where ISI = International sensitivity index of the reagent (Refer reagent vial label).'

It is recommended by the WHO that MNPT should be established for each lot of PT reagents by each laboratory, since PT results are dependent on the combination of reagent lot, instrument and technique followed at each laboratory. Usually plasma from at least 20 normal healthy individuals should be used to establish the MNPT. The average of such PT results in seconds = MNPT.

Expected Values

Normal values using Liquiplastin® are between 10 and 14 seconds. Between manual and Turbodensitometric instrument results a variation of 1 to 2 seconds may be expected. For photo-optical instruments, it is recommended that each laboratory must establish their own normal range. It is mandatory that each laboratory must establish its own MNPT for each lot of Liquiplastin®.

Oral anticoagulant therapeutic range : INR = 2.0-3.5.

Remarks

(1) It is recommended that controls with known factor activity should be run simultaneously with each test series to validate test run. (2) Incorrect mixture of blood and Trisodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glassware, etc. are potential source of errors. (3) Oxalated plasma may induce prolonged clotting times. (4) Since the PT test functions correctly only at 37 ± 0.5°C, temperature of all equipment must be calibrated daily. (5) Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose. (6) Turbid , icteric, lipemic or grossly hemolyzed samples may generate erroneous PT results. (7) Glasswares and cuvettes used in the test must be scrupulously clean and free from even traces of acids/alkalies or detergents. (8) Plasma samples held at 4-8° C may undergo 'cold activation' leading to a marked shortening of the PT. (9) The PT may be shortened during acute inflammatory conditions, which are accompanied by increase in Fibrinogen levels and also by agents, such as antihistamines, butabarbital, phenobarbital, caffeine, oral contraceptives and vitamin K. The PT may be prolonged by corticosteroids, EDTA, oral contraceptives, asparaginase, clofibrate, ethanol, tetracycline, aspirin and anticoagulants such as heparin and warfarin. (10) It is important that each laboratory express the results in terms of INR for patients on oral anticoagulant therapy for the clinician to adjust the dosage based on INR. (11) Since the test uses platelet poor plasma, each laboratory must calibrate the necessary force and time required during centrifugation to yield the PPP. Contamination of plasma with excess platelets could falsely elevate levels of some of the factors. (12) Homogenization of Liquiplastin® reagent suspension before use is important to achieve accurate and consistent results.

SENSITIVE THROMBOPLASTIN REAGENT FOR PROTHROMBIN TIME (PT) DETERMINATION (ISI=1.0) UNIPLASTIN®

(Courtesy: Tulip Group of Companies)

Summary

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues. Tissue thromboplastin, in the presence of calcium, is an activator, which initiates the extrinsic pathway of coagulation, which includes plasma coagulation factors VII, X, V, prothrombin and fibrinogen. During oral anticoagulant therapy most of the vitamin K-dependent factors, such as II, VII, IX, X, protein C and protein S are depressed, as also during the deficiencies of clotting factor activity which may be hereditary or acquired. Prothrombin time determination is the preferred method for presurgical screening, as a liver function test, determination of congenital deficiency of factors II, V, VII and X and for monitoring of patients on oral anticoagulant therapy.

Reagent

Uniplastin is a novel, highly-sensitive, low opacity, ready to use liquid calcified thromboplastin reagent, which is derived from rabbit brain. Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Reagent Storage and Stability

a. Store the reagent at 2 to 8°C. Do not freeze.
b. The shelflife of the reagent is as per the expiry date mentioned on the reagent vial label. The uncontaminated reagent is stable for: 1 year at 2–8°C, 1 week at 18–25°C, 2 days at 37°C.

Principle

Tissue thromboplastin in the presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When Uniplastin reagent is added to normal citrated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period. The time required for clot formation would be prolonged if there is acquired or congenital deficiency of factors/factor activity in the extrinsic pathway of the coagulation mechanism or reduction in the activity of vitamin K-dependent clotting factors during oral anticoagulant therapy.

Note

1. *In vitro* diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Uniplastin reagent is not from human source, hence contamination due to HBsAg and HIV is practically excluded.
3. Reagent contains 0.01% Thimerosal as preservative.
4. It is very important that scrupulously clean and dry micropipette tips be used to aspirate/dispense the reagent.
5. Avoid exposure of the Uniplastin reagent to elevated temperatures, contamination and undue stress due to high and low temperature exposure cycles. Immediately replace reagent cap after use and store at recommended temperatures only.
6. On prolonged storage at 2–8°C, the thromboplastin suspension has a tendency to settle down. Homogenize the reagent by resuspending before use.

Additional Material Required

12 × 75 mm test tubes (plastic tubes are preferred), 0.1 mL and 0.2 mL precision pipettes, stopwatch, water bath or heating block at 37°C, fresh normal plasmas for establishing MNPT.

Sample Collection and Preparation of PPP

Though no special preparation of the patient is required prior to sample collection by approved techniques, it is preferable that patients are not heavily exercised before blood collection. Fasting or only light non-fatty meals prior to blood collection provide samples with a desirable lower opacity. Withdraw blood without undue venous stasis or frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean one' and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into anticoagulated tubes, after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant. Avoid foam formation during mixing.

Mix exactly nine parts of freshly collected blood with one part of Trisodium citrate (0.11 mol/L, 3.2%). For occasional patients with hematocrit less than 20% or greater than 55%, this ratio must be readjusted to ensure valid results. Centrifuge immediately for 15 minutes at 1500-2000 rpm (approximately 1500 g) on a laboratory centrifuge and transfer the plasma into a clean test tube. It should be ensured that the plasma is free from platelets (PPP). Cap the test tubes to prevent deterioration of samples. Plasma must be tested preferably immediately. However, if the specimen is held at 22 to 24°C then they may be tested within 2 hours and if the specimen is held at 2 to 4°C then they may be tested within 3 hours.

Test Procedure

Manual Method

1. Bring the reagent vial to room temperature (20 to 30°C). Mix the contents of the vial to homogenize the suspension completely.
2. Aspirate from the reagent vial enough reagent for immediate testing requirements in a thoroughly clean and dry test tube (plastic test tubes are preferred).
3. Prewarm the reagent and bring to 37°C before use in test procedure (5-10 minutes may be required depending on the reagent volume to attain 37°C before testing).
4. Recap the reagent vial and replace immediately to 2 to 8°C.
5. To a 12 × 75 mm tube add 0.1 mL of plasma (PPP) and place the tube in a water bath for 3 to 5 minutes at 37°C.
6. To the tube forcibly add 0.2 mL of Uniplastin reagent (prewarmed at 37°C for at least 3 minutes) and simultaneously start a stopwatch. Shake the tube gently to mix contents.
7. Gently tilt the tube back and forth and stop the stopwatch as soon as the first fibrin strand is visible and the gel/clot formation begins. Record the time in 'seconds'.
8. Repeat steps 4 to 6 for a duplicate test on the same sample.

9. Find the average of the duplicate test values. This is the prothrombin time (PT).

If a coagulation instrument is being used to perform the tests, the instrument manufacturer's instructions must be strictly adhered to.

Calculation of Results

Manual Method

The results may be reported directly in terms of the mean of the double determination of PT of the test plasma in 'seconds'.

Or as a ratio 'R':

$$R = \frac{\text{Mean of the patient plasma PT in seconds}}{\text{MNPT for the reagent}}$$

Or as international normalized ratio (INR), $INR = (R)^{ISI}$, where ISI = International sensitivity index of the reagent (Refer reagent vial label).

It is recommended by the WHO that MNPT should be established for each lot of PT reagents by each laboratory, since PT results are dependent on the combination of reagent lot, instrument and technique followed at each laboratory. Usually plasma from atleast 20 normal healthy individuals should be used to establish the MNPT. The average of such PT results in seconds = MNPT.

Expected Values

Normal values using Uniplastin are between 11–15 seconds. Between manual and turbodensitometric instrument results a variation of 1–2 seconds may be expected. For photo-optical instruments, it is recommended that each laboratory must establish their own normal range. It is mandatory that each laboratory must establish its own MNPT for each lot of Uniplastin.

Oral anticoagulant therapeutic range: INR = 2.0–3.5.

Remarks

1. It is recommended that controls with known factor activity should be run simultaneously with each test series to validate test run.
2. Incorrect mixture of blood and trisodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glassware, etc. are potential source of errors.
3. Oxalated plasma may induce prolonged clotting times.
4. Since the PT test functions correctly only at 37 ± 0.5°C, temperature of all equipment must be calibrated daily.
5. Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose.
6. Turbid, icteric, lipemic or grossly hemolysed samples may generate erroneous PT results.
7. Glasswares and cuvettes used in the test must be scrupulously clean and free from even traces of acids/alkalies or detergents.
8. Plasma samples held at 4 to 8°C may undergo 'cold activation' leading to a marked shortening of the PT.
9. The PT may be shortened during acute inflammatory conditions which are accompanied by increase in Fibrinogen levels and also by agents such as antihistamines, butabarbital, phenobarbital, caffeine, oral contraceptives and vitamin K. The PT may be prolonged by corticosteroids, EDTA, oral contraceptives, asparaginase, clofibrate, erythromycin, ethanol, tetracycline, aspirin and anticoagulants such as heparin and warfarin.
10. It is important that each laboratory express the results in terms of INR for patients on oral anticoagulant therapy for the clinician to adjust the dosage based on INR.
11. Since the test uses platelet poor plasma, each laboratory must calibrate the necessary force and time required during centrifugation to yield the PPP. Contamination of plasma with excess platelets could falsely elevate levels of some of the factors.
12. Homogenization of UNIPLASTIN reagent suspension before use is important to achieve accurate and consistent results.

THROMBOPLASTIN REAGENT FOR PROTHROMBIN TIME (PT) DETERMINATION, LYOPLASTIN® (LYOPHILIZED REAGENT, ISI=1.0)

(*Courtesy:* Tulip Group of Companies)

Summary

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues. Tissue thromboplastin, in the presence of calcium, is an activator, which initiates the extrinsic pathway of coagulation, which includes plasma coagulation factors VII, X, V, prothrombin and fibrinogen. During oral anticoagulant therapy most of the vitamin K dependent factors such as II, VII, IX, X, protein C and protein S are

depressed, as also during the deficiencies of clotting factor activity which may be hereditary or acquired. Prothrombin time determination is the preferred method for presurgical screening, as a liver function test, determination of congenital deficiency of factors II, V, VII and X and for monitoring of patients on oral anticoagulant therapy.

Reagent

Lyoplastin is a sensitive, lyophilized calcified thromboplastin reagent which is derived from rabbit brain.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Storage and Stability

a. Store the reagent at 2-8°C. Do not freeze.
b. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label.
c. The reconstituted Lyoplastin reagent can be used for 10 days when stored at 2–8°C provided it is not contaminated.
d. It is strongly recommended that enough reconstituted reagent should be retrieved for the days use and the unused reagent should be immediately replaced to 2–8°C.

Principle

Tissue thromboplastin in the presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When Lyoplastin reagent is added to normal citrated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period of time. The time required for clot formation would be prolonged if there is acquired or congenital deficiency of factors/factor activity in the extrinsic pathway of the coagulation mechanism or reduction in the activity of vitamin K dependent clotting factors during oral anticoagulant therapy.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Lyoplastin reagent is not from human source, hence contamination due to HBsAg and HIV is practically excluded.
3. It is very important that scrupulously clean and dry micropipette tips be used to aspirate/dispense the reagent.
4. Avoid exposure of the Lyoplastin® reagent to elevated temperatures, contamination and undue stress due to high and low temperature exposure cycles. Immediately replace reagent cap after use and store at recommended temperatures only.

Additional Material Required

12 × 75 mm test tubes (plastic tubes are preferred), 0.1 mL and 0.2 mL precision pipettes, 1 mL precision pipette, distilled water, stop watch, water bath or heating block at 37°C, fresh normal plasmas for establishing MNPT.

Reagent Preparation

Bring the lyoplastin® reagent to room temperature (25–30°C) prior to reconstitution. Lyoplastin® reagent is reconstituted with 3 mL de-ionized, distilled water as follows: (a) Add accurately 3 mL of distilled water to the lyophilized Lyoplastin® reagent, (b) Gently mix to dissolve, (c) Keep for 10 minutes and mix again gently ensuring complete resuspension of the lyophilized reagent. Avoid froth formation, (d) Thorough mixing should be ensured before withdrawing material every time for test purposes.

Sample Collection and Preparation of PPP

Though no special preparation of the patient is required prior to sample collection by approved techniques, it is preferable that patients are not heavily exercised before blood collection. Fasting or only light non-fatty meals prior to blood collection provide samples with a desirable lower opacity. Withdraw blood without undue venous stasis or frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into anticoagulated tubes, after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant. Avoid foam formation during mixing.

Mix exactly nine parts of freshly collected blood with one part of Trisodium citrate (0.11 mol/L, 3.2%) or Profact available from Tulip. For occasional patients with hematocrit less than 20% or greater than 55%, this ratio must be readjusted to ensure valid results. Centrifuge immediately for 15 minutes at 1500–2000 rpm (approximately 1500 g) on a laboratory centrifuge and transfer the plasma into a clean test tube. It should be ensured that the plasma is free from platelets (PPP). Cap the test tubes to prevent deterioration of samples. Plasma must be tested preferably immediately. However, if the

specimen are held at 22–24°C then they may be tested within 2 hours and if the specimen is held at 2–4°C then they may be tested within 3 hours.

Test Procedure

Manual Method

1. Aspirate from the reagent vial enough reagent for immediate testing requirements in a thoroughly clean and dry test tube (plastic test tubes are preferred).
2. Bring the reagent to room temperature before prewarming at 37°C for testing purpose.
3. Recap the reagent vial and replace immediately 2–8°C.
4. To a 12 × 75 mm tube add 0.1 mL of plasma (PPP) and place the tube in a water bath for 3 to 5 minutes at 37°C.
5. To the tube forcibly add 0.2 mL of Lyoplastin reagent (prewarmed at 37°C for at least 3 minutes) and simultaneously start a stopwatch. Shake the tube gently to mix contents.
6. Gently tilt the tube back and forth and stop the stopwatch as soon as the first fibrin strand is visible and the gel/clot formation begins. Record the time in 'seconds.'
7. Repeat steps 4–6 for a duplicate test on the same sample.
8. Find the average of the duplicate test values. This is the prothrombin time (PT). If a coagulation instrument is being used to perform the tests, the instrument manufacturer's instructions must be strictly adhered to.

Calculation of Results

Manual Method

The results may be reported directly in terms of the mean of the double determination of PT of the test plasma in 'seconds.' Or as a ratio 'R':

$$R = \frac{\text{Mean of the patient plasma PT in seconds}}{\text{MNPT for the reagent*}}$$

Or as international normalized ratio (INR), $INR = (R)^{ISI}$, where ISI = International sensitivity index of the reagent (Refer reagent vial label).

*It is recommended by the WHO that MNPT should be established for each lot of PT reagents by each laboratory, since PT results are dependent on the combination of reagent lot, instrument and technique followed at each laboratory. Usually plasma from at least 20 normal healthy individuals should be used to establish the MNPT. The average of such PT results in seconds = MNPT.

Expected Values

Normal values using Lyoplastin® are between 11-15 seconds. Between manual and turbo densitometltric instrument results a variation of 1-2 seconds may be expected. For photo-optical instruments, it is recommended that each laboratory must establish their own normal range. It is mandatory that each laboratory must establish its own MNPT for each lot of Lyoplastin®.

Oral anticoagulant therapeutic range: INR = 2.0-3.5.

Remarks

1. lt is recommended that controls with known factor activity should be run simultaneously with each test series to validate test run.
2. Incorrect mixture of blood and Trisodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glassware, etc. are potential source of errors.
3. Oxalated plasma may induce prolonged clotting times.
4. Since the PT test functions correctly only at 37 $\pm$ 0.5°C temperature of all equipment must be calibrated daily.
5. Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose.
6. Turbid, icteric, lipemic or grossly hemolyzed samples may generate erroneous PT results.
7. Glasswares and cuvettes used in the test must be scrupulously clean and free from even traces of acids/alkalies or detergents.
8. Plasma samples held at 4–8°C may undergo 'cold activation' leading to a marked shortening of the PT.
9. The PT may be shortened during acute inflammatory conditions which are accompanied by increase in Fibrinogen levels and also by agents such as antihistamines, butabarbital, phenobarbital, caffeine, oral contraceptives and vitamin K. The PT may be prolonged by corticosteroids, EDTA, oral contraceptives, asparaginase, clofibrate, erythromycin, ethanol, tetracycline, aspirin and anticoagulants such as heparin and warfarin.
10. It is important that each laboratory express the results in terms of INR for patients on oral anticoagulant therapy for the clinician to adjust the dosage based on INR.

11. Since the test uses platelet poor plasma, each laboratory must calibrate the necessary force and time required during centrifugation to yield the PPP. Contamination of plasma with excess platelets could falsely elevate levels of some of the factors.
12. Homogenization of Lyoplastin reagent suspension before use is important to achieve accurate and consistent results.

Clinical Implications

Conditions accompanied by an increased prothrombin time (PT) include:

- Prothrombin deficiency
- Vitamin K deficiency
- Hemorrhagic disease of the newborn
- Liver disease (e.g. alcoholic hepatitis)
- Anticoagulant therapy
- Biliary obstruction
- Salicylate intoxication
- Hypervitaminosis A
- DIC disease.

Interfering Factors

1. *Diet*: Excessive amounts of green, leafy vegetables will increase body's absorption of vitamin K.
2. *Alcohol*: PT is increased due to liver disease.
3. *Diarrhea and vomiting*: These increase PT.
4. *Quality of venipuncture*: It is important that a clean and careful venipuncture is done, otherwise the PT can be shortened.
5. Many drugs can alter PT.

Clinical Alert

1. If PT is excessively prolonged, vitamin K is given intramuscularly. Ordinarily, intramuscular injections are contraindicated during anticoagulant therapy because large painful hematomas may form at the injection site. As values get into danger zones, assess carefully for bleeding, including: (i) craniotomy checks, (ii) lung auscultation (especially of upper lobes), and (iii) occult blood in the urine.
2. Patients who are being monitored by PT for long-term anticoagulant therapy should not take any drugs unless absolutely necessary.
3. When unexpected changes in anticoagulant doses are needed to maintain a stable PT, or when there is a consistent change in PT, a drug interaction should be suspected.
4. Blood for PT should be drawn for a base line and prior to administration of anticoagulants.
5. Protamine sulphate is the antidote for heparin.

The INR Method of Reporting Results

By definition INR represents the PT ratio which would have been obtained for a particular patient sample as if the WHO reference thromboplastin itself (ISI=1.0) had been used in the PT determination.

$$INR = [R]^{ISI}$$

$$INR = \left[\frac{\text{Patient PT in seconds}}{\text{Mean of the normal range}}\right]^{ISI}$$

A PT ratio is obtained by dividing the patient PT in seconds by the "mean of the normal range" (MNPT). This ratio is then "normalized" by raising the results to the power of the ISI of the PT reagent used. Lower the ISI of the reagent used, closer will be the INR to the observed PT ratio. Ideally, when the ISI of the reagent is 1.0 then the INR is a simple PT ratio since $(R)^{1.0} = R$.

Currently many coagulation instruments are available that can perform this exponential calculation by entering the ISI of the reagent in use. Alternatively a table is provided by reagent manufacturers for reading off "INR" directly for the given patient PT ratio, corresponding to the ISI value of the reagent used.

Recommended Therapeutic Ranges for Oral Anticoagulant Therapy

Indications	INR	Intensity
• Prophylaxis of venous thrombosis (high risk surgery) • Treatment of venous thrombosis • Treatment of pulmonary embolism • Prevention of systemic embolism – Tissue heart values – Acute myocardial infarction (to prevent systemic embolism) – Valvular heart disease	2.0–3.0	
• Atrial fibrillation • Mechanical prosthetic values (high risk) • Prevention of myocardial infarction recurrent	2.5-3.5	High

INR CONVERSION TABLE

ISI							
R	1.00	1.05	1.10	1.15	1.20	1.25	1.29
1.0	1.00	1.00	1.10	1.00	1.00	1.00	1.00
1.1	1.10	1.11	1.11	1.12	1.12	1.13	1.13
1.2	1.20	1.21	1.22	1.23	1.24	1.26	1.27
1.3	1.30	1.32	1.33	1.35	1.37	1.39	1.40
1.4	1.40	1.42	1.45	1.47	1.50	1.52	1.54
1.5	1.50	1.53	1.56	1.59	1.63	1.66	1.69
1.6	1.60	1.64	1.68	1.72	1.76	1.80	1.83
1.7	1.70	1.75	1.79	1.84	1.89	1.94	1.98
1.8	1.80	1.85	1.91	1.97	2.02	2.08	2.13
1.9	1.90	1.96	2.03	2.09	2.16	2.23	2.29
2.0	2.00	2.07	2.14	2.22	2.30	2.38	2.45
2.1	2.10	2.18	2.26	2.35	2.44	2.53	2.60
2.2	2.20	2.29	2.38	2.48	2.58	2.68	2.77
2.3	2.30	2.40	2.50	2.61	2.72	2.83	2.93
2.4	2.40	2.51	2.62	2.74	2.86	2.99	3.09
2.5	2.50	2.62	2.74	2.87	3.00	3.14	3.26
2.6	2.60	2.73	2.86	3.00	3.15	3.30	3.43
2.7	2.70	2.84	2.98	3.13	3.29	3.46	3.60
2.8	2.80	2.95	3.10	3.27	3.44	3.62	3.77
2.9	2.90	3.06	3.23	3.40	3.59	3.78	3.95
3.0	3.00	3.17	3.35	3.54	3.74	3.95	4.13
3.1	3.10	3.28	3.47	3.67	3.89	4.11	4.30
3.2	3.20	3.39	3.59	3.81	4.04	4.28	4.48
3.3	3.30	3.50	3.72	3.95	4.19	4.45	4.67
3.4	3.40	3.61	3.84	4.09	4.34	4.62	4.85
3.5	3.50	3.73	3.97	4.22	4.50	4.79	5.03
3.6	3.60	3.84	4.09	4.36	4.65	4.96	5.22
3.7	3.70	3.95	4.22	4.50	4.81	5.13	5.41
3.8	3.80	4.06	4.34	4.64	4.96	5.31	5.60
3.9	3.90	4.17	4.47	4.78	5.12	5.48	5.79
4.0	4.00	4.29	4.59	4.92	5.28	5.66	5.98
4.1	4.10	4.40	4.72	5.07	5.44	5.83	6.17
4.2	4.20	4.51	4.85	5.21	5.60	6.01	6.37
4.3	4.30	4.63	4.98	5.35	5.76	6.19	6.58
4.4	4.40	4.74	5.10	5.50	5.92	6.37	6.76
4.5	4.50	4.85	5.23	5.64	6.08	6.65	6.96
4.6	4.60	4.96	5.36	5.78	6.24	6.74	7.16
4.7	4.70	5.03	5.49	5.93	6.40	6.92	7.36
4.8	4.80	5.19	5.62	6.07	6.57	7.10	7.56
4.9	4.90	5.31	5.74	6.22	6.73	7.29	7.77
5.0	5.00	5.42	5.87	6.37	6.90	7.48	7.97
5.1	5.10	5.53	6.00	6.51	7.06	7.66	8.18
5.2	5.20	5.65	6.13	6.66	7.23	7.85	8.39
5.3	5.30	5.76	6.26	6.81	7.40	8.04	8.60
5.4	5.40	5.88	6.39	6.95	7.57	8.23	8.81
5.5	5.50	5.99	6.52	7.10	7.73	8.42	9.02
5.6	5.60	6.10	6.65	7.25	7.90	8.61	9.23
5.7	5.70	6.22	6.78	7.40	8.07	8.81	9.44
5.8	5.80	6.33	6.91	7.55	8.24	9.00	9.66
5.9	5.90	6.45	7.05	7.70	8.41	9.20	9.87
6.0	6.00	6.56	7.18	7.85	8.59	9.39	10.09

Other Factors Influencing the INR

The variability in the responsiveness of the PT reagents, is corrected through the "ISI" calibration, however, three additional technical factors influence the INR:

- Derivation of MNPT
- Magnitude of difference in the ISI value of test thromboplastin and IRP (ISI=1.0)
- Method of clot detection employed during PT test.

MNPT

MNPT is a critical requirement in the derivation of INR. Ideally each laboratory must derive its own MNPT from 20 or more normal patients for a given PT reagent and lot under use. This corrects within laboratory test variables that influence PT results. If "normal control plasmas" are used in place of patient plasma for arriving at the MNPT it can effect the evaluation of the patients level of anticoagulation. For example,

Reagent ISI=2.5	*Test Day 1*	*Test Day 2*	*Test Day 3*
Patient PT (sec)	16.0	16.0	16.0
Normal Control (10.4–12.3 sec)	11.5	10.4	12.3
INR Formula $[R]^{ISI}$	$\frac{16.0^{2.5}}{\quad}$	$\frac{16.0^{2.5}}{\quad}$	$\frac{16.0^{2.5}}{\quad}$
Resulting INR	2.27	2.89	1.92

If the control time is greater than the mean normal range (MNPT), the PT ratio for any patient PT will be smaller, potentially leading to over coagulation. If the control time is lesser than MNPT the ratio for any patient PT will be greater, leading to under coagulation.

On the other hand MNPT for a particular laboratory using the same combination of methodology, reagent and instrument would remain constant.

ISI Value of PT Used and Method of Clot Detection

INR loses some precision when comparisons are made with thromboplastins with markedly different ISI values as against the IRP (ISI=1.0) and different methods of clot detection, e.g. manual, mechanical, optical, etc.

Therefore, manufacturers must provide ISI values adapted to the method used for clot detection. Also the reagent used for reporting results should be ideally as close to 1.0 as possible.

Advantages of the INR system

- Major advantage of the INR system is that it helps alleviate confusion in the interpretation of PT results. Usually laboratory changes like change in thromboplastin and/or equipments could go unnoticed

by the attending physicians. the INR remains constant even with such changes.
- INR system affords comparison of PT results between laboratories.
- INR system provides a more accurate and convenient mean of monitoring patients who travel extensively.
- INR therapeutic ranges for different clinical conditions are based on international collaborative studies. Usage of standardized dosage reduces the risk of thrombotic episodes or secondary bleeding.

Disadvantages of the INR System

- The prothrombin time test is always a part of the preoperative screening panels. It is also frequently used to evaluate other hemostatic disorders, such as liver disease, DIC, LA, hereditary factor deficiencies and acquired vitamin K deficiency. Since these disorders have been excluded from the derivation of the ISI, INR has a diagnostic and therapeutic value mainly applicable for patients stabilized on oral anticoagulants. Therefore, laboratories may prefer to report both the INR and patients time in seconds depending on clinical application.
- The INR systems effectiveness would still depend on the calibration of the coagulation instruments as well as thromboplastin reagents used.
- Derivation of the correct MNPT and use of the mean normal range in each laboratory.
- Usage of thromboplastin reagents with ISI of preferably 1.0 or as close to 1.0 as possible.
- The correct use of the formula to compute the INR.
- Uniform understanding of the INR system by clinicians as well as laboratorians.

Patient Variables in PT/INR Testing

There are many factors that can influence the results of the PT/INR tests so that they do not reflect the patient's usual coagulation state. Coagulation tests are susceptible to errors introduced by suboptimal specimen quality because of a number of factors such as blood collection technique, labile state of several coagulation proteins, and laboratory transportation factors. In order to get acceptable accuracy it is important to understand and control these factors as much as possible.

Factors that Influence Coagulation Test Results

Age and Gender

Age specific reference ranges are critical for correct interpretation of coagulation data. Bleeding time declines with age and many coagulation factors increase with age as do markers of coagulation activation. Age and gender can also influence platelet function. Females tend to have longer bleeding times than males.

Blood Type

Type O individuals have significantly lower von Willebrand factor and factor VIII activity than subjects with type A, B, or AB. This causes increased bleeding and dotting times.

Within Day Variation

Incidences of platelet activation are highest in the mornings, resulting in increased coagulation activation.

Seasonal Variation

Increased coagulation activity has been described in cold weather.

Intraindividual Variability

Many coagulation analytes are less precise than other analytes and thus can give variable results within the same individual.

Diet, Alcohol and Smoking

Cardiac risk factors can increase coagulation factor level/activation. Smoking elevates plasma fibrinogen. Von Willebrand factor, thrombin generation and platelet activation may all have an effect causing variability. Moderate ethanol intake inhibits platelet reactivity and increases fibrinolysis and INR.

Medications

A number of other medications, including hormone replacement therapy, selective estrogen receptor, modifiers and oral contraceptives can alter coagulation and raise the INR. In addition, non-steroidal anti-inflammatory drugs, antibiotics and fluoroquinolones can also alter the INR.

Menstrual Cycle, Pregnancy

Significant hormonally determined changes in coagulation factors, inhibitors, fibrinolysis and activation markers must be considered as interpretation of the results.

Diseases

States, which lead to anemia, polycythemia or hemolysis or uremia, can also interfere with coagulation tests.

Physical and Emotional Stress

These are commonly associated with increased coagulation and platelet activation.

Posture

Values can change from supine to upright positions due to the shift of water and subsequent reduction in plasma volume. Hence, standardization of posture is recommended.

Venous Occlusion

Traumatic or prolonged phlebotomy accentuates the hemostatic activation, producing artificially altered coagulation times.

Vitamin K

Certain fat substitutes in some snackitems contain unspecified amount of vitamin K. Green, leafy vegetables and green tea also contain high levels of vitamin K. This can have an impact on serum vitamin K levels and the INR can drop as a result. Alternative medicines: According to the AANA (American Association of Nurse Anesthetists) some sources, certain herbal drugs can cause interference in coagulation cycles, falsely elevating the INR.

Anticoagulant Therapy

It is of utmost importance to bear in mind that patients on heparin will show inaccurate INR results.

While certain pre-analytical factors are not entirely controllable, every effort must be made to ensure that most conditions have been stable for a period of time. Patient preparation and blood collection should be standardized according to the guidelines.

Prothrombin Determination (Two-stage Method)

Principle

Prothrombin in the presence of optimal procoagulants and calcium will form thrombin. The amount of thrombin formed can be calculated by determining the dilution of plasma that will clot a standard fibrinogen reagent in a specific period of time. The amount of thrombin formed is a measure of the amount of prothrombin present in the starting sample.

The test consists of two stages. In the first stage, prothrombin is incubated with a standard mixture containing thromboplastin, calcium, a buffer and a source of procoagulants. In the second stage, samples of the incubating mixture are added to a standard fibrinogen solution and the clotting time is determined.

Results

1. The object of the procedure is to determine the dilution of plasma from which will evolve one unit of thrombin under optimal conditions. A unit of thrombin is defined as that amount which will form a clot of 1 mL of fibrinogen in 15 seconds under standard conditions.
2. If varying amounts of thrombin are added to standard amounts of fibrinogen the clotting time of the mixture is an index of the thrombin concentration within a specific range. When thrombin concentrations are plotted against clotting times, the results describe a hyperbolic curve. With thrombin concentrations between 0.80 and 1.34 units, there is good correlation between thrombin concentration and clotting time. With greater amounts of thrombin, there is little change in the speed of clotting, with relatively large changes in thrombin concentration. With lesser amounts of thrombin, small changes in thrombin concentration result in large changes in the speed of clotting.

APTT/PTTK CEPHALOPLASTIN REAGENT FOR PARTIAL THROMBOPLASTIN TIME (APTT) DETERMINATION USING ELLAGIC ACID AS ACTIVATOR LIQUICELIN-E®

(Courtesy: Tulip Group of Companies)

Summary

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of a series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues. Activated partial thromboplastin time is prolonged by a deficiency of coagulation factors of the intrinsic pathway of the human coagulation mechanism such as factor XII, XI, IX, VIII, X, V, II and fibrinogen. Determination of APTT helps in estimating abnormality in most of the clotting factors of the intrinsic pathway including congenital deficiency of factor VIII, IX, XI and XII and is also a sensitive procedure for generating heparin response curves for monitoring heparin therapy.

Reagent

Liquicelin-E® is a liquid ready to use activated cephaloplastin reagent for the determination of activated partial thromboplastin time. It is a phospholipid preparation derived from rabbit brain with ellagic acid as an activator.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Reagent Storage and Stability

a. Store the reagent at 2-8°C. Do not freeze.
b. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label. The reagent is stable for: 1 year at 2–8°C, 1 week at 18–25°C, 2 days at 37°C.

Principle

Cephaloplastin activates the coagulation factors of the intrinsic pathway of the coagulation mechanism in the presence of calcium ions. APTT is prolonged by a deficiency of one or more of these clotting factors of the intrinsic pathway and in the presence of coagulation; inhibitors like heparin.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Liquicelin-E, reagent is not from human source hence, contamination due to HBsAg and HIV is practically excluded.
3. Reagent contains 0.01% thimerosal as preservative.
4. It is very important that clean and dry micropipette tips be used to dispense the reagent.
5. Avoid exposure of the reagent to elevated temperatures and contamination. Immediately replace cap after use and store at recommended temperatures only.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Withdraw blood without undue venous stasis and without frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into tubes, after detaching the needle from the syringe.

Mix exactly nine parts of freshly collected blood with one part of Trisodium citrate (0.11 mol/L, 3.2%) or Profact available from Tulip; Centrifuge immediately for 15 minutes at 3000 rpm (approximately 2000 g) and transfer the plasma into a clean test tube. Plasma must be tested within three hours of blood collection. For heparin determination, platelet deficient plasma should be used, hence higher centrifugation time is required.

FNP Collection

Prepare a plasma pool (FNP) of freshly collected blood from at least five normal healthy donors and process as above. Plasma must be tested within three hours of blood collection.

Additional Material Required*

12 × 75 mm test tubes; 0.1 mL, 0.2 mL and 2.0 mL precision pipettes; Stopwatch; Water bath or heating block 37°C; Fresh normal pooled plasma; $CaCl_2$ (0.02 mol/L).

*Available from Tulip Diagnostics.

Test Procedure

Manual Method

1. Before use, the reagent should be mixed well by gentle swirling. Do not shake.
2. Aspirate from the reagent vial enough reagent for the immediate testing requirement in a thoroughly clean and dry test tube. Bring this reagents to room temperature before prewarming at 37°C for testing purposes.
3. Separate test tubes containing Liquicelin-E® and Tulip's calcium chloride solution should be brought to 37°C (depending on volume, approximately 5 to 10 minutes required). Do not incubate the test plasma.
4. To a 12 × 75 mm test tube, add 0.1 mL test plasma and 0.1 mL Liquicelin-E®. Shake tube briefly to mix the reagent and plasma, place tube at 37°C for 3 to 5 minutes.
5. Following incubation period, add forcibly 0.1 mL prewarmed calcium chloride into the plasma and Liquicelin-E® mixture, simultaneously start a stopwatch. Shake tube briefly to mix contents, keep at 37°C for 20 seconds.
6. Following 20 seconds incubation, remove the tube, gently tilt back and forth until a gel clot forms, stop the watch, record time.
7. Repeat steps 2–4 for a duplicate test using the same test plasma.
8. Find the average from the duplicate test values. This is the activated partial thromboplastin time (APTT of patient plasma).
9. Similarly repeat steps 2-4 twice, and record duplicate values using FNP in place of test plasma (APTT of FNP).

If a coagulation instrument is being used to perform the tests, the instrument manufacturer's instructions must be strictly adhered to.

Calibration Curve Method (For determination of heparin concentration):

1. Dilute heparin (as used for treatment) with physiological saline to a concentration of 10 U/mL.
2. Mix 0.2 mL of 10 U/mL diluted heparin with 1.8 mL of FNP to give a heparin standard of 1 U/mL concentration.
3. Dilute the heparin standard as prepared above (1 U/mL) with FNP as follows :

Test tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Heparin standard (1 U/mL) in mL	0.5	0.4	0.3	0.2	0.1	0.1	-
FNP in mL	-	0.1	0.2	0.3	0.4	0.9	0.5
Heparin concentration (U/mL)	1	0.8	0.6	0.4	0.2	0.1	0.0

4. Pipette 0.1 mL each of the seven heparin dilutions into clean test tubes.
5. Add 0.1 mL Liquicelin-E® reagent to each test tube.
6. Mix well and incubate each test tube at 37°C for exactly 3 minutes before testing.
7. Forcibly add 0.1 mL calcium chloride (prewarmed at 37°C) to each test tube, one by one and simultaneously start the stopwatch.
8. Gently tilt the tube back and forth and stop the stopwatch as the first fibrin strand is visible and the gel/clot formation begins. Record the time in seconds.
9. Repeat steps 4–8 for each dilution for duplicate test, and find the average of the duplicate test values.
10. Plot the mean of the double determination in 'seconds' against each heparin concentration using Liquicelin-E® graph paper.
11. Clotting times (APTT) of test specimens can be interpolated against the heparin concentration to determine the heparin concentration of the sample in U/mL.

Calculation and Reporting of Results

Manual Method

a. The result may be reported directly in terms of the mean of the double determination of the APTT of the test plasma.

OR

b. As a ratio R as follows:

$$R = \frac{\text{APTT of patient plasma (in seconds)}}{\text{APTT of FNP (in seconds)}}$$

Calibration Curve Method

Heparin concentration in the test sample can be directly obtained from the Liquicelin-E® calibration curve by interpolating the test plasma clotting time against the heparin concentration in U/mL.

Expected Values

Normal values using Liquicelin-E® reagent are between 21 and 29 seconds at 3 minutes activation time. Between manual and turbodensitometric instrument results a variation of 1-2 seconds may be expected. For photo-optical instruments, it is recommended that each laboratory must establish their own normal range.

Remarks

1. Due to inter and intralaboratory variations users must establish their own normal population range as well as normal and abnormal range.
2. It is recommended that controls with known factor activity should be run simultaneously with each test series routinely.
3. Incorrect mixture of blood and trisodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glassware, etc. are potential source of errors.
4. Incorrect dilutions of heparin is also a potential source of error.
5. Oxalated plasma may induce prolonged clotting times.
6. Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose.
7. Abnormalities of coagulation factor VII, factor XIII and platelets are not detected by this test procedure.
8. For automated equipment, it is strongly recommended that the equipments manufacturer's methodology is strictly adhered to.
9. In heparin monitoring time of collection of blood sample is important since the in vivo half-life of heparin is approximately 1.5 hours. When it is administered intravenously, it has an immediate anticoagulant effect but its efficacy decreases rapidly with time.
10. Platelet factor IV, a heparin-neutralizing factor can be released due to platelet aggregation or damage. In order to prevent this phenomenon in vitro the specimen should be collected with a minimum of trauma.
11. Decrease in APTT time is observed in males under estrogen therapy and oral contraceptive administration in females.

Clinical Implications of APTT

1. The APTT is prolonged in all coagulation defects of stage I (includes platelet activity and thromboplastin).
2. The APTT is usually prolonged in Willebrand's disease and is accompanied by a consistently diminished factor VIII level.
3. The APTT and PT will detect 95% of coagulation abnormalities. When APTT is performed in conjunction with a prothrombin time (PT), a further clarification of

coagulation defects is possible. For example, a normal PT and abnormal APTT means that the defect lies in the first stage of the clotting mechanism.

Causes of prolonged APTT

- Hemophilia
- Vitamin K deficiency
- Liver disease
- Presence of circulating anticoagulants
- DIC disease (chronic or acute).

Shortened APTT occurs in:

- Extensive cancer, except when liver is involved
- Immediately after acute hemorrhage
- Very early stages of DIC.

Circulating Anticoagulants

Usually occurs as an inhibitor of a specific factor (e.g. factor VIII). Most commonly seen in the development of anti-factor VIII or anti-factor IX in 5 to 10% of hemophiliacs. Anticoagulants that develop in the treated hemophiliac are detected by prolonged APTT. Circulating anticoagulants also can be detected in some cases:

- Following repeated plasma transfusions
- Drug reactions
- Tuberculosis
- Chronic glomerulonephritis
- Systemic lupus erythematosus
- Rheumatoid arthritis.

NORMAL AND ABNORMAL CONTROL PLASMAS FOR COAGULATION ASSAYS PLASMATROL H-I/II®

(*Courtesy:* Tulip Group of Companies)

Summary

Tulip Plasmatrol H-l and Plasmatrol H-ll are two level human plasma controls that are suitable for use as normal and abnormal control plasma for PT, APTT, TT and fibrinogen testing using clot based methods. Coagulation controls provide a means of day-to-day quality control in the hemostasis laboratory for control of accuracy and precision.

Reagent

Plasmatrol is a stabilized and freeze dried preparation of selected human plasma with values determined and assigned for specific clot based tests, which are lot specific. The plasma controls are assayed using Tulip coagulation reagents.

Reagent Storage and Stability

Unopened vials should be stored at 2–8°C and are stable up to the expiry date mentioned on the vial labels. After reconstitution the shelf life of the control plasma is 3 hours at 25–30°C and 8 hours when stored at 2–8°C.

Principle

The properties of the control plasma are similar to those of pooled fresh plasmas. Since, the plasma controls have assigned values, when substituted in place of a sample, in clot based coagulation assays, they can be used for laboratory quality assurance.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The source material used for preparation of the reagent is screened by third generation assays for HBsAg, HCV and HIV antibodies and are found to be non-reactive. However, handle the material as if it is infectious, as no known test method can assure that infectious agents are absent.

Preparation of the Reagent

1. Reconstitute the control plasma with exactly 1 mL of bi-distilled water. Avoid using water-containing preservatives.
2. Re-stopper the vial and allow to stand until, the hydration is complete (usually 5–7 minutes).
3. Mix by gently swirling and inversion, avoiding froth formation. Do not shake.
4. Allow to stand and equilibrate for a further 15 minutes before use.
5. Use the reconstituted plasma within 3 hours of reconstitution.

Test Procedure

1. Use the reconstituted Plasmatrol controls in the same manner as freshly prepared titrated platelet poor plasma from a patient.
2. Use the procedure as laid out in the Uniplastin, Liquiplastin®, Liquicelin-E®, Fibroscreen, Fibroquant pack inserts.

Expected Values

1. The expected value of specific assays are provided on the assay value sheet accompanying each kit, and are lot specific.

2. The expected values are obtained using replicate assay of each manufactured lot of Plasmatrol, manually and using mechanical coagulometers such as Hemostar, Hemostar XF.
3. The individual laboratory values should fall within the expected values.
4. It must however be noted that each laboratory should establish its own normal values and reference range according to GLP.

Remarks

1. When used appropriately, Plasmatrol controls are subjected to the limitations of the assay system deployed.
2. If proper values are not obtained it may indicate problems with one or more variables of the assay system.
3. Stability of the reagent is dependent on storage and handling conditions. Since these can vary between laboratories, each laboratory should determine the stability of the reagent under usual operating conditions.
4. Incorrect mixing of control plasma and reagent, insufficient preparation of plasma/reagent, contaminated reagents and glassware, etc. are a potential source of error.
5. Due to interlaboratory variations in techniques, standardization of test procedures and calibration of equipments, some variation from assigned mean values may be expected.

FIBROSCREEN THROMBIN TIME TEST FOR QUALITATIVE ESTIMATION OF FIBRINOGEN FIBROSCREEN®

(*Courtesy:* Tulip Group of Companies)

Summary

At present there are known to be at least eleven factors in circulating blood, which are required for normal hemostasis. Deficiency in any of these factors viz. Factors I, II, V, VII, VIII, IX, X, XI and XIII results in a notable hemorrhagic condition, and the severity of the bleeding is proportional to the degree of deficiency. In order to treat the hemorrhagic condition, it is important to identify and quantify the deficient factor.

Fibroscreen reagent is one such test reagent, which can identify the deficiency of factor I (fibrinogen). The reagent is used as a source of thrombin to determine the qualitative reactivity of fibrinogen.

Reagent

Fibroscreen reagent is a lyophilized preparation of bovine thrombin of 50 NIH/mL. Reconstitute with 1 mL of distilled water; wait for 5 minutes, do not shake and mix gently by swirling till the solution attains homogeneity. Further keep aside for 10 minutes to attain equilibrium. Gently swirl the vial while drawing the reagent for use. Once reconstituted it is ready to use reagent for the thrombin time test.

Storage and Stability

1. Store the unopened reagent vials at 2–8°C. Do not freeze.
2. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label and carton label.
3. Once reconstituted the Fibroscreen reagent is stable for 6 days when stored at 2–8°C and for 4 hours at room temperature (20–25°C), provided it is not contaminated. Extreme care has to be taken to maintain aseptic precautions while reconstituting, retrieving and handling reagents to prevent contamination. The Fibroscreen reagent vial must be replaced at 2–8°C immediately upon retrieving the reagent for the day's work.

Principle

When a known quality and concentration of Fibroscreen reagent is added to citrated plasma, by observing the time required for clot formation and the quality of clot formed, a qualitative estimation of fibrinogen in the sample can be obtained.

Note

1. In vitro diagnostic reagent for laboratory and professional use. Not for medicinal use.
2. The reagent contains 0.1% sodium azide as preservative.
3. Fibroscreen thrombin reagent is not from a human source, hence contamination due to HBsAg, HIV and HCV is practically excluded.
4. It is very important that absolutely clean and dry micropipettes be used to aspirate and dispense the reagent.
5. Avoid exposure of the reagent to elevated temperatures, direct light and contamination. Immediately replace cap after use and store at recommended temperature.

Quality Control

A known normal control should be run in parallel with each batch of tests. This control may be Tulip plasma coagulation control Plasmatrol-I or freshly drawn normal plasma.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Withdraw blood without undue venous stasis and without frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into tubes, after detaching the needle from the syringe. Mix nine parts of freshly collected blood with one part of sodium citrate (0.109-M mol/L, 3.2%). Centrifuge immediately for fifteen minutes at 3000 rpm (approximately 2000 g) and transfer the plasma into a clean test tube. Plasma must be tested within 3 hours of collection.

Additional Material Required

10 ×75 mm glass test tubes, 0.2 mL precision pipettes, stop-watch, distilled water, fresh plasma.

Procedure

Bring all the reagents and samples to room temperature before testing.

Manual Method

Testing should be done in duplicate at room temperature (20-25°C):

1. To a clean and dry 10 × 75 mm test tube add 0.2 mL of plasma to be tested and 0.2 mL of the reconstituted Fibroscreen reagent.
2. Start a stopwatch simultaneously with the addition of the Fibroscreen reagent.
3. Shake the tube gently to mix the contents and then tilt the tube back and forth.
4. Note the time at the first appearance of the clot and for the remaining portion of 60 seconds for consistency and character of the clot formed.

Interpretation of Results

Normal plasma begins to show clot formation within 15 seconds after Fibroscreen reagent has been added. Because time of clot formation may be influenced by additional factors in the test system, estimation of approximate concentration of fibrinogen cannot be made from the initial clotting time alone but must be also made from observations of the consistency and character of the clot at 60 seconds. At 60 seconds, samples with normal fibrinogen levels will form a firm clot that adheres to the walls of the test tube when the tube is inverted. If either of these parameters are not met, (i.e. clotting time below 15 seconds or formation of a firm adhering clot after inversion of the test tube) abnormality (less than 100 mg%) of the fibrinogen reactivity should be suspected. In such cases quantitative estimation of fibrinogen using Fibroquant is strongly recommended.

Expected Values

A normal value using Fibroscreen reagent is the formation of a solid gel clot in 5-15 seconds, which adheres to the test tube wall on inversion at 60 seconds.

Remarks

1. Fibroscreen thrombin time remains normal in deficiencies of factor XIII (fibrin stabilizing factor).

INTERPRETATION OF FIRST LINE TESTS:

Test				*Condition*
PT	*APTT*	*TT*	*Platelet count*	
N	N	N	N	Disorder of platelet function, factor XIII deficiency, disorder of vascular hemostasis, normal hemostasis
Long	N	N	N	Factor VII deficiency, early oral anticoagulation
N	Long	N	N	Factor VIII: C, IX, XI, XII, prekallikrein, HMWK deficiency,von Willebrand's disease, circulating anticoagulant
Long	Long	N	N	Vitamin K deficiency, oral anticoagulants factor V, VII and II deficiency
Long	Long	N	N	Heparin, liver disease, fibrinogen deficiency, hyperfibrinolysis
Long	Long	N	N	Thrombocytopenia
Long	Long	N	Low	Massive transfusion, liver disease
Long	Long	Long	Low	DIC, acute liver disease

N-Normal

2. Fibrin gels may form in plasma with a fibrinogen concentration below normal. However, these gels are not firm, extrude considerable serum, and tend to slide on the side walls of the tilted test tube. Careful comparison of such gels with the firm clot with normal plasma used as a control will eliminate the possibility of confusion.
3. Fibroscreen thrombin time test is usually performed first before any specific assays are attempted, when a prolongation of (PT and APTT) cannot be explained.

FIBRINOGEN ESTIMATION-QUANTITATIVE FIBROQUANT, REAGENT FOR QUANTITATIVE ESTIMATION OF FIBRINOGEN

(*Courtesy:* Tulip Group of Companies)

Summary

At present there are known to be atleast eleven factors in circulating blood, which are required for normal hemostasis. Deficiency in any of these factors viz factors I, II, V, VII, VIII, IX, X, XI and XIII, results in a notable hemorrhagic condition, and the severity of the bleeding is proportional to the degree of deficiency. In order to treat the hemorrhagic condition, it is important to identify and quantify the deficient factor.

Fibrinogen (Factor I) is a high molecular weight glycoprotein synthesized in the liver, which plays an important role in hemostasis. For normal hemostasis to occur in response to injury or tissue damage, a sufficient concentration of fibrinogen must be present in plasma. Fibrinogen is converted into fibrin by the action of thrombin and is a key component of clot formation.

Fibroquant kit contains lyophilized thrombin and fibrinogen calibrator to determine the quantitative reactivity of fibrinogen. Since the reagent system contains heparin neutralizing substances, heparin levels up to 0.4 IU/mL does not interfere with test results.

When used as a front line test with PT, APTT, platelet count and thrombin time, fibrinogen assay helps in investigating acute hemostatic failure.

Reagent

Fibroquant kit contains:

1. Thrombin reagent, which is a lyophilized preparation from bovine source ~50 NIH units per vial.
2. Fibrinogen calibrator, which is a lyophilized preparation of human plasma equivalent to stated amount of fibrinogen on a mg basis (refer Fibroquant graph paper supplied with each kit for the value of each lot).
3. Owren's buffer, ready to use (pH 7.35).

Storage and Stability

1. Store the unopened reagent vials at 2–8°C. Do not freeze.
2. The shelf-life of the reagents is as per the expiry date mentioned on the reagent vial labels.
3. Once reconstituted the Fibroquant thrombin reagent is stable for 6 days when stored at 2-8°C and for 4 hours at room temperature (20–25°C), provided it is not contaminated. Extreme care has to be taken to maintain aseptic precautions while reconstituting, retrieving and handling reagents to prevent contamination. The reagent vial must be replaced to 2–8°C immediately upon retrieving the reagent for the day's work.
4. The reconstituted Fibroquant fibrinogen calibrator is stable for 6 hours at 2–8°C and for 2 hours at room temperature (20–25°C).

Principle

The addition of thrombin coagulates fresh citrated plasma. The coagulation time is proportional to the fibrinogen concentration. This allows the estimation of plasma fibrinogen by functional clotting assay.

Note

1. In vitro diagnostic reagent for laboratory and professional use. Not for medicinal use.
2. The individual reagents contain 0.1% sodium azide as preservative.
3. Fibroquant thrombin reagent is not from a human source hence, contamination due to HBsAg, HIV and HCV is practically excluded.
4. Fibrinogen calibrator provided in the Fibroquant kit is from a human source, which was tested and found to be non-reactive for HBsAg, HCV and HIV. However, no known test methods can assure that infectious agents are absent. Handle all human products as potentially infectious.
5. It is very important that absolutely clean and dry micropipettes be used to aspirate and dispense the reagent.
6. Avoid exposure of the reagent to elevated temperatures, direct light and contamination. Immediately replace the cap after use and store at recommended temperature.

Quality Control

A known normal control should be run in parallel with each batch of tests. This control may be Tulip plasma coagulation control Plasmatrol-I or freshly drawn normal plasma.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Withdraw blood without undue venous stasis and without frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into tubes, after detaching the needle from the syringe. Mix nine parts of freshly collected blood with one part of sodium citrate (0.109 mol/L, 3.2%). Centrifuge immediately for 15 minutes at 3000 rpm (approximately 2000 g) and transfer the plasma into a clean test tube. Plasma must be tested within 3 hours of collection.

Additional Material Required

10 × 75 mm glass test tubes, 0.2 mL and 0.1 mL precision pipettes, stopwatch, water bath at 37°C, distilled water, automated, semiautomated/mechanical/optical instrument if applicable.

Procedure

Bring all the reagents and samples to room temperature before testing.

Procedure for Fibrinogen Calibration Curve Preparation

1. The Fibroquant thrombin reagent vial must be reconstituted exactly with one mL of distilled water; wait for 5 minutes, do not shake but gently swirl the vial till the solution attains homogeneity. Further keep the vial aside for 10 minutes to attain equilibrium. Once reconstituted it is ready to use for the fibrinogen test.
2. The Fibroquant fibrinogen calibrator vial must be reconstituted with exactly one mL of distilled water; wait for 5 minutes, do not shake, gently swirl the vial till the solution attains homogeneity. Further keep the vial aside for 10 minutes to attain equilibrium. This is the fibrinogen calibrator stock solution.
3. Dilute fibrinogen calibrator stock solution with Owren's buffer as follows:

Test tube no.	*I*	*II*	*III*
Owren's buffer	NIL	0.8 mL	0.9 mL
Fibrinogen calibrator	0.2 mL	0.2 mL	0.1 mL
Dilution (calibrator)	NIL	1:5	1:10

- Pipette 0.2 mL of each fibrinogen calibrator dilution into clean test tubes and prewarm for 3 minutes at 37°C.
- Add 0.1 mL of reconstituted thrombin reagent (prewarmed at 37°C for one minute) and simultaneously start stopwatch.
- Stop the stopwatch at the first appearance of the fibrin web, as the gel clot begins to form and record the time in seconds.
- Repeat steps 1 to 3 for a duplicate test one each calibrator dilution.
- Plot the average of the duplicate test values on Tulip firbrinogen graph paper*.
- Connect the points, which should produce a straight line.
- The calibration curve may be extended beyond the lowest and highest point.

*The calibration curve is valid only for the same lot of Fibroquant thrombin reagent.

Test Procedure for Sample

1. Prepare a 1:10 dilution of plasma specimen with Owrens buffer solution.
2. To a 10 × 75 mm test tube at 37°C add 0.2 mL of 1:10 dilution of plasma sample to be tested.
3. Incubate at 37°C for one minute.
4. To the test tube add 0.1 mL of Fibroquant thrombin reagent (prewarmed at 37°C for one minute) and start the stopwatch simultaneously.
5. Stop the stopwatch at the first appearance of the fibrin web, as the gel clot begins to form and record the time in seconds.
6. Repeat steps 1-5 for a duplicate test.
7. If at the sample dilution of 1:10 the observed clotting time is usually between 8 and 25 seconds, the fibrinogen content is normal (Fibrinogen content between 150 and 400 mg/dL). Assay results can be read off directly from the graph paper provided with the Fibroquant kit for the fibrinogen concentration.
8. If the fibrinogen content is high the clotting time will be less than 8 seconds. In such cases repeat the test at 1:20 dilution of the sample or 1:30 dilution of the sample. The results read off the graph will be multiplied by a factor 2 or 3 for the respective dilution.

9. Conversely, if fibrinogen content is low, the clotting time will be over 25 seconds. Repeat the assay at 1:5 dilution, or if necessary at 1:2 dilution. In this case the results read off the graph will be divided by a factor of 2 or 5 for the respective dilutions.

This procedure can also be performed on an automated/semiautomated mechanical/optical instrument but the equipment manufacturer's methodology should be strictly adhered to.

Remarks

1. Significant levels of heparin and elevated levels of fibrinogen degradation products (FDP) in the patient plasma can cause falsely low fibrinogen results.
2. Insufficient prewarming of plasma and reagent or contaminated glassware may cause erroneous results.
3. EDTA should not be used as an anticoagulant.
4. Use reagents of the same lot for performing the test.
5. Do not interchange reagents from different lots.

FIBRINOLYTIC ACTIVITY

The three methods presented below are measures of fibrinolytic activity in general and are influenced by many factors. These serve as screening procedures, but the specific contribution of the various factors must be determined by other means. The lack of suitable standards makes quantitative measurements unavailable for most diagnostic laboratories.

Euglobulin Lysis Time

Principle

Euglobulin fraction of plasma contains fibrinogen and all the plasminogen activator and plasminogen of plasma but only traces of the anti-plasmins. The lysis of a fibrin clot formed by the addition of thrombin is a measure of the fibrinolytic activity.

Requirements

1. Equipment for collection of blood
2. Centrifuge
3. Topical thrombin
4. Serological pipettes
5. Carbon dioxide. A tank of CO_2 fitted with a valve to allow control of the rate of flow.

Method

1. Blood is collected in the usual manner and mixed immediately with 0.11 M sodium citrate in a ratio of 1 part citrate solution to 9 parts blood.
2. Plasma is obtained by centrifugation.
3. 0.4 mL plasma is placed in a test tube and 7.6 mL distilled water is added.
4. CO_2 is bubbled into the solution through a capillary tube for 30 seconds.
5. The precipitate which forms is collected by centrifugation at about 3,000 rpm for 15 minutes.
6. The precipitate is dissolved in 1 mL M/15 phosphate buffer, pH 7.2.
7. To the euglobin in phosphate buffer, 0.1 mL thrombin (topical thrombin diluted to 100 units per mL with saline) is added. The solution is mixed.
8. Clotting should be rapid. After clotting has occurred, the tube is placed in water bath (37°C) and observed for lysis of clot, which is the end-point.

Result

In normal plasma, a period of 2 to 4 hours is required for euglobin clot lysis to occur (the technique should be standardized in each laboratory).

Dilute Blood Clot Lysis Time

Principle

Plasmin inhibitors lose activity on dilution to a greater extent than fibrinolytic activity. Whole blood is diluted with a buffer solution and clotted by the addition of thrombin. The clot is observed for lysis of the clot.

Requirements

1. Equipment for collection of blood sample
2. Test tube
3. Timer
4. Phosphate buffer, pH 7.4. To 1000 mL distilled water, 9.47 g Na_2HPO_4 is added and dissolved. This is mixed with 250 mL distilled water containing 3.02 g KH_2PO_4
5. Topical thrombin (or any other make) diluted to 100 units per mL with normal saline.

Method

1. Tubes containing 1.70 mL buffer and 0.1 mL thrombin solution are placed in an ice bath.
2. Collect blood sample in standard manner using a syringe that can deliver accurately 0.2 mL aliquots of blood.
3. Add 0.2 mL blood to each of two tubes containing buffer and thrombin and mix.
4. Clotting should occur promptly.
5. Tubes are placed in refrigerator (4°C) for 30 minutes and then transferred to a water bath at 37°C.

6. The clots are observed for lysis. The endpoint is fragmentation rather than complete dissolution of the clot.

Result

Blood from a normal subject should not lyse in less than 6 to 10 hours. The test is qualitative. If results indicate rapid lysis of the clot, more quantitative methods are necessary.

FDPS A QUALITATIVE AND SEMIQUANTITATIVE LATEX SLIDE TEST FOR DETECTING CROSS LINKED FIBRIN DEGRADATION PRODUCTS IN HUMAN PLASMA X-L FDP

(*Courtesy:* Tulip Group of Companies*)*

Summary

During coagulation sequence of reactions occur in the body in response to variety of external and or internal stimuli. The enzymatic cascade reaction terminates in the conversion of fibrinogen to fibrin, by the enzyme thrombin. The fibrin gel is then converted to a stable fibrin clot by thrombin activated factor XIII.

Finally, the fibrin network is dissolved by the enzyme plasmin to generate cross-linked fibrin degradation products (XL FDP). D dimer comprising of two D fragments cross linked together, is the smallest plasmin resistant molecular unit present within XL FDP. Detection of D dimer is invaluable as a diagnostic marker for thrombotic conditions such as disseminated intravascular coagulation (DIC), deep vein thrombosis (DVT) and pulmonary embolism (PE). D dimer levels can also be used to monitor thrombolytic therapy with tPA and with streptokinase, thrombotic complications in pregnancy, acute myocardial infarction, sickle cell crisis, severe septic infections, liver disease, DIC accompanying snake bite and prognosis and response to therapy in cancer.

Reagent

1. **XL FDP reagent**: A uniform suspension of polystyrene latex particles coated with mouse monoclonal anti D-dimer antibody (DD-3B6/22). The reagent is standardized to detect XL FDP $\geq$ 200 ng/mL.
2. **Positive control,** reactive with XL FDP latex reagent.
3. **Negative control,** non-reactive with XL FDP latex reagent.
4. **Phosphate buffer,** for performing semi-quantitative test. All the reagents contain 0.1% sodium azide as preservative.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2-8°C. Do not freeze.
2. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial labels.

Principle

XL FDP slide test for detection of cross-linked fibrin degradation products is based on the principle of agglutination. The test specimen (plasma) is mixed with XL FDP latex reagent. The sensitivity of the reagent is ~ 200 ng/mL, below which samples are negative and above which samples give a positive agglutination reaction.

The crosslinked fibrin degradation products, D dimer, D dimer E, and high molecular weight derivatives are all recognized by Tulip XL FDP reagent incorporating the monoclonal antibodies. No binding was found to the fibrinogen degradation products XYD and E to 20 mg/L or to fibrinogen up to 1000 mg/L.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagents contain 0.1% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. All the reagents derived from human source have been tested for HBsAg and anti-HIV antibodies and are found to be non-reactive. However, handle the material as if infectious.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperature conditions. It is recommended that the performance of reagent be verified with positive and negative controls supplied with the kit.
5. Shake the XL FDP latex reagent vial before use to disperse the latex particles uniformly and improve test readability.
6. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection. Plasma samples are recommended for use with XL FDP test. Fresh EDTA, citrate or heparinized anticoagulated plasma specimens are suitable for performing the test.

Sample storage:	20–25°C	—	8 hours
	2–8°C	—	4 days
Frozen	(–20°C)	—	2 months.

Thaw frozen specimens at 37°C and centrifuge plasma before testing.

KIT Composition

1. XL FDP latex reagent, positive control, negative control, PBS buffer.
2. Glass slide with six reaction circles, disposable sample dispensing dropper, mixing sticks, rubber teat, package insert.

Additional Material Required

Stopwatch, test tubes high intensity direct light source.

Test Procedure

Bring all the reagents and sample to room temperature before performing the test.

Qualitative Method

1. Pipette one drop of plasma specimen onto the glass slide using the disposable dropper provided with the kit. Hold the dropper exactly in vertical position to dispense the drop accurately.
2. Add one drop of XL FDP latex reagent adjacent to the drop of plasma specimen, taking care to hold the dropper in a vertical position while dispensing the drop. Do not let the dropper tip touch the plasma specimen on the slide.
3. Using a mixing stick, mix the plasma and latex reagent uniformly over the entire circle.
4. Immediately start a stopwatch, rock the slide gently, back and forth, and observing for agglutination macroscopically at three minutes.
5. Do not read the test result beyond 3 minutes.

Semiquantitative Method

1. Using PBS buffer solution prepare serial dilutions of the plasma sample 1:2, 1:4, 1:8, 1:16, 1:32 and so on.
2. Pipette each dilutions of plasma specimen on to the separate reaction circles.
3. Add one drop of XL FDP latex reagent to each drop of diluted plasma specimen on to the slide. Do not let the dropper tip touch the diluted plasma specimen on the slide.
4. Immediately start the stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at three minutes.

Interpretation of Results

Qualitative Method

- Agglutination is a positive result indicating D dimer level above 200 ng/mL.
- No agglutination is a negative result indicating absence of clinically significant D dimer levels in the plasma specimen.

Semiquantitative Method

Agglutination in the highest plasma dilution corresponds to the approximate amount of D dimer level in ng/mL.

To calculate D dimer level in ng/mL in the sample, use the following formula,

D dimer level (ng/mL) = 200 × d

Where, d = highest dilution of plasma showing agglutination during the semi-quantitative test of the sample.

Note

Activation of the coagulation system with subsequent microvascular fibrin deposition, and lysis has been reported in diverse clinical conditions such as trauma, surgery, inflammation and malignancy. Elevated levels of plasma XL FDP may be expected to occur in such conditions.

Remarks

1. D dimer half-life is approximately 6 hours in circulation of individuals with normal renal function. Patients with stabilized clots and not undergoing active fibrin deposition and plasmin activation may not give detectable D dimer elevations.
2. In PE, larger the clot size, higher the expected level of circulating D dimer. Conversely, the amount of D dimer released from very small clots may be diluted by the circulation and may not give a detectable increase.
3. Fibrinolysis is a highly regulated process and in delicate dynamic balance. In case of hereditary, acquired deficiency and dysfunction of fibrinogen, the rate of fibrinolysis will be altered there by not giving a detectable D dimer level.
4. As with any laboratory test, detection of elevated levels of XL FDP in a specimen should be correlated with clinical findings.

Screening Tests for Diagnosis of Procoagulant Deficiency

Principle

Most of the coagulation disorders can be classified on the basis of the prothrombin time used in conjunction with the partial thromboplastin time. The effect of $BaSO_4^-$ adsorbed

normal plasma on the results of the abnormal test is of diagnostic importance.

Requirements

1. All reagents and equipment necessary for the prothrombin time and partial thromboplastin time.
2. $BaSO_4^-$ treated normal plasma.

Method

1. The plasma under study is tested in the usual manner by both the prothrombin time and the partial thromboplastin time.
2. Mix equal volumes of the test plasma and normal control plasma. About 0.5 mL of the mixture is needed.
3. Mix equal volumes of the test plasma and $BaSO_4^-$ treated normal plasma. About 0.5 mL of the mixture is needed.
4. Determine the prothrombin time and the partial thromboplastin time each of the mixtures.

Results

1. If both the prothrombin time and the partial thromboplastin time are normal, it is doubtful that clinically significant coagulation disorder is present.
2. If the normal plasma fails to correct an abnormal prothrombin time or partial thromboplastin time, it is likely that the patient has a circulating anticoagulant.
3. If normal plasma corrects either the prothrombin time or the partial thromboplastin time, the presumptive diagnosis is as indicated in the table.
4. Factor XII deficiency will have a pattern similar to that of factor VIII deficiency. The correct diagnosis can be further elucidated by the lack of a real bleeding tendency in the factor XII deficiency. Definite diagnosis can be established only by having samples of plasma known to be specifically deficient in factor VIII and factor XII. Failure of correction of the patient's partial thromboplastin time by plasma known to be deficient in factor VIII establishes the diagnosis of hemophilia. Likewise, failure of correction of the patient's partial thromboplastin time by plasma known to be deficient in factor XII establishes the diagnosis of Hageman trait.
5. Factor XI deficiency may have a pattern similar to that of factor VIII or factor IX deficiency. The correct diagnosis can be further elucidated by the sex-linked recessive transmission of factor VIII or factor IX deficiency. Definitive diagnosis can be established only by having available sample of plasma known to be deficient in factors VIII, IX, and XI. Failure of correction of the patient's partial thromboplastin time by plasma known to be deficient in factor XI establishes the diagnosis of factor XI deficiency.

The two-stage prothrombin method is needed to distinguish a true deficiency of prothrombin from the pattern indicated for factor X deficiency.

A severe deficiency of fibrinogen will have a pattern similar to that of factor V deficiency. A quantitative method for fibrinogen is required to determine the fibrinogen level.

For delving further into details of coagulation disorder diagnosis, Tulip diagnostics provides kits for estimation of—antiplasmin, antithrombin III, A2, antitrypsin, factor II reagent, factor V reagent, factor VII reagent, factor VIII reagent, factor VIII R:AG antiserum, factor IX, factor X reagent, factor XIII, fibrinogen reagent,—macroglobulin, PTT reagent, thrombin reagent, thrombin coagulase and *Staphylococcus* clumping test.

LABORATORY DIAGNOSIS OF COAGULATION DISORDERS

Some properties of the coagulation factors are given below:

1. *Fibrinogen group*: Factors, I, V, VIII, XIII.
   - Thrombin interacts with them all

	Prothrombin	*Partial thromboplastin time*		*Presumptive diagnosis*
Test plasma alone	Test plasma	Test plasma	Test plasma	Deficiency of
	+$BaSO_4$ plasma	alone	+$BaSO_4$ plasma	factor
Long	Normal	Long	Normal	V
Long	Long	Normal	Normal	VII
Normal	Normal	Long	Normal	VIII
Normal	Normal	Long	Long	IX
Long	Long	Long	Long	X

- Activity lost in coagulation process (not present in serum)
- Increase during inflammation, in pregnancy and women on oral contraceptives
- Factors V and VII lose activity in stored plasma
- Liver parenchyma synthesis (except factor VIII).

2. *Prothrombin group:* Factors II, VII, IX and X.
   - Activated form contains an active serine center
   - Liver parenchymal cell synthesis
   - All except prothrombin (II) are not consumed during coagulation (present in serum)
   - Dependent on vitamin K for synthesis
   - Stable, well preserved in stored plasma.
3. *Contact group*: Factors XI, XII.
   - Activated forms contain an active serine center
   - Not dependent on vitamin K for synthesis
   - Stable, well preserved in stored plasma.

Factor VIII is most important as far as coagulation disorders are concerned. Its structure is as follows:

Abbreviations

VIII C = Part has coagulant activity.
VIII R: AG = Factor VIII Related Antigen, effects platelet function also.
VIII WF = von Willebrand factor
(VIII C does not function in the absence of other parts).

- In hemophilia A the VIII C portion is defective so there is lack of clotting activity but platelet function is normal.
- In von Willebrand's disease VIII R: AG is missing so there is abnormal platelet function alongwith lack of clotting activity (VIII C portion does not function alone).

Laboratory Diagnosis of Hemophilia

- Activated partial thromboplastin time raised.
- Whole blood coagulation time prolonged in severe cases.
- Factor VIII clotting assay (VIII C) lowered.
- Immunological methods show normal VIII R: AG.
- Bleeding time and prothrombin time tests are normal
- Carrier females have half the clotting activity (VIII-C) expected for the level of VIII R: AG.

Christmas Disease (Hemophilia B)

- Activated partial thromboplastin time prolonged
- Whole blood clotting time raised (in severe cases)
- Factor IX clotting assay diminished
- Both bleeding time and prothrombin time tests are normal.

von Willebrand's Disease

- Prolonged bleeding time
- Low levels of factor VIII clotting activity (VIII C)
- Low levels of factor VIII related protein VIII R:AG
- Defective platelet aggregation with ristocetin. The aggregation response to other agents (collagen, thrombin, adrenaline) is normal.
- In some patients there is defective retention of platelets in glass bead columns.

Hemorrhagic Disease of Newborn

- The prothrombin time and activated partial thromboplastin time are both prolonged
- The platelet counts and fibrinogen levels are normal with absent fibrin degradation products.

AUTOMATION IN COAGULATION ANALYSIS

Hemostar

Sleek, user-friendly coagulation analyzer based on turbophotometric detection of clot-based assays. Hemostar has ten inbuilt incubation positions for sample and one location for reagents (Fig. 10.1).

- It allows the user to make all basic tests for the analysis of plasmatic hemostatic phase, e.g. Quick test (PT), aPTT, TT, fibrinogen, defective factors, etc.
- It is a totally open instrument to be used with all kinds of quality reagents, simply by following the producers instructions.

FIG. 10.1: Hemostar
(*Courtesy:* Tulip Group of Companies)

- It includes a magnetic stirrer system in order to mix well the reagents and plasma.
- Optical group: Made up of a Wolfram lamp at low voltage and long self-life, an optical light pass, the reaction cell holder and a solid state photocell which registers all absorption changes produced.
- Thermostatic block: Thermostated device at 37 ± 0.1°C with magnetic stirrer motor built-in.
- Screen: Four digit indicator with tenth of second precision, thermostatic block control temperature LED (37°C) and two operation keys, the chronometer can be used seperately to control incubation time.
- Tests: Quick Test (PT), aPTT, TT, Fibrinogen, deficiency factors, etc.
- Power requirements: AC 220 V. 50 Hz 120 VA
- Dimensions: 180 × 300 × 140 nm
- Weight: 2, 3 kg.

Technical Features

System	Automatic single channel coagulometer based on turbo-photometric clot detection system
Measurement	Opto-mechanical measuring system
	Automatic start of measurement on addition of reagent/sample for precise result
	On screen digital chronometer to measure tenth of a second accurately
Sensitivity	Highly-sensitive in all measuring range because of Opto-mechanical measuring system (combination of optical controls with mechanical movement of magnetic stirrers)
Incubator	Inbuilt Incubator (solid state) fixed at 37°C
	Incubator lodges 10 reaction cuvettes and one reaction vial
Testing mode	PT, APTT, TT, fibrinogen and factor assays
General	
Lamp source	Low voltage tungsten lamp
Detector	IR detector
Motor	For stirring action of magnetic stirrer
Display	Back illuminated LCD with single row and 14 characters.
Power supply	220V, 50 Hz/60 Hz
Power consumption	50 VA
Serial output	RS 232 standard
Working temperature	15 to 35°C
Dimensions	18 × 30 × 40 cm
Weight	2.2 kg

Hemostar XF

Single channel coagulometer with turbo-photometric clot detection principle with an opto-mechanical measuring system which improves sensitivity of detecting weak fibrin polymers. Hemostar XF has 10 open locations which enables to perform PT, APTT, TT, fibrinogen assays and factors assays. This walk away plug and play system has been designed to make complicated clotting analysis of hemostasis system into a simple error free task (Fig. 10.2).

- Single-channel coagulometer, fully-controlled by built-in microprocessor
- Turbophotometric detection of fibrin polymer formation (clots)
- Solid state thermostat set at 37°C
- Keyboard for programing and data entry
- Digital control and results display
- Integral printer for easier transcription of results
- Enables performance of all types of hemostasis plasma phase studies and particularly: Quick, APPT, TT, fibrinogen, factor studies
- Optics: Low voltage photometry lamp
- Truncated cone reading cell
- Photofeed detection
- Magnetic stirrer motor
- Thermostat: Metal block thermostated to 37 ± 0.1°C by solid state elements
- Capacity for 10 cells and a reagent bottle
- Programing: The CLOT 1A has a permanent memory for programing the parameters for each coagulation

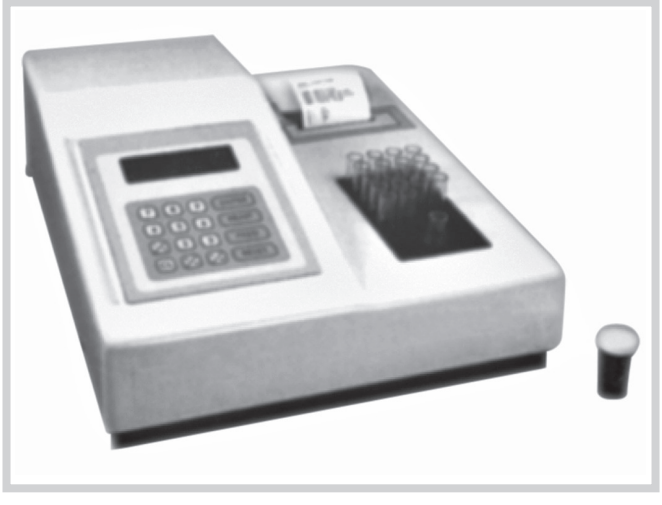


FIG. 10.2: Hemostar XF
(Courtesy: Tulip Group of Companies)

technique. These parameters remain stored in the memory until they are reprogramed, even when the unit is switched off
- The keypad enables the user to sequentially access the programing and speeds up to date entry during routine use of the instrument
- The digital display functions as a programing and results monitor
- Results: Displayed on the screen and in printout form, containing all parameters and the relevant identification number
- Size: 36 × 15 × 35 cm
- Weight: 3.7 kg
- Power supply: 220 V 50 Hz 100 VA.

Technical Features

System	Microprocessor controlled automatic single channel coagulometer based on turbophotometric clot detection system
Measurement	Opto-mechanical measuring system
	Automatic start of measurement on addition of reagent/ sample for precise result
	On screen digital chronometer to measure tenth of a second accurately
Sensitivity	Highly-sensitive in all measuring range because of opto-mechanical measuring system (combination of optical controls with mechanical movement of magnetic stirrers).
Incubator	Inbuilt Incubator (solid state) fixed at 37°C.
	Having 19 incubation positions
Testing Mode	PT, APTT, TT, fibrinogen and factor assays
General	
Programing	10 Locations for programing parameters
Key board	Key board for programing and data entry
Printer	Built-in quiet thermal printer for direct reporting of result in INR, per cent activity, PTR and APTT ratio
Serial output	RS 232 standard
Lamp source	Low voltage tungsten lamp
Detector	IR detector
Motor	For stirring action of magnetic stirrer
Display	Back illuminated LCD with 4 rows of 20 characters
Working temperature	15 to 35° C
Power supply	220 V, 50 Hz, 60 Hz
Power consumption	100 VA
Dimensions	31 × 15 × 35 cm
Weight	3.7 kg

CoaLAB 6000

Fully automated compact and versatile bench top coagulation analyzer for clotting assays based on the patented turbodensitometric principle of clot detection. CoaLAB 6000 is engineered to operate in random access mode for PT, APTT and fibrinogen with an option for batch and stat mode. CoaLAB 6000 has the agility for throughput of 130 PT tests per hour. CoaLAB is an ideal coagulation analyzer designed to simplify complicated coagulation analysis into a walkway task for laboratories with high and medium throughput (Fig. 10.3).

Compact

- Bench top coagulation analyzer
- Built-in computer with display and keypad
- Internal thermal printer
- On-board waste box for used cartridges.

Versatile

- Single and double determination adjustable
- Primary and secondary tubes can be used
- All reagent positions can be stirred
- On or off switch option allowing real time or patient oriented printing of results
- Stat-program
- Automatic reagent level control
- All test parameters can be reprogramed and/or changed by the user.

User Friendly

- Easy installation of the analyzer
- Optimal user guidance through routine software

FIG. 10.3: CoaLAB 6000
(*Courtesy:* Tulip Group of Companies)

- Easy method of change via chip cards
- Optimized cuvette cartridge consumption
- Preprogramed method settings and evaluation procedures for: PTT, aPTT and fibrinogen assays
- Updating and/or upgrading via chipcards.

Technical Specifications

CoaLAB 6000	Fully automated coagulation analyzer for clotting assays
Measuring principle	Turbodensitometric. This opto-mechanical principle is able to measure lipemic and turbid plasma samples and reagents containing kaolin
Measuring station	6 measuring channels (parallel working mode)
Loading station	18 sample positions (for primary and special cups), 6 reagent positions (for 4 mL/10 mL vials) at room temperature. All reagent positions can be stirred. 6 positions for cuvette cartridges, each cartridge includes 6 cuvettes
Cuvette	Cuvette cartridges with 6 cuvettes including mixers, reagent vials, sample-cups. Test volume: max 250 µL
Traverse	Movement into 2 directions X-Z, for pipetting and transport of plasma and reagent Pipettor heated to 37°C. A level sensor detects the presence of liquid
Dilutor	250 µL syringe, 1 pipetting tube
System liquid	Distilled water for washing and cleaning of pipettor
Washing solution	Special washer to avoid carry-over of thrombin
Display	Alphanumeric LCD-display, 2 lines, 20 characters each
Operating mode	Random access mode for PT/aPTT and fibrinogen, batch mode selectable
Throughput	>120 PT tests/hour
Printer	Built-in thermal printer
Printout	Patient and result oriented (selectable)
Barcode reader	Optional, for fast introduction of patient/sample numbers
Interfaces	1 × RS232 C host/service, 1× barcode scanner
Power supply	100–240 V, 57–s63 Hz, 150 VA

Contd...

Contd...

Dimensions	72 × 45 × 55 cm (~28.3 × 17.7 × 21.7 in)
Weight	17 Kg (~ 35 pounds)
Software features	Optimal user guidance through routine software
	Method menu for PT, aPTT and fibrinogen
	Additional assays evaluated on the analyzer: Factor II, V, VII, VIII, IX, X, XII and thrombin time. Documentation of all test parameters, e.g. calibration curves, date, time, conversions of percent, g/L, mg/dL, ratio, INR and incubation time can automatically be loaded via chipcard
	Easy method change via chipcard
	Two software languages (German/English) on board
	Consecutive award of sample/patient ID's or by optional barcode scanner or manually by user
	External service and research program
Special features	Single and double determination adjustable
	On board waste box for used cartridges, for hygienic disposal of used cuvette cartridges
	Plasma or reagent start (can be configured). Optimized cuvette cartridge consumption to avoid waste of unused cuvettes
	On or off switch option allowing "real time" or patient oriented printing
	User oriented result management (print-out, LCD and host)
	Advanced paper feed button. Wide range power supply 100 - 240 V, 47-63 Hz
	Easy installation of analyzer ("plug and play")
	Highlighted status/error messages in printout
	Special check of liquid level in washing station. Optimized liquid check at pipettor
	Optimized cuvette cartridge guidance
	Software controlled cuvette cartridge detection
	Acoustic status signals of analyzer for convenient user information. Easy change of analyzer tubings

TROUBLESHOOTING

General Instructions for Coagulation Test

Possible causes	*Solutions*
Preparation of patients	
1. Patients heavily exercised before blood colletction	Although no special preparation of patients is required prior blood collection, fasting patients or patients on a light non-fatty meal are preferable. Fasting samples collected provide desirable lower opacity, which improves the sensitivity of clot detection especially when photo-optical instruments are being used
2. Erroneous results are obtained due to varying opacity	Turbid, lipemic or grossly hemolyzed samples vary in opacity and therefore, generate erroneous results
Sample collection techniques	
1. Frothing of blood	Blood should be withdrawn without undue venous stasis and without frothing into a plastic syringe with a short needle of 19 to 20 SWG. The venepuncture must be a 'clean' one besides the tourniquet should not be placed too tightly or for extended lengths of time. Patting the venepuncture site should also be avoided
2. Delay in mixing blood with anticoagulant	Distribute blood into test tubes after detaching the needle from the syringe. Mix blood with the anticoagulant immediately by gentle inversion of the tube
3. Formation of microclots leading to artificially prolonged results	Clean' venepuncture is essential to avoid formation of microclots at the site of venepuncture and consumption of factors, which will lead to artificially prolonged results.
4. Use of improper needle for withdrawal of blood	With smaller bore longer needles blood will remain in contact with metal surface for a longer time leading to initiation of clotting or partial consumption of factors being assayed causing error in results. Therefore, short bigger bore needles are recommended since it allows free flow of blood within the syringe and reduces blood contact with metal surface.
Sample preparation	
1. Choice of anticoagulant	The anticoagulant used for most coagulation procedures is sodium citrate or preferably buffered sodium citrate
2. Shift in the ratio of citrate to blood	The optimum ratio of citrate to blood is 1 part of anticoagulant to 9 parts of blood • More blood, less citrate: Leads to formation of clots, consumption of factors and subsequent prolongation of results during test • More citrate, less blood: Consumption of calcium from the reagents giving prolonged test results
Sample processing and storage	
Possible causes	*Solutions*
1. Improper containers used for blood collection and processing	Containers for blood collection and processing should be clean and dry, free of detergents; acids and alkalies ideally made of plastic or siliconized glass tubes
2. Samples used for a long time are used for testing	Fresh samples should be used for testing. If specimen are kept at 22–24°C then they must be tested within 2 hours and kept at 2–4°C, then within 3 hours
3. Sample not stored properly	Samples stored for future testing should be capped tightly to ensure accuracy of results
4. Centrifugation time and speed not maintained	Excessive centrifugation may destroy clotting factors due to heat generation. Maintain proper centrifugation speed and time to avoid erroneous results

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Calibration of instruments/equipments	
1. Water baths not preset properly	To achieve accuracy and reproducibility, preset water bath at 37 ± 0.5° C
2. Improperly calibrated pipettes	Prior to testing, check whether the pipettes so used are being regularly calibrated or not
Storage and procedure	
1. Deterioration of reagents due to thermal stress and high ambient temperatures	Most coagulation reagents are extremely delicate. For them to maintain their sensitivity and performance over the usage period recommended storage temperatures must strictly adhered to
2. Error in performing the test procedure	Ensure that the procedure and addition sequence is followed as indicated in the package insert

Critical requirement of MNPT in the derivation of INR

- MNPT should ideally be derived by each laboratory from 20 or more normal patients for a given PT reagent and lot under use. If "normal control plasmas" are used in place of patient plasma for arriving at MNPT, it can effect the evaluation of the patients' level of anticoagulation

ISI value of PT used and method of clot detection:

- INR looses some precision when comparisons are made with thromboplastins with different ISI values. ISI values should be adapted to the methods used for clot detection ideally close to 1.0 as possible

INR System

Accuracy of the INR system depends on:

- The INR system effectiveness would still depend on the calibration of the coagulation instruments as well as thromboplastin reagents used
- Derivation of the correct MNPT and use of mean normal range in each laboratory
- Usage of thromboplastin reagents with ISI of preferably 1.0 or as close to 1.0 as possible
- The correct use of formula to compute the INR
- Uniform understanding of the INR system by clinicians as well as laboratarians

PROTHROMBIN TIME

Uniplastin®/Liquiplastin®/Lyoplastin®

Problem: Prolonged Clotting Time

Possible causes	*Solutions*
1. Oxalated plasma may be used	Factor V and factor VII are more labile in sodium oxalate. Buffered 3.2% sodium citrate is an ideal anticoagulant since factor V and factor VII are more stable in citrate. Therefore, citrate plasma should be used
2. Plasma not tested immediately after blood collection	Plasma must be tested within 3 hours of blood collection
3. Insufficient prewarming of the plasma and reagents	Plasma should be incubated for 3–5 minutes at 37°C and the reagents should be incubated for at least 10 minutes at 37°C before commencing the testing procedure. Do not incubate the entire vial. Incubate only the requisite quantity
Possible causes	*Solutions*
4. Incorrect mixture of blood and sodium citrate. The concentration of sodium citrate, which is used for the test, may be incorrect	Ensure that nine parts of freshly collected blood are mixed with one part of sodium citrate for normal hematocrit or PCV. For occasional patients with PCV less than 20% (e.g. Microcytic hypochromic anemia) and greater than 55% (e.g. Polycythemia vera), the anticoagulant to blood ratio must be readjusted using the formula, $C = 1.85 \times 10^{-3}(100\text{-}H)V$

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	Where, C = Volume of sodium citrate in mL V = Volume of whole blood + sodium citrate in mL H = Hemocrit in percentage
5. The manufacturer's test protocol not followed in case of automated instruments	Adhere to the instrument manufacturer's instructions and test protocol
6. Temperature of water bath not set correctly	The temperature of the water bath should be preset at 37 ± 1°C
7. Expired reagents are used for testing	Check the expiry date of the reagents before use
8. Incorrect addition of reagent	The test requires extreme precision, besides the addition of the reagent should be followed as per the instructions given in the package insert. Exactly 0.1 mL of plasma and 0.2 mL of liquiplastin/uniplastin reagent prewarmed at 37°C should be used. Well-calibrated micropipettes should be used for dispensing
9. Contaminated reagents and glassware used for testing	Check the working of the reagents with normal control plasmas. Check the reagents for turbidity. Ensure that clean and dry glassware and micropipettes are used
10. Contaminated/wet micro-pipette tips used for retrieving the reagent	Ensure that clean and dry tips are used for retrieving the reagent
11. Clotting times of patients on anticoagulant therapy depends on the type and dosage of anticoagulants and also on the time and last dose of anticoagulant	The history of the patient must be taken into consideration to determine the anticoagulant used as well as the time lag between specimen collection and last dose of anticoagulant
12. Error in reading and interpretation of test results: a. The stopwatch is not stopped as soon as the gel clot is seen b. Clot formation is observed in inadequate light c. Water droplets are present at the base of the tube	The test protocol should be adhered to. The results should be read and interpreted as per the test protocol The stopwatch should be stopped as soon as the gel clot is seen since there is a difference of 2–3 seconds between the beginning and end of clot formation There should be adequate light while observing for clot formation Wipe the base of the tube to remove additional water as it hampers reading of the test results.
13. Improper storage condition leading to reagent precipitation	To maintain the sensitivity and performance of the reagent, avoid thermal stress due to exposure to high ambient temperature thereby causing precipitation, ensure that the reagent is stored at the recommended temperature
14. Improper mixing of the reagent after prolonged storage at 2-8°C	On prolonged storage at 2-8°C, thromboplastin suspension tends to settle down. Homogenize the reagent by resuspending before use
15. In case of automated instruments, the entire reagent vial is incubated thereby leading to deterioration of the reagent on repeated incubation	Only the requisite quantity of the reagent for performing the test should be incubated

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Problem: Shortened Clotting Time

Possible causes	*Solutions*
1. Broken glassware used allowing silica to trigger the clotting reaction	Ensure that broken glassware is not used for testing purposes
2. Administration of drugs/other clinical conditions	PT tests are shortened because of the administration of drugs such as antihistamines, butabarbital, phenobarbital, caffeine, oral contraceptives and vitamin K. It is therefore essential to know the patient history to determine the type of anticoagulant used and the time lag between the last dose of the anticoagulant and specimen collection

DPTT/PTTK

Liquicelin-E®

Problem: Prolonged Clotting Time

Possible causes	*Solutions*
1. Oxalated plasma may be used	Factor V and factor VII are more labile in sodium oxalate. Buffered 3.2% sodium citrate is an ideal anticoagulant since factor V and factor VII are more stable in citrate. Therefore, citrate plasma should be used
2. Plasma not tested immediately after blood collection	Plasma must be tested within 3 hours of blood collection
3. Insufficient prewarming of the plasma and reagents	Plasma should be incubated for 3–5 minutes at 37°C and the reagents should be incubated for at least 10 minutes at 37°C. Do not incubate the entire vial. Incubate only the requisite quantity
4. Incorrect mixture of blood and sodium citrate. The concentration of sodium citrate, which is used for the test, may be incorrect	Ensure that nine parts of freshly collected blood are mixed with one part of sodium citrate for normal hematocrit or PCV. For occasional patients with PCV less than 20% (e.g. Microcytic hypochromic anemia) and greater than 55% (e.g. polycythemia vera), the anticoagulant to blood ratio must be readjusted using the formula, $C = 1.85 \times 10^{-3} (100\text{-}H)V$. Where, C = volume of sodium citrate in mL V = volume of whole blood + sodium citrate in mL H = Hemocrit in percentage
5. Molarity of calcium chloride	Check the molarity of the calcium chloride being used. It should be 0.025 M
6. Contaminated/wet micropipette tips used for retrieving the reagent	Ensure that clean and dry tips are used for retrieving the reagent.
7. The mixture of plasma and reagent is not incubated for minimum time period of 3 minutes	0.1 mL of plasma and 0.1 mL of Liquicelin-E reagent should be mixed well and incubated for exactly three minutes at 37°C for effective contact activation and to obtain accurate results
8. Error in reading and interpretation of test results. a. The stopwatch is not stopped as soon as the gel clot is seen	The test protocol should be adhered to. The results should be read and interpreted as per the test protocol The stopwatch should be stopped as soon as the gel clot is seen since there is a difference of 2-3 seconds between the beginning and end of clot formation

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Possible causes	*Solutions*
b. Clot formation is observed in inadequate light	There should be adequate light while observing for clot formation
c. Water droplets are present at the base of the tube	Wipe the base of the tube to remove additional water as it hampers reading of the test results
9. Incorrect addition of reagent	The test requires extreme precision, besides the addition of the reagents should be as per the instructions given in the package insert. Exactly 0.1 mL of plasma, 0.1 mL of Liquicelin-E reagent and 0.1 mL of calcium chloride prewarmed at 37°C should be used. Well-calibrated micropipettes should be used for dispensing
10. Temperature of water bath not set correctly	The temperature of the water bath should be set at 37 ± 1°C
11. Cotaminated reagents (APTT and calcium chloride) and glassware	Check the working of the reagents with normal control plasmas. Check the reagents for turbidity. Ensure that clean and dry glassware and micropipettes are used
12. Clotting times of patients on anticoagulant therapy depends on the type and dosage of anti-coagulants and also on the time lag between specimen collection and the last dose of anticoagulant	The history of the patient must be taken carefully to determine the anticoagulant used as well as the time lag between the specimen collection and the last dose of anticoagulant
13. Improper storage condition leading to reagent precipitation	To maintain the sensitivity and performance of the reagent, avoid thermal stress due to exposure to high ambient temperature thereby causing precipitation, ensure that the reagent is stored at the recommended temperature

Problem: Shortened Clotting Time

Possible causes	*Solutions*
1. Broken glassware allowing silica to trigger the clotting reaction	Ensure that broken glassware is not used for testing purposes
2. Administration of drugs/other clinical conditions	APTT tests are shortened because of the administration of drugs, oral contraceptives or conjugated estrogen therapy. It is, therefore, essential to know the patient history to determine the type of anticoagulant used and the time lag between the last dose of the anticoagulant and specimen collection

FIBRINOGEN ESTIMATION

Fibroscreen®/Fibroquant®

Problem: Prolonged Clotting Time

Possible causes	*Solutions*
1. Oxalated plasma may be used	Factor V and factor VII are more labile in sodium oxalate. Buffered 3.2% sodium citrate is an ideal anticoagulant since factor V and factor VII are more stable in citrate. Therefore, citrate plasma should be used
2. Plasma not tested immediately after blood collection	Plasma must be tested within 3 hours of blood collection.
3. Insufficient prewarming of the plasma and reagents	Plasma should be incubated for 3–5 minutes at 37°C and the reagents should be incubated for at least 10 minutes at 37°C Do not incubate the entire vial. Incubate only the requisite quantity.
4. Incorrect mixture of blood and sodium citrate. The concentration of sodium citrate, which is used for the test, may be incorrect	Ensure that nine parts of freshly collected blood are mixed with one part of sodium citrate for normal hematocrit or PCV. For occasional patients with PCV less than 20% (e.g. Microcytic hypochromic anemia) and greater than 55% (e.g. polycythemia vera), the anticoagulant to blood ratio must be readjusted using the formula, $C = 1.85 \times 10^{-3} (100\text{-}H) V$ Where, C = volume of sodium citrate in mL V = volume of whole blood-sodium citrate in mL H = Hemocrit in percentage
5. Error in reading and interpretation of test results • The stopwatch is not stopped as soon as the gel clot is seen • Clot formation is observed in inadequate light • Water droplets are present at the base of the tube	The test protocol should be adhered to. The results should be read and interpreted as per the test protocol The stopwatch should be stopped as soon as the gel clot is seen since there is a difference of 2–3 seconds between the beginning and end of clot formation There should be adequate light while observing for clot formation Wipe the base of the tube to remove additional water as it hampers reading of the test results
6. Temperature of water bath not set correctly	The temperature of the water bath should be set at 37 ± 1°C
7. Contaminated reagents and glassware	Check the working of the reagents with normal control plasmas. Check the reagents for turbidity. Ensure that clean and dry glassware and micropipettes are used
8. Improper storage condition leading to reagent precipitation	To maintain the sensitivity and performance of the reagent, avoid thermal stress due to exposure to high ambient temperature thereby causing precipitation, ensure that the reagent is stored at the recommended temperature
9. Contaminated/wet micropipette tips used for retrieving the reagent	Ensure that clean and dry tips are used for retrieving the reagent
10. Improper plotting of calibration curve	The calibration points must be plotted correctly for getting the exactly values of test fibrinogen levels
11. Significant levels of heparin and elevated levels of fibrinogen degradation products (FDP) in the patient plasma	

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12. Clotting times of patients on anticoagulant therapy depends on the type and dosage of anticoagulants and also on the time lag between specimen collection and the last dose of anticoagulant	The history of the patient must be taken carefully to determine the anticoagulant used and FDP levels in the patient plasma as well as the time lag between the specimen collection and the last dose of anticoagulant

Problem: Shortened Clotting Time

Possible causes	*Solutions*
1. Broken glassware allowing silica to trigger the clotting reaction	Ensure that broken glassware is not used for testing purposes
2. With fibroscreen, fibrin gels may form in the plasma with a fibrinogen concentration below normal	These gels are not firm, extrude considerable serum, and tend to slide on the sidewalls of the tilted test tube. Careful comparison of such gels with a firm clot with normal plasma as a control will eliminate the possibility of confusion.

Fibrinogen Degradation Products (D-Dimer) Estimation XL-FDP®

Problem: False Positive Results

Possible causes	*Solutions*
1. Markedly lipemic, hemolyzed and contaminated serum samples could produce nonspecific results	Avoid using lipemic, hemolyzed and contaminated samples for testing
2. Drying of reagent on the slide	Do not read results beyond 3 minutes. The test should not be carried out directly under the fan
3. Presence of dust or debris on the glass slide used	Dust or debris could be misinterpreted as agglutination, therefore, only clean and dry glass slides must be used for testing
4. Latex particles contaminated with positive control/positive sample	Care must be taken to see that the reagent dropper tip does not touch the sample taken on the slide during dispensing of the reagent
5. Dried latex particles observed in the latex reagent • During slide test with negative control • In the dropper of the vial (due to freezing of the latex reagent during storage) • Improper dispensing of the entire reagent from dropper	Immediately after performing the test, transfer the contents of the reagent dropper back into the reagent vial Ensure that no reagent is left behind in the dropper. Close the cap of the reagent vial properly and store it back at 2–8°C Do not freeze the reagent vial
6. Activation of coagulation system with subsequent microvascular fibrin deposition and lysis has been reported in diverse clinical conditions such as trauma, surgery, inflammation and malignancy	Elevated levels of plasma XL FDP may be expected to occur in such conditions. Note the clinical history of the patient before arriving at a final diagnosis

Problem: Delayed Agglutination

Possible causes	*Solutions*
1. Cold reagents are used for testing	Bring all reagents and samples to room temperature before commencing the testing procedure

Problem: False Negative Results

Possible causes	*Solutions*
1. Serum is used as test specimen	Always use plasma as test specimen for performing the test
2. The reagent may be damaged due to microbial contamination or exposure to extreme temperatures	Check the performance of the latex reagent using known positive samples, if the latex reagent is working then the positive control may have deteriorated
3. Weak agglutination is difficult to interpret	Shake the latex reagent well before use to disperse the latex particles uniformly and improve the test readability
4. Excess sample dispensed leading to prozoning	Dilute the plasma and check for agglutination. If no agglutination is observed with the neat sample but agglutination is observed with the diluted sample, then it may be due to prozoning. Determine the titer. Dispense exact amount of sample as mentioned in the package insert
5. Samples stored for a long period of time are used as specimens	Fresh samples are preferable for testing, however; samples can be stored for up to a week at 2–8°C
6. In PE, if the clots are of small size. The amount of D dimmer released from very small clots may be diluted by the circulation	
7. Patients with stabilized clots and not undergoing active fibrin desposition and plasmin activation may not give detectable D dimmer elevations	The clinical history of the patient must be taken consideration to determine the D dimmer levels in the patient
8. In case of hereditary, acquired deficiency and dysfunction of fibrinogen	
9. If the conclusion of false negative result has been arrived at by comparison with another kit, then this other kit could be giving a false positive reaction	Run the test with a third kit to validate results

Problem: Positive Control Giving Negative Reaction

Possible causes	*Solutions*
1. The positive control may have deteriorated due to contamination or exposure to extreme temperatures	Check the performance of the latex reagent; using known positive samples, if the latex reagent is working then the positive control may have deteriorated

CHAPTER 11

Blood Banking (Immunohematology)

The ABO and Rh are the major (clinically significant) blood group antigens though almost 400 of them have been recognized. Given below are the more important blood group systems:

1. ABO, 2. Rhesus, 3. Dell, 4. Duffy, 5. Kidd, 6. Lutheran, 7. Lewis, 8. P, 9. MNS, 10. I. (All of the above if mismatched can cause hemolytic transfusion reaction and 1 to 6 can be responsible for hemolytic disease of the newborn).

BLOOD GROUP ANTIBODIES

Naturally Occurring Antibodies

Under normal circumstances, the newborn has no ABO antibodies. However, after 10–20 weeks later, moderate amount of antibodies are present which appear without any specific antigenic stimulus. So, these are called naturally occurring antibodies. Anti-A and anti-B are important examples of this class. They are IgM immunoglobulins, they react optimally at room temperature, they are also called cold antibodies. They are complete antibodies in serological behaviors, because these antibodies readily agglutinate the red cells carrying the corresponding antigen in saline.

Immune antibodies are produced in response to immunization by either transfusion or pregnancy. They are usually IgG antibodies, they react best at body temperature—37°C and are called warm antibodies. Rh antibody (anti-D) is important immune antibody. They are often called incomplete antibodies as they do not cause agglutination of red cells with corresponding antigen in saline. These antibodies cause only sensitization or coating of the red cells.

In 1900, Karl Landsteiner discovered the blood groups ABO and classified blood into A, B and O groups. A fourth blood group 'AB' was discovered by Landsteiner's associates, Von Decastello and Struli in 1902.

The four blood groups are determined by the presence or absence of blood group antigens (agglutinogens) on red blood cells and accordingly an individual's group is A, B, AB or O (O denotes absence of A or B antigens). In addition, it has been shown that corresponding to antigen A and B, there are naturally occurring antibodies anti-A and anti-B (agglutinins) in the plasma/serum of individuals whose red cells lack the corresponding antigen. Group A individuals have anti-B, group B individuals have anti-A, group, O individuals have both anti-A and anti-B and group AB individuals have no agglutinins in plasma/serum.

It was further shown that Group A could be subdivided into two principal subgroups A_1 and A_2. On the basis of this, ABO system is divided in six main groups A_1, A_2, B, A_1B, A_2B and O.

Genetics of ABO System (Table 11.1)

The ABO system follows Mendelian law of inheritance. The locus for ABO grouping is a chromosome 9, which is occupied by one of three major allelic genes namely, A, B and O. Each individual has a pair of chromosomes (one from each parent). The A and B genes are dominant, while O gene is recessive, thus, not detected directly and accordingly absence of A and B antigens on red cells indicates 'O' blood group (Fig. 11.1).

TABLE 11.1: The ABO antigens and corresponding antibodies

Antigen on RBC	*Antibody in Plasma/serum*	*Bloodgroup*
A	anti-B	A
B	anti-A	B
AB	none	AB
None	anti-A and anti-B	O

Biochemistry

The expression of A and B genes appears to be dependent on another gene called H gene. H gene is inherited, independent of A, B and O genes. H gene is expressed as both homozygous (HH) and heterozygous (Hh). When no H gene is inherited, a (hh) phenotype results which is extremely rare. This is commonly called Bombay group. Bombay group individuals are homozygous for hh gene.

As shown in Figure 11.2, there is a basic precursor substance which is converted to H substance by an enzyme L. Fucosyltransferase (a product of H gene).

H substance is next acted upon by A and/or B gene specified transferase enzyme and is converted to A and/or B antigens. The 'O' gene is an amorph (no gene product) and thus, group 'O' cells enter only H substance.

The persons with genetic configuration of 'hh' cannot act on the precursor substance and the precursor substance remains unaltered. Since no H substance is produced, the ABO genes remain inactive and there is no conversion to A, B or H antigen (These are the persons called Bombay Group).

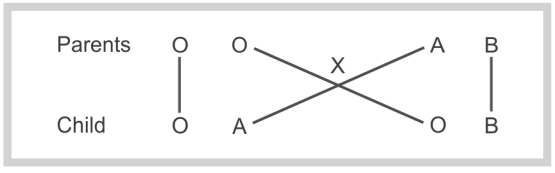

FIG. 11.1: The possible phenotypes and genotypes in ABO group system

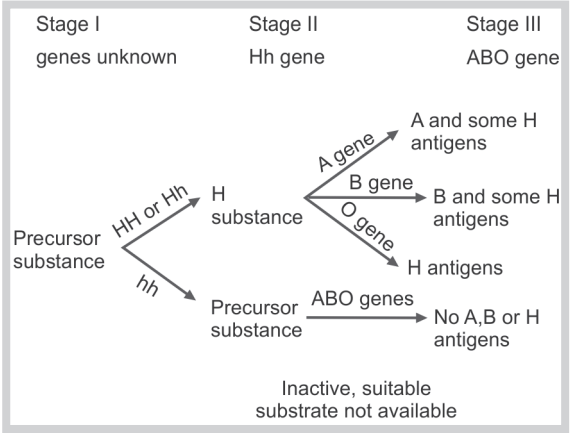

FIG. 11.2: Stages in production of blood group determinants of red cells

TABLE 11.2: The possible phenotypes and genotypes in ABO group system

Phenotypes	*Genotypes*
A_1	A_1A_1, A_1,A_2A_1,O
A_2	A_2A_2,.A_2O
B	BB, BO
A_1B	A_1B
A_2B	A_2B
O	OO

Subgroups of A and AB

Anti-A serum very seldom differentiates between A_1 and A_2. For this reason, we use human anti-A_1 and lectin anti-A which agglutinate A_1 and A_1B cells but not A_2 and A_2B cells. About 20% of persons with A antigen in A or AB group are A_2 or A_2B.

It is not necessary to classify group A patient or donors as A_1 or A_2 except when the individual serum contains anti-A_1. Anti-A_1 occurs in the serum of 1–8% of A_2 group persons and 22–35% of A_2B group persons.

Anti-A_1 causes discrepancies between ABO cell and serum groupings and may cause crossmatch incompatibilities, but it is considered clinically significant if it reacts at 37°C.

Subgroups weaker than A_2 occur infrequently. They are characterized by declining number of 'A' antigens on red cells and reciprocal increase in H reactivity. Weaker variants of A are A_3, Ax, Am and A intermediate.

Subgroups of B

Subgroups of B are clinically not significant, but they can be Bx and Bm type.

Antibodies of ABO System

The antibodies in ABO are usually naturally occurring and are mostly IgM. However, IgG classes are also present. IgG anti-A and IgG anti-B are found more commonly in group O individuals.

Anti-A and anti-B are usually not produced in infants up to age of 3–6 months. However, they reach a maximum titer by 5–10 years and then gradually become weaker as the individual ages. The antibodies found in the serum of infants at birth are almost of maternal origin. The serum grouping of a newborn is, therefore, not recommended.

Anti-H

Anti-H very rarely occurs as cold reactive agglutinin in individuals with very low levels of H antigens on their cells and has little clinical significance. However, anti-H found in Bombay blood group (Oh) is an all antibody and is clinically significant. It occurs as a hemolysin and agglutinates cells at 37°C.

ABO Testing Procedures

- For demonstration of true ABO group of an individual, it is important to do both cell grouping (forward typing) and serum grouping (reverse typing). Both forward and reverse typing must match to confirm the true ABO type of an individual.
- Serum should always be added before adding the cells and examine each tube after serum has been added to ensure that none has been left.
- ABO grouping test should be done at room temperature, or below. Testing at 37°C weakens reaction.
- Tubes, slides and microplates should be labeled properly.

ABO Antibody Reagents

The development of monoclonal antibodies obtained from cultures of cells secreting antibodies called hybridomas has made available a new source of ABO typing reagent. Before the advent of hybridoma technology, the ABO grouping reagent was derived from human donors with or without immunization and are called polyclonal reagent. The monoclonal reagents anti-A, anti-B and anti-AB have significant advantage over earlier traditional polyclonal reagent in terms of specificity, potency, consistency and should be free from virus such as HIV and hepatitis.

Red Cells Reagents

The red cells used in ABO grouping are pooled A cells, B cells and O cells. The cells should be washed in saline to remove serum or plasma. The supernatant of last wash should be clear. Use 2–4% cell suspension for tube, microplate typing and 30–40% for slide typing.

Group 'O' cells are used in serum grouping to detect antibodies other than anti-A or anti-B in some donors. These antibodies are not naturally occurring and are called irregular antibodies. They occur due to immunization either by:

- Transfusion
- Pregnancy.

Supplementary reagent used are:

- Anti-A lectin, which reacts strongly with A individuals
- Anti-H reacts selectively with ABO group according to H substance. Group O individuals which contain only H react strongly with anti-H while A, B contains very little H and thus, reacts very weakly or negatively with anti-H. Other supplementary reagent that may be used to resolve the discrepancies of ABO grouping may include
- Group A_2 Cells
- Group O reagent screen cells.

Group O Reagent Screen Cells

Group O reagent screen cells contain red cells with various antigen specificities. These 2 or 3 group O cells are complimentary to each other to provide antigens for detection of most of clinically significant antibodies. They are used to rule out ABO typing discrepancy caused by cold antibodies.

Preparation of Red Cell Suspensions

Depending upon the specific technique employed, 2, 5, 10 or 50% red cell suspensions are required. These can be prepared by transferring freshly obtained blood from a skin puncture into saline or suspending in saline the packed red cells obtained from citrated or oxalated blood. Preservative anticoagulant solutions are also available that permit preservation of red cells for one month or longer. This is most useful for controls and panel cells of known antigenic composition (reagent red cells). Most frequently, suspensions are made by gently breaking up blood clots with an applicator stick and transferring the red cell aggregates into saline or other suspending media.

Method (for 2% suspension)

1. To about 5 mL of normal saline, add several drops of whole blood (fresh, citrated, oxalated or fragments of clots).
2. Centrifuge, in order to pack the red cells.
3. Withdraw the supernatant fluid as completely as possible.
4. Add 0.1 mL of packed red cells to a test tube containing 4.9 mL of normal saline and mix well. This represents a 2% suspension of red cells in saline.

All red cell suspensions must be refrigerated when not in use. They are unsuitable if they show hemolysis and should be used within 12 hours of preparation.

Blood Grouping Sera should Meet the following Requirements

1. It should have titer of recommended potency.
2. It should be free of cold agglutinins.
3. It should be free of so-called irregular agglutinins.
4. It should not form rouleaux when mixed with red cells.
5. It should be clear, of normal color (except when a dye is added for identification), and free of cells or any other particles, not hemolyzed, icteric or chylous.
6. It should be free of complement.
   - Anti-A serum (minimum titer with A_1 cells 256)
   - Anti-B serum (minimum titer with B cells 256)

- Anti-AB serum (serum of group O) (minimum titer 256 with A_1 and B cells)
- Anti-A_1 reagent (absorbed anti-A serum, plant lectins)
- Anti-Rh (D) serum (minimum titer 32)
- Anti-A serum is colored blue
- Anti-B serum is colored yellow.

ANTI-A, ANTI-B, ANTI-AB Blood Grouping Antisera for Slide and Tube Tests

(*Courtesy:* Tulip's Erybank Range)

Summary

Human red blood cell antigens can be divided into four groups A, B, AB and O depending on the presence or absence of the corresponding antigens on the red blood cells. Approximately, 41% of the Caucasian population have the A-antigen, 9% have the B antigen, 4% have both A and B antigens, while the remaining have neither A nor B antigen.

Reagents

Erybank anti-A, anti-B and anti-A/B are ready to use reagent prepared from human serum. These reagents of the immunoglobulin class IgM are a pool of specific human serum obtained from certified selected donors who are found to be negative for HBsAg and anti-HIV antibody. Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and performance.

Reagent Storage and Stability

a. Store the reagent at 2–8°C. Do not freeze.
b. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing A and/or B antigen will agglutinate in the presence of antibody directed towards the antigen. Agglutination of red blood cells with anti-A, anti-B and anti-AB reagent is a positive test result and indicates the presence of the corresponding antigen. Absence of agglutination of red blood cells with anti-A, anti-B and anti-AB reagent is a negative test result and indicates the absence of corresponding antigen.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Erybank reagents are from human source and the source material used in its manufacture is tested by approved techniques and found negative for HBsAg and HIV, HCV antibodies.
3. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
4. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2-8°C if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or heparin	: 2 days
Sodium citrate or sodium oxalate	: 14 days
ACD or CPD	: 28 days

Clotted whole blood should be tested within 14 days.

Additional Material Required for Slide and Tube Tests

Glass slide (50 × 75 mm), test tubes (10 × 75 mm), Pasteur pipettes, isotonic saline, centrifuge, timer, and mixing sticks.

Test Procedure

Bring reagent and samples to room temperature before testing.

Slide Test

1. Place one drop of Erybank reagent anti-A or anti-B or anti-AB on a clean glass slide.
2. To each reagent drop, add one small drop of whole blood.
3. Mix well with a mixing stick uniformly over an area of approximately 2.5 cm^2.
4. Rock the slide gently back and forth.
5. Observe for agglutination macroscopically at 2 minutes.

Tube Test

1. Prepare a 2–3% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Erybank anti-A, anti-B, anti-AB into correspondingly labeled test tubes.
3. Pipette into each of the test tubes, one drop of the test red cell suspension and mix well.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g) or incubate at room temperature for 20–30 minutes.

5. Gently resuspend the cell button, observing for agglutination macroscopically.

Interpretation of Results

Slide and Tube Tests

Agglutination is a positive test result and indicates the presence of A and/or B antigen.

Do not interpret peripheral drying or fibrin strands as agglutination.

No agglutination is a negative test result and indicates the absence of A and/or B antigen.

Remarks

1. In the tube test procedure, it is recommended that tubes with negative reactions should be recentrifuged and results read again after 5 minutes so that weak antigens are not overlooked.
2. Anti-A, lectin can be used to differentiate between A, and A_1 subgroups.
3. A_3 cells demonstrate a mixed field agglutination.
4. Certain weaker subgroups of A and B may produce weak reactions with anti-A, B.
5. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrate its own equipment and determine the time required for achieving the desired results.

ANTI-A, ANTI-B, ANTI-AB Monoclonal Blood Grouping Antibodies for Slide and Tube Tests

(*Courtesy:* Tulip's Eryclone Range)

Summary

Monoclonal antibodies are derived from hybridoma cell lines, created by fusing mouse antibody producing B lymphocytes with mouse myeloma cells. Each hybridoma cell line produces homogeneous antibodies of only one immunoglobulin class, which are identical in their chemical structure and immunological activity. Human red blood cell antigens can be divided into four groups A, B, AB and O depending on the presence or absence of the corresponding antigens on the red blood cells. Approximately, 41% of the Caucasian population have the A antigen, 9% have the B antigen, 4% have both A and B antigens, while the remaining have neither A nor B antigen.

Reagent

Eryclone® anti-A, anti-B and anti-AB are ready-to-use reagent prepared from supernatants of mouse hybridoma cell cultures. These antibodies of immunoglobulin class IgM are a mixture of several monoclonal antibodies of the same specificity but having the capability of recognizing different epitopes of the human red blood cell antigens A and B. Each batch of reagent undergoes quality control at various stages of manufacture for its specificity, avidity and performance.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing A and/or B antigen will agglutinate in the presence of antibody directed towards the antigen. Agglutination of red blood cells with anti-A, anti-B, anti-AB reagent is a positive test result and indicates the presence of the corresponding antigen. Absence of agglutination of red blood cells with anti-A, anti-B, anti-AB reagent is a negative test result and indicates the absence of the corresponding antigen.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
3. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.
4. Eryclone® reagent are not from human sources, hence, contamination due to HBsAg and HIV is practically excluded.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2–8°C if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or Heparin	: 2 days
Sodium citrate or sodium oxalate	: 14 days
ACD or CPD	: 28 days

Clotted whole blood should be tested within 14 days.

Additional Material Required for Slide and Tube Tests

Glass slides (50 × 75 mm), test tubes (12 × 75 mm), Pasteur pipettes, isotonic saline, centrifuge, timer, and mixing sticks.

Test Procedure

Bring reagent and samples to room temperature before testing.

Slide Test

1. Place one drop of Eryclone® anti-A or anti-B or anti-AB reagent on a clean glass slide.
2. To each reagent drop, add one small drop of whole blood.
3. Mix well with a mixing stick uniformly over an area of approximately 2.5 cm^2.
4. Rock the slide gently, back and forth.
5. Observe for agglutination macroscopically at 2 minutes.

Tube Test

1. Prepare a 2–3% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Eryclone® anti-A, anti-B, anti-A, B into correspondingly labeled test tubes.
3. Pipette into each of the test tubes, one drop of the test red cell suspension and mix well.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g) or incubate at room temperature for 30 minutes.
5. Gently resuspend the cell button, observing for agglutination macroscopically.

Interpretation of Results

Slide and Tube Tests

Agglutination is a positive test result and indicates the presence of A and/or B antigen. Do not interpret peripheral drying or fibrin strands as agglutination. No agglutination is a negative test results and indicates the absence of A and/or B antigen.

Remarks

1. (a) Eryclone® anti-A, anti-B and anti-AB reagent do not show a reaction with crypt antigens (T, Tn, Tk activated cells) (b) Eryclone® anti-B is truly negative reacting with acquired B characteristics.
2. In the tube test procedure, it is recommended that tubes with negative reactions should be recentrifuged and results read again after 5 minutes so that weak antigens are not overlooked.
3. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrates its own equipment and determine the time required for achieving the desired results.
4. Results of forward grouping obtained by using anti-A, anti-B, anti-A, B reagent should always be reconfirmed by performing reverse grouping with known red cells.
5. It is strongly recommended that red cells with known ABO characteristics should be occasionally run, preferably on a daily basis so as to control reagent performance and validate test results.
6. After usage, the reagent should be immediately recapped and replaced to 2–8°C storage.

ANTI-A1 LECTIN
Dolichos Biflorus Lectin for Slide and Tube Tests

(*Courtesy:* Tulip's Erybank Range)

Summary

Human red blood cells possessing the A antigen can be broadly subdivided into two main subgroups namely A_1 and A_2 based on their reaction with A_1 lectin. A_2 subgroups comprises of weaker subgroups of A such as A_1, A_2, A_3, A_4, A_5, A_6, A_0, etc.

Group A red blood cells which agglutinate with anti-A_1 lectin are classified as subgroup A_1, whereas red blood cells which do not agglutinate with anti-A_1 lectin are classified as subgroup A_2. It is estimated that about 80% of the group A population are A_1 and the remaining A_2 or weaker. Anti-A_1 lectin is especially useful in selecting blood for an A_2 or A_2B recipient whose blood may contain anti-A_1 antibodies.

Reagent

Erybank® anti-A_1 lectin is a ready-to-use purified extract of *Dolichos biflorus* seeds that is carefully calibrated to differentiate most A_1 cells from A_2 cells. It contains a phytohemagglutinin, which is virtually specific for A_1 antigen on the human red blood cells.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and titer.

Reagent Storage and Stability

a. Store the reagent at 2–8°C. Do not freeze.
b. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing A_1 antigen will agglutinate in the presence of seed extract (lectins) containing phytohemagglutinin specifically directed toward it.

Agglutination of red cells with Erybank® anti-A_1 lectin is a positive test result and indicates the presence of A_1 antigen. No agglutination with Erybank® anti-A_1 lectin is a negative test result and indicates the absence of the A_1 antigen. Red blood cells that are positive with anti-A reagent and negative with A_1 lectin are classified as A_2.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. Extreme turbidity may indicate microbial contamination/reagent deterioration. Such reagent should be discarded.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2-8°C, if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or Heparin	:	2 days
Sodium citrate or sodium oxalate	:	14 days
ACD or CPD	:	28 days

Clotted whole blood should be tested within 14 days.

Additional Material Required for Slide and Tube Tests

Glass slides (50 × 75 mm), test tubes (12 × 75 mm), pasteur pipettes, isotonic saline, centrifuge, timer, mixing sticks.

Test Procedure

Bring all reagent and samples to room temperature before testing.

Slide Test

1. Prepare a 10% suspension of the red blood cells to be tested in isotonic saline.
2. Place one drop of Erybank® anti-A_1 lectin on a clean glass slide.
3. Pipette two drops of the cell suspension on the slide.
4. Mix well with a mixing stick uniformly over an area of approximately 2.5 cm^2.
5. Rock the slide gently, back and forth.
6. Observe for agglutination macroscopically at one minute.

Tube Test

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Erybank® anti-A_1 lectin into a labeled test tube.
3. Pipette into the test tube, one drop of the test red cell suspension.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
5. Gently resuspend the cell button, observing for agglutination macroscopically.

Interpretation of Results

Slide and Tube Tests

Agglutination is a positive test result and indicates the presence of A_1 antigen. Do not interpret peripheral drying or fibrin strands as agglutination. No agglutination is a negative test result and indicates the absence of A_1 antigen.

Remarks

1. A_1 antigen is not fully expressed on the red blood cells of newborns below one year of age, hence, false negative results may occur.
2. It is strongly recommended that known A_1 and A_2 cells should be occasionally run, preferably on a daily basis to control reagent performance and validate test results.
3. A_1 A_2 (A_{int}) cells may agglutinate moderately with Erybank®. Anti-A_1 lectin. These should be tested further with Anti-H lectin to confirm A_{int} cells.
4. As undercentrifugation or overcentrifugation can lead to erroneous results, it is recommended that each laboratory calibrate its own equipment and determine the time required for achieving the desired result.

ANTI-H LECTIN
Ulex Europaeus Lectin for Slide and Tube Tests

(*Courtesy:* Tulip's Erybank Range)

Summary

The H antigen is a basic blood group antigen present in human beings. There is considerable variation in the H antigen content in different individuals of the same ABO group, but the general pattern indicates their strength as $O>A_2>A_2B>B>A_1>A_1B$. Water-soluble H substance can also be demonstrated in saliva or body fluids of individuals

who are secretors. Human red blood cells that do not agglutinate with Anti-H lectin are classified as Bombay phenotype (Oh). The Bombay phenotype is more common in India than other parts of the world and the estimated gene frequency of Oh phenotype in Bombay is 0.0066%.

Reagent

Erybank Anti-H lectin is a ready-to-use purified extract of *Ulex europaeus* seeds. It contains a phytohemagglutinin which is virtually specific for the H antigen on human red blood cells. Erybank Anti-H lectin is used for recognition of the H antigen on human red blood cells. It is useful, especially for assessing the H secretor status of group 'O' individuals and also in differential grouping of A_{int} subgroup along with anti-A lectin.

Reagent Storage and Stability

a. Store the reagent at 2–8°C. Do not freeze.
b. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing the H antigen will agglutinate in the presence of seed extract (lectins) containing phytohemagglutinin specifically directed towards it. Water-soluble H substance present in saliva neutralizes anti-H lectin. Agglutination of red blood cells/neutralization of anti-H lectin by saliva is a positive test result and indicates the presence of H substance on/in the red cell/saliva respectively. No agglutination/neutralization of anti-H lectin is a negative test result and indicates the absence of H substance on/in the red cell/saliva respectively.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. Extreme turbidity may indicate microbial contamination/reagent deterioration. Such reagent should be discarded.

Sample Collection and Preparation

For Recognition of H Antigen on Human Red Blood Cells

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2–8°C, if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or heparin	:	2 days
Sodium citrate or sodium oxalate	:	14 days
ACD or CPD	:	28 days

Clotted whole blood should be tested within 14 days.

For Assessing Secretor Status in Human Saliva

a. Collect about 2 mL of fresh saliva in a glass tube and incubate in a boiling water bath for 10 minutes.
b. Centrifuge at 3400 rpm (1000 g) for 10 minutes.
c. Use the clear supernatant immediately for the study or freeze immediately if to be tested later.

Additional Material Required for Slide and Tube Tests

Glass slides (50 × 75 mm), test tubes (10 × 75 mm), Pasteur pipettes, isotonic saline, centrifuge, timer, mixing sticks, red blood cells positive for H antigen, red blood cells negative for H antigen, saliva positive for H antigen, Saliva negative for H antigen.

Procedure

Bring all reagent and samples to room temperature before testing.

Slide Test

1. Place one drop of Erybank anti-H lectin on a clean glass slide.
2. Add one drop of whole blood to be tested on the slide and mix well with a mixing stick uniformly over an area of approximately 2.5 cm^2.
3. Rock the slide gently, back and forth.
4. Observe for agglutination macroscopically at 2 minutes.

Tube Test

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Erybank anti-H lectin into a test tube.
3. Pipette into the test tube, one drop of the test red cell suspension and mix well.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
5. Gently resuspend the cell button, observing for agglutination macroscopically.

Tube Test (Secretor Status)

1. Place two drops of anti-H lectin into two clean glass tubes.
2. Pipette two drops of saliva into the tubes and mix well.
3. Incubate at room temperature for 10 minutes.
4. Add one drop of negative and positive cell suspensions into the tubes, mix well and incubate at room temperature for 5 minutes.
5. Mix well and centrifuge for one minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).

6. Gently resuspend the cell button, observing for agglutination macroscopically.

Interpretation of Results

Slide and Tube Tests

Agglutination is a positive test result and indicates the presence of H antigen. No agglutination is a negative test result and indicates the absence of H antigen and the red cells being of Bombay phenotype (Oh).

Tube Test (Secretor Status)

Agglutination of the red cells indicates that the anti-H has not been neutralized and the patient is a non-secretor. No agglutination of the red cells indicates the anti-H has been neutralized and the patient is a secretor.

Remarks

1. Do not interpret peripheral drying or fibrin strands as agglutination.
2. It is recommended that known negative and positive cells must be included as controls with each test series.
3. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrate its own equipment and the time required for achieving the desired results.

Other Requirements

1. Normal saline solution.
2. Bovine or human albumin (22% or 30%).
3. Reagent red blood cells: Suspensions of red cells in which presence or absence of significant blood group antigens has been determined may be collected periodically from suitable donors or be purchased commercially wherever available.
4. Enzymes: Bromelin, ficin, papain, trypsin.

PHYSIOLOGICAL SALINE SOLUTION FOR SEROLOGICAL APPLICATIONS (Sodium Chloride 0.9% w/v)

(*Courtesy:* Tulip's Erybank Range)

Summary

In blood group serology, for detection of either antigens or antibodies, physiological saline (0.85% to 0.9% w/v) is being extensively used. However, it is important that the physiological saline used should be compatible with red blood cell membrane integrity.

Reagent

Erybank natrium chloride 0.9% w/v solution is standardized for its serological applications. Reagent contains 0.1% sodium azide as preservative.

Principle

Red blood cell lysis or shrinkage is observed in case of diluents used which contain very low salt concentration or very high salt concentration respectively. So optimal salt concentration is very essential to maintain the red cell membrane integrity. Sodium chloride with 0.9% w/v concentration is observed as the optimal salt concentration. It is routinely used as diluent for serological applications.

Note

1. Store the reagent at RT, it also can be stored at 4–8°C.
2. The reagent contains 0.1% sodium azide as preservative.
3. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.

Uses

1. The reagent is used for the suspension of red blood cells.
2. The reagent is used as a diluent for the antibodies.
3. Washing of red blood cells.
4. Making dilutions of reagent or samples for testing.

BOVINE SERUM ALBUMIN 22% SOLUTION FOR SEROLOGICAL APPLICATIONS

(*Courtesy:* Tulip's Erybank Range)

Summary

Bovine serum albumin is mainly used to enhance the reactivity of blood grouping and typing antibodies in direct agglutination tests. bovine albumin also enhances the reactivity and sensitivity of indirect antiglobulin test which is used for compatibility testing, antibody screening, identification and titration.

Reagent

Erybank® bovine serum albumin is manufactured from selected raw bovine serum, its protein concentration and pH adjusted to 22% and 7.1 (± 0.1) respectively. Its conductivity is controlled specifically for serological applications.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Agglutination of antibody coated red cells depends upon the class and type of antibody involved and the characteristics of the reaction medium such as

ionic strength and pH. Incomplete antibodies of IgG class, especially those with Rh specificity, agglutinate red cells if the zeta potential between the red cells is adjusted by addition of colloids and salts such as bovine serum albumin (BSA). Addition of BSA enhances such immunological reactions and increases test sensitivity.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2-8°C if not tested immediately. Do not use hemolyzed samples. Donor units can be tested up to the end of their dating. For the indirect antiglobulin test, serum from fresh clotted whole blood should be used.

Additional Material Required for Compatibility Testing

Test tubes (10 × 75 mm), 0.2 mL serological pipettes, Pasteur pipettes, human red blood cells with specific antigen reacting with the antibody to be titrated, centrifuge, incubator, isotonic saline, anti-humanglobulin reagent such as Eryclone® anti-human globulin reagent, Coomb's control cells (Refer Eryclone® anti-human globulin pack insert), AB neutral human serum.

Broad-spectrum Compatibility Test

Major Crossmatch Procedure

Initial Phase

1. Label two test tubes as A (for albumin) and B (for saline), depending upon the number of donors to be cross matched, as many pairs of such labeled tubes would be required.
2. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
3. Pipette two drops of recipient serum in both the labeled test tubes.
4. Pipette one drop of donor red cells in both the labeled test tubes and mix well.
5. Only to the albumin tube (A), add two drops of Erybank® bovine serum albumin reagent and mix well.
6. Centrifuge both the tubes for one minute at 1000 rpm (125 g) or for 20 seconds at 3400 rpm (1000 g).
7. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.
8. Proceed to incubation phase.

Incubation Phase

1. Incubate the saline tube at room temperature and the albumin tube at 37°C for 15 minutes.
2. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.
3. Proceed to the antiglobulin phase.

Antiglobulin Phase

1. Only the albumin tubes (A) are tested in the antiglobulin phase.
2. Wash the mixture of red blood cells and serum thoroughly with isotonic saline for a minimum of three times. Decant completely after the last wash
3. Place two drops of Eryclone® anti-human globulin reagent into the test tube containing the sedimented cells and mixwell.
4. Centrifuge for one minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
5. Very gently, resuspend the cells and observe for agglutination macroscopically.

Antibody Titration Test

1. a. Prepare a 5% suspension of red blood cells with specific antigen reacting with antibody to be titrated, in Erybank® bovine serum albumin reagent.
   b. Also, prepare a 5% suspension of patient red cells in Erybank® bovine serum albumin reagent.
2. Label ten test tubes (1 to 10) and make progressive dilutions of the patients serum as indicated below:
   a. Pipette 0.1 mL of AB neutral serum into each test tube except the first tube.
   b. Pipette 0.1 mL of the patient serum into first two tubes only.
   c. After mixing the contents of the second tube thoroughly, transfer 0.1 mL of the mixture to the third tube. Continue the serial dilution by transfer up to tube No. ten. Discard 0.1 mL of the mixture from the last tube.
3. To tubes No. one through to nine, add one drop of albumin suspended selected red blood cells, (as prepared in point No. 1 (a) above) and mix well.
4. To tube No. ten, add one drop of patient's red cells suspended in albumin (as prepared in point No.1 (b) above) and mix well.
5. lncubate all the tubes at 37°C for a minimum of 15 minutes.

6. Centrifuge all the tubes for one minute at 1000 rpm or 20 seconds at 3400 rpm (1000 g).
7. Very gently, resuspend the cell buttons and observe for agglutination macroscopically.
8. Antiglobulin test should be performed on all tubes which do not show a very strong agglutination.

Interpretation of Results

Compatibility Test

In all phases of the compatibility test, if no agglutination or hemolysis is observed, then the patient and the donor may be considered compatible. If hemolysis or agglutination at any point till the completion of the antiglobulin phase is observed, the patient and donor are considered incompatible.

Antibody Titration Test

The end point of the titration is the reciprocal of the dilution in the last tube showing agglutination.

Remarks

1. If plasma is used in the indirect antiglobulin test, the complement-dependent antibodies may not be detected due to the absence of calcium.
2. To all negative test results, after the antiglobulin test phase, one drop of Coomb's control cells should be added. If the Coomb's control cells do not agglutinate, then the compatibility test must be repeated.
3. Red blood cells showing a positive direct antiglobulin test should not be used for the indirect antiglobulin test.
4. Bovine serum albumin will not bring about agglutination of red cells by all IgG blood grouping typing antibodies.
5. As undercentrifugation or overcentrifugation can lead to erroneous results, it is recommended that each laboratory calibrates its own equipment and determine the time required for achieving the desired results.
6. After usage, the reagent should be immediately recapped and replaced at 2–8°C storage.

CONCENTRATED ISO-OSMOTIC PHOSPHATE BUFFERED SALINE FOR SEROLOGICAL APPLICATIONS

(Osmosol from Tulip)

Summary

The pH of reaction medium is an important factor in antigen-antibody interaction. It has been observed that irrigation/infusion saline solution of low pH, isotonic saline autoclaved and stored in plastic containers could severely compromise the sensitivity and specificity of antiglobulin test when used as wash solutions/resuspension medium in immunohematological procedures. Hence, careful consideration should be given to the source, pH and storage container of isotonic saline solutions intended for use in immunohematological procedures. Usage of buffered isotonic phosphate saline such as Osmosol maintains the pH at 7.0–7.2 thereby improving the sensitivity and specificity of tests employed in immunohematological procedures.

Reagent

Osmosol is a concentrated 20X buffered isotonic phosphate saline useful for preparing iso-osmotic saline preparation, especially for immunohematological use. Inclusion of sodium azide in the final formulation prevents contamination during use.

Reagent Storage and Stability

Store the reagent at room temperature. Once opened, store at 2–8°C.

The shelf-life of the concentrated Osmosol reagent is as per the expiry date mentioned on the reagent vial label.

Upon dilution, the isotonic buffered saline solution so obtained is stable for at least a month provided it is not contaminated during use.

Principle

Osmosol with osmolarity similar to blood serum or plasma, incorporating phosphate buffer maintains red blood cell membrane integrity and optimum pH of the reaction medium for antigen-antibody reaction during immunohematological tests thereby improving the sensitivity and specificity of the test.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains 0.2% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. Extreme turbidity may indicate contamination. Such reagent must be discarded.

Additional Material Required

Distilled water for blood bank use, pH paper capable for reading pH at 6.5–7.5 or pH meter, sterile and scrupulously clean glasswares for preparing the isotonic buffered saline solution.

Method of Preparation

1. Invert the contents of Osmosol vial into a scrupulously clean, sterile glass jar/bottle. Make the volume to 500 mL with distilled water. Gently mix the contents. Alternatively, if lesser quantity of isotonic buffered saline is required then dilute 1 part of concentrated Osmosol with 19 parts of distilled water. The glasswares used for preparing the isotonic buffered saline should be sterile and scrupulously clean.
2. Check the pH of isotonic buffered saline. The pH of the isotonic buffered saline should be in the range 6.9–7.2.
3. The isotonic buffered saline so obtained is ready-to-use for washing and preparing red blood cell suspension, dilution medium for antibodies in serological applications.

Remarks

1. Erroneous test results can occur from microbial or chemical contamination of buffered saline. The final reagent so obtained should be a clear solution.
2. Isotonic buffered saline obtained from Osmosol should not cause hemolysis of red blood cells, gel formation with serum under test. Any observable change in serum or cellular elements, the reagent must be discarded.
3. Occasionally, it is recommended that the pH of the isotonic buffered saline obtained from Osmosol should be checked before using for serological applications. The pH should be in the range 6.9–7.2. Any change in pH value out of the specified range, the reagent should be discarded.
4. The isotonic buffered saline obtained from Osmosol should be strictly stored in scrupulously clean, sterile beakers/glass wares and not in plastic containers.
5. The 20X concentrated Osmosol vial may show fine particulate appearance if stored at 2–8°C. This can be overcome by gently warming the concentrated solution to 25°C, before dilution.

Media for Collection and Preservation of Reagent Red Cells

1. *Modified Alsever's solution:*
   Dextrose—2.05 g
   Sodium citrate—0.8 g
   Citric acid—0.05 g
   Sodium chloride—0.45 g
   Distilled water—100 mL
   (Expected pH = 6.1).
2. *Serum lactose solution*:
   Lactose—10 g
   Dextrose—1.53 g
   Sodium citrate—1.38 g
   Citric acid—0.5 g
   Distilled water—100 mL.

This 100 mL solution is mixed with 100 mL of serum from a person of group AB (serum must be free of irregular and cold agglutinins).

Precautions

Antisera must be refrigerated when not in use. Reagents and blood specimens for hemagglutination test must be handled aseptically since bacterial contamination may cause both, falsely negative and falsely positive results. Positive and negative controls must be run daily with all antisera.

RED CELL PRESERVING SOLUTION FOR SEROLOGICAL APPLICATIONS

(Erywell from Tulip)

Summary

In blood group serology, known red cell panels are of immense value in confirming the results of forward grouping, antibody screening and detection of rare phenotypes. A red cell preserving solution is utilized to preserve the red cells of interest. Erywell red cell preservation solution is formulated specifically for enhanced preservation of red cells carrying clinically important phenotype or genotype, which are required for routine immunohematological practice.

Reagent

Laboratory reagent. Ready-to-use solution.

Erywell red cell preserving solution is a standardized Alsever's solution for maintaining red cell integrity and survival.

Principle

Red blood cell shrinkage and loss of antigenic properties are observed on storage. To preserve the red cells for a longer time and ensure enhanced usage life for serological procedures, the Erywell solution supplies the necessary nutrients, salts and preservatives for maintaining red cell integrity and antigenic properties useful during serological procedures.

Storage and Stability

Store the reagent at 2–8°C.

Stability of unopened vial: 12 months from the date of manufacturing.

Additional Material Required

1. Freshly collected red blood cells in EDTA dipotassium salt (1.5 mg/mL of whole blood).
2. Test tubes 12 × 100 mm, scrupulously clean and dry.
3. 10 mL pipettes, scrupulously clean and dry.
4. 500 µL micropipette and micropipette tips.
5. Freshly prepared normal saline (0.9% NaCl).
6. Table centrifuge.
7. Sterile 10 mL vials.

Procedure

Quick Method for Whole Blood Preservation

1. Collect 5 mL whole blood in EDTA dipotassium salt (1.5 mg/mL of whole blood). Add equal volume of Erywell solution to it. Gently mix the solution.
2. Before using the red cells prepared by the quick method, it is recommended to wash the red cells three times with normal saline before use for testing purpose.

Preparation of 2/3/5% Stabilized Red Blood Cell Suspension in Erywell Solution

1. Collect 2 mL of freshly drawn venous blood in a clean and dry test tube containing 3 mg of EDTA di-potassium salt.
2. Add 5 mL of normal saline solution and mix well.
3. Centrifuge the tube at 3000 rpm for 2–3 minutes to form a red cell button.
4. Discard the supernatant.
5. Resuspend the red cell button in normal saline solution.
6. Centrifuge the tube at 3000 rpm for 2–3 minutes.
7. Repeat the washing of the red cells (steps 4 and 5) one more time in normal saline.
8. After the centrifugation, remove the supernatant without disturbing the red cell button.
9. Now resuspend the red cell button in 5 mL of Erywell solution.
10. Centrifuge the tube at 3000 rpm for 2–3 minutes.
11. After the centrifugation, remove the supernatant without disturbing the red cell button.
12. Take 0.2/0.3 mL of packed red cells from the above cell button and resuspend them in 10 mL of Erywell solution for preparation of 2/3% red cell suspension. To obtain a 5% red cell suspension resuspend 0.5 mL of packed red cells from the above cell button in 10 mL of Erywell solution.
13. Store the red cell suspension in a sterile 10 mL vial.
14. The red cell suspension so obtained is ready-to-use for testing.

Storage

1. The whole blood preserved in Erywell solution prepared by the quick method can be stored up to 4 weeks at 2–8°C.
2. The ready-to-use 2–3–5 % stabilized red blood cell suspension in Erywell solution should be stored at 2–8°C and can be utilized for weeks from the date of preparation.

Precautions

1. Store Erywell at 2–8°C with cap tightly closed.
2. Do not contaminate the solution as it may subsequently affect the stability of red cell suspension.
3. Glassware used to retrieve Erywell red cell suspension should be scrupulously clean and sterile.

ABO GROUPING

Slide ABO Grouping Test

1. Prepare a 10% suspension in saline of the red cells to be tested.
2. Mark the left side of a clean glass slide 'anti-A' and the right side 'anti-B'.
3. Add to the left side a drop of anti-A grouping serum and to the right side a drop of anti-B grouping serum.
4. Next to the antisera drops, place 1 drop of 10% saline suspension of unknown red cells. The contents on the left and right sides should not get mixed up.
5. With one-half of an applicator stick, mix the red cell suspension with the anti-A serum; and with the other half-mix the red cell suspension with the anti-B serum.
6. Gently rock the slide back and forth and observe the mixture for one minute unless agglutination occurs earlier.
7. It is necessary to confirm the slide grouping by testing the unknown serum with the known red cell suspensions of groups A, B, and O as indicated for six-tube method. (Instead of 10% saline suspension, citrated/oxalated blood or direct blood from skin puncture may be taken. Slow and weak agglutinations may occur in A subgroups).

Six-tube Method (Fig. 11.3)

1. Separate the serum from red cells or clot of unknown specimen.

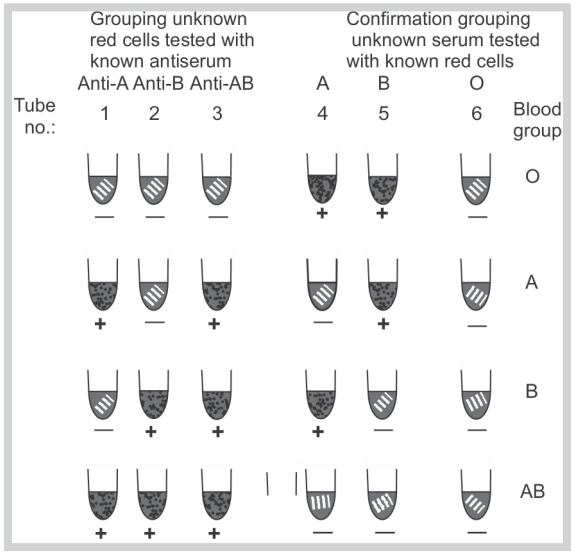

FIG. 11.3: ABO blood grouping—tube method

2. Prepare a 2% suspension in saline of the red cells of the unknown specimen.
3. For each specimen, six test tubes are labeled showing in addition to proper identification of the unknown specimen the undermentioned information: Tube 1, anti-A; tube 2, anti-B; tube 3, anti-AB; tube 4, A; tube 5, B; tube 6, O.
4. Place 1 drop of anti-A serum in tube 1, 1 drop of the anti-B serum into tube 2, and 1 drop of the anti-A, B serum into tube 3.
5. Add two drops each of the serum of the unknown specimen to tubes 4, 5, and 6.
6. Add one drop each of the 2% red cell suspension in saline of the unknown specimen to tubes 1, 2, and 3.
7. To tube 4, add 1 drop of a 2% red cell suspension in saline of group A cells; to tube 5, add 1 drop of a 2% red cell suspension in saline of group B cells; and to tube 6, add 1 drop of a 2% suspension of red cells in saline of group O cells.
8. Mix the contents of all tubes by shaking the test tube rack.
9. Depending on the speed necessary for completing the test, one of the two alternatives may be chosen: (i) the test tubes may be left at room temperature for at least 2 hours, or (ii) after 2 to 3 minutes, the tubes may be centrifuged (1 minute at 1500 rpm in a clinical centrifuge).
10. After incubation or centrifugation, the red cell suspensions are redispersed by tapping the tubes. Presence of agglutination is checked with the naked eye, with the scanning lens of a microscope and if the results are doubtful or negative, microscopically (10X objective) after placing a drop on a slide.

Interpretation

The results of an ABO grouping test can be accepted as valid only if the findings obtained in the first three test tubes with known antisera agree with those obtained in the second three test tubes with known red cell suspensions; this second, part of the test is called confirmation, check or reverse grouping. Given on previous page are the result in the four main ABO groups in such tests when both the unknown red cells and unknown serum are tested. Agglutination is indicated by the +sign, –sign indicates no agglutination.

Absent or weak agglutination with the unknown serum may be simulated by hemolysis of the known red cells, this suggests the presence of hemolytic anti-A or anti-B antibody. The assumption that hemolysis is due to anti-A or anti-B must be confirmed by repeating the test with the serum after its inactivation in a water bath at 56°C for 30 minutes—use of the inactivated serum is expected to result in agglutination instead of hemolysis.

If discrepancies in the two parts of ABO grouping are observed, the test should be redone. If a second test reveals the same discrepancy, the following possibilities are to be considered:

1. Cold agglutinins may cause agglutination of the known cells by the unknown serum regardless of its ABO group. Confirm this by testing the unknown serum with its own red cells at 4°C for 1 to 2 hours. Under those conditions cold agglutinins produce agglutination, which disappears after transfer of the specimen to a water bath at 37°C for 5 to 10 minutes.
2. Agglutination of the unknown red cells by known antisera that conflict with the results obtained in the confirmation grouping may be due to coating of the red cells by autoantibodies. Confirmation of this phenomenon is obtained by performing on the unknown red cells the direct antiglobulin test and obtaining positive results. In order to obtain in such cases, a reliable ABO grouping, the red cells should be washed several times with large amounts of isotonic saline solution, following which they are more likely to give adequate result.
3. Unexpected agglutination obtained with the unknown serum may reflect presence of irregular agglutinins, such as Rh antibodies, which react with the corresponding blood factor in the suspensions of the known red cells.

4. Discrepancies in the two parts may sometimes be present due to A subgroup. In which anti-A_1 serum, anti-A_2 serum, and A_1 and A_2 reagent red cells are used for confirmation.
5. Serum of newborn and young infants may not contain the isoagglutinins expected from the reactivity of their red cells; hence, in infants, the use of unknown serum is not a reliable method. Much less frequently isoagglutinins may be absent in older children and adults due to hypo or agammaglobulinemia or for unknown reasons.

Briefly the Reasons can be Described as:

a. Improper identification of specimen.
b. Improper techniques like:
   - Failure to add proper reagent
   - Incorrect cell to serum ratio
   - Failure to identify hemolysis
   - Incorrect reading, recording or interpretation of test results.
c. Failure of equipment.
d. Poor standardized or stored reagent.
e. Patients problems.
   - Patient may fail to express ABO antigens on red cells, e.g.
     1. Age (newborn or old age).
     2. Disease states, i.e. leukemia or lymphomas.

This Leads to False Negative Results

➢ Acquired B-antigen can occur
1. Gram-negative septicemia
2. Carcinoma colon.

This may Cause False Positive Reaction

➢ Rouleaux formation: This is an aggregation of red cells in the form of piles of coins and can be misinterpreted as agglutination
➢ Acquired antibodies, e.g. anti-A_1, in A_2 persons anti-H in Bombay phenotype, cold autoantibodies, all unexpected antibodies
➢ Absence or weakening of antibodies, e.g. immune deficiency states, agammaglobulinemias, etc.

Solving Problems of Discrepancies

Once a discrepancy is detected in ABO cells and serum grouping, repeat the test before additional investigations are carried out. Quality assurance of reagent, correct technique, careful observation and interpretation of results resolve many problems.

Repeat Preliminary Procedures

1. Obtain a fresh blood sample from donor unit or patient to rule out discrepancy due to contamination or unidentified samples.
2. Wash the cells 3–4 times in normal saline to rule out rouleaux formation and prepare 2–5% cell suspension.
3. Perform direct antiglobulin test on the cells, to detect if cells are coated with antibody as in HDN and AIHA.
4. Retest the cells with fresh and potent anti A, anti-B, anti-AB, anti-A or anti-H as appropriate for individual problem.
5. Test the serum against appropriate A_1, A_2 and B cells. Group O cells and autologous cells should be used as controls to detect alloagglutinins and autoagglutinins.
6. Use group O cord cells if anti-I is suspected.

RH BLOOD GROUP SYSTEM

It had been suspected for a long time that cause of many transfusion reactions was due to specific differences in blood other than the four main blood groups originally described by Landsteiner. In 1939, Levine and Stetson described an antibody in the serum of a group O mother who delivered stillborn fetus and subsequently developed symptoms of hemolytic transfusion reaction when transfused with her husband's group O blood. They noted that the responsible antibody developed in the mother through an antigenic factor from the fetus. The antibody was not named at that time.

In 1940, Landsteiner and Wiener immunized rabbits and guinea pigs with red cells of rhesus monkeys. The serum of immunized rabbits contained an antibody named anti-Rh which agglutinated red cells in approximately 85% of white population tested. Its antigenic determinant was called Rh factor. The antibody discovered by Levine and Stetson in the mother was subsequently reexamined and found identical in activity as the anti-Rh antibody of Landsteiner and Wiener. This work led to the discovery of Rh system.

Clinical Importance of Rh

Rh blood group system is important because of:

1. Hemolytic disease of newborn (HDN) may occur in Rh-negative pregnant women with Rh-positive fetus.
2. Rh antibodies may develop in Rh-negative patient if given Rh-positive blood.

Present Status and Nomenclature

The genes of the Rh system are present on chromosome 1. There are three pairs of genes called Cc, Dd, and Ee but only five antigens (C, c, D, E, e) as there is no antigen produced by the 'd' gene. Rh gene travels in the set of three, e.g. CDC, CDE, cDE, etc. with one set being received from each parent. There are thus, eight possible chromosomes each of which carries genes for three factors (Table 11.3).

TABLE 11.3: The eight basic chromosomes of fisher and race

DCe(R_1)	dce(r)
DcE(R_2)	dCe(r')
Dce ($R_{0)}$	dcE (r")
DCE(R_z)	dCE (r^y)

Any two chromosomes, one from each parent may be inherited by an individual so that 36 different genotypes are possible Table 11.4 gives the most common genotypes.

Out of various gene combinations as shown in Table 11.4, it is presence or absence of D gene which is most important. When a person inherits 'D' antigen its red cells react with anti-D and these are called Rh-D Positive. If a person does not inherit D antigen, the red cells do not react with anti-D and thus, called Rh-D negative.

Rh D^u Antigen

It is defined as weakened expression of the normal D antigen, i.e. there are fewer than normal D antigens per red cells. There are two grades of D^u:

High grade D^u

Low grade D^u

High grade D^u red cells are agglutinated by certain anti-D sera while low grade D^u are mostly detected by (Anti-human-globulin) AHG test.

It has been shown that D^u positive bloods may immunize the Rh-negative patient resulting in the formation of anti-D antibodies, hence, it is important to exclude D^u individuals from Rh negative blood donor list.

Rh Antibodies

Rh antibodies are usually immune IgG type but may be IgM or even IgA. Most Rh antibodies result from exposure to Rh positive red cells, either due to pregnancy or transfusion in individuals who lack the corresponding antigen.

Auto Rh antibodies may be found in individual with warm autoimmune hemolylic anemia and anti-e is the most commonly found antibody.

Reagents for Rh(D) Grouping

Both polyclonal and monoclonal reagents are available in different combinations.

I. *Polyclonal human and D serum*
   a. Anti-Rh(D) serum for saline or rapid tube test (high protein medium).
      This contains macromolecular additives and gives rapid reliable results.
   b. Anti-D for saline tube test
      1. Anti-D-IgM
      2. Anti-D-IgG.

TABLE 11.4: Common genotypes

DCe/dce	(R_1r)	dce/dce (rr)
DCe/DCe	(R_1R_1)	
DCe/DcE	(R_1R_2)	
DcE/dce	(R_2r)	dcE/dce (r"r)
DCe/DCe	(R_1R_0)	
DcE/DcE	(R_2R_2)	
Dce/dce	(R_0r),	
DCe/dcE	(R_1r")	
DCe/dCe	(R_1r')	dCe/dce (r'r)

II. *Monoclonal anti-D reagents*
   1. IgM anti-D monoclonal reagent
   2. IgG anti-D monoclonal reagent
   3. IgM and IgG (Blend) anti-D monoclonal reagent
   4. Blend of IgM monoclonal and IgG polyclonal reagent.

The IgM anti-D monoclonal is highly specific, saline reacting working well at room temperature and at 37°C. It is good for emergency slide test, or immediate spin tube test as well as routine Rh-D typing, but IgM anti-D are unreliable for detecting weak D by antiglobulin test, while IgM and IgG (Blend) monoclonal reagent or IgM anti-D monoclonal and IgG anti-D (polyclonal) blend can be used for weak D testing by antiglobulin test.

Rh(D) Grouping Procedures

Rh(D) grouping is done along with ABO grouping using same techniques as used for ABO grouping:

1. Slide or tile method
2. Tube method
3. Microplate method.

Slide Testing

Slide test is not recommended for routine test because it is not reliable especially for weak reactive cells.

Method

1. Place one drop of anti-Rh(D) on a labeled slide.
2. Place one drop of 22% albumin on another labeled slide to serve as control.
3. Put one drop of 40–50% red cells on both the slides.
4. Mix the cell suspension and reagent, using a clean stick for each slide and spread the mixture evenly on the slide over area of 15 mm diameter.
5. Place both slides on a view box (lighted), till gently and continuously for two minutes. Observe for agglutination.

A positive test has agglutination with anti-Rh(D) in the 'test' and a smooth suspension of cells in control. A negative test has smooth suspension of cells in both 'test' and 'control.

Tube Method

1. Place one drop of anti-Rh(D) serum in a tube labeled test
2. Place one drop of 22% albumin in a tube labeled 'control'.
3. Add 1 drop of 2–5% cell suspension in plasma or serum in each tube.
4. Mix well and keep at 37°C for 1 hour (sedimentation method).
   In case of emergency, incubate the tube for 10 minutes at 37°C and then centrifuge at 1000 rpm for 1 minute (spin tube).
5. Gently resuspend the cell button and observe for agglutination. All negative results must be confirmed under microscope.

Interpretation

A positive test has agglutination with anti-Rh(D) in the test and a smooth suspension of cells in the control. A negative test has smooth suspension of cells in both 'test' and 'control'.

Testing for D^u

Method

1. Take one drop of anti-Rh(D) serum in a clean labeled test tube.
2. Place one drop of appropriate control in a labelled tube.
3. Add 1 drop of 2–5% cell suspension to be tested to both the tubes.
4. Mix and incubate both the tubes at 37°C for 15–30 minutes.
5. Centrifuge at 1000 rpm for 1 minute.
6. Gently resuspend the cell button and examine for agglutination. If there is strong agglutination of cell in 'test' tube, then sample is Rh(D) positive and there is no need to proceed with antiglobulin phase of test.
7. If no agglutination or doubtful reaction is observed, wash the cells 3–4 times with saline and decant the last washing.
8. Add 1–2 drops of antiglobulin reagent (Coomb's), mix gently and centrifuge at 1000 rprn for 1 minute.
9. Resuspend the cell button gently and examine for agglutination and record the results.
10. If the test is negative, the reaction can be confirmed by adding known IgG sensitised cells, recentrifuge and reexamine for agglutination. Presence of agglutination confirms the test result.

Interpretation

Agglutination in the 'test' tube and none in negative control tube constitutes a positive test result and blood is accordingly labeled Rh-D^u positive.

ANTI-D (Rho) Human (IgG) Polyclonal Blood Typing Antibodies for Slide and Modified Tube Tests

(*Courtesy:* Tulip's Erybank Range)

Summary

Polyclonal antibodies are derived from hyperimmune human serum containing Anti-D (Rho) antibodies directed towards the human D (Rho) antigen. Human red blood cells are classified as RhoD positive or RhoD negative depending on the presence or absence of the D antigen on them. Approximately 85% of the Caucasian population is RhoD positive. The D^u phenotype is a variant of the D antigen and is recognized by performing the antiglobulin test.

Reagent

Erybank anti-D polyclonal is a ready-to-use high-protein reagent prepared from pools of hyperimmune human serum containing antibodies directed towards the human D (Rho) antigen. These antibodies of the immunoglobulins class IgG are a mixture of several polyclonal antibodies of the same specificity but having the capability of recognizing different epitopes of the human red blood cell antigen D (Rho).

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and titer. The reagent is suitable for slide and modified tube tests.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing D antigen will agglutinate in the presence of antibody directed towards the antigen. Agglutination of red blood cells with Erybank anti-D polyclonal reagent is a positive test result and indicates the presence of D antigen. No agglutination with anti-D reagent is a negative test result and indicates absence of D antigen. All negative test results should be further tested for D^u by performing the D^u test procedure as described later.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Erybank anti-D polyclonal reagent is from human source and the source material used in its manufacture is tested by approved techniques and found negative for HBsAg and HIV, HCV antibodies.
3. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.

Sample Collection and Storage

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2–8°C if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or heparin	: 2 days
Sodium citrate/sodium oxalate	: 14 days
ACD or CPD	: 28 days

Clotted whole blood should be tested within 14 days.

Additional Material Required for Slide and Tube Tests

Glass slides (50 × 75 mm), test tubes (10 × 75 mm), Pasteur pipettes, isotonic saline, centrifuge, timer, mixing sticks, Rl viewing box with 40–45°C surface temperature, Eryclone anti-human globulin (Coomb's) reagent, Eryclone Rh-hr control.

Test Procedure

Slide Test

1. Place one drop of Erybank anti-D polyclonal reagent on a clean prewarmed glass slide (40–45°C surface temperature).
2. Add one equal drop of whole blood.
3. Mix well with a mixing stick uniformly over an area of approximately 2.5 cm^2.
4. Rock the slide gently, back and forth.
5. Observe for agglutination macroscopically at 2 minutes.

Tube Test

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Erybank Anti-D polyclonal reagent into a labeled test tube.
3. Pipette into the test tube, one drop of the 5% cell suspension and mix well.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
5. Gently resuspend the cell button, observing for agglutination macroscopically.

D^u Test Procedure

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Erybank anti-D polyclonal reagent into a labeled test tube.
3. Pipette into the test tube one drop of the 5% cell suspension and mix well. Incubate at 37°C for 15 minutes.
4. Wash the contents of the tube thoroughly, at least three times, with isotonic saline and decant completely after the last wash.
5. Add two drops of Eryclone anti-human globulin re-agent and mix well.
6. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
7. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Interpretation of Results

Slide and Tube Tests

a. Agglutination with reagent and no agglutination with control is a positive test result and indicates the presence of D antigen. Do not interpret peripheral drying or fibrin strands as agglutination. No agglutination with reagent and control is a negative test result and indicates absence of D antigen.
b. Agglutination in Rh-hr (negative) control indicates the presence of autoantibodies or rouleaux formation. In such cases, it is recommended that the determination of Rh factor should be made in a saline reacting anti-D such as RHOFINAL anti-D (IgM + IgG).
c. Cord cells, heavily sensitized with anti-D, may give a false negative immediate spin test result.

D^u Test Procedure

a. Agglutination with reagent and no agglutination with control indicate the presence of D^u antigen. No agglutination with reagent and control indicates absence of D^u antigen.
b. Mixed field agglutination in D^u test on red cells from a recently delivered woman may indicate a mixture of maternal Rh negative and fetal Rh positive blood.
c. Red cells demonstrating a positive direct antiglobulin test cannot be accurately tested for D^u antigen.

Remarks

As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that, each laboratory calibrates its own equipment and the time required for achieving the desired results.

ANTI-D (RhO) (IgM) Monoclonal Blood Typing Antibodies for Slide and Tube Tests

(*Courtesy:* Tulip's Eryclone Range)

Summary

Monoclonal antibodies are derived from hybridoma cell lines, created by fusing mouse antibody producing B lymphocytes with mouse myeloma cells or are derived from a human B cell line through Epstein–Barr Virus (EBV) transformation. Each hybridoma cell line produces homogeneous antibodies of only one immunoglobulin class, which are identical in their chemical structure and immunological activity.

Human red blood cells are classified as RhoD positive or RhoD negative depending on the presence or absence of D antigen on them. Approximately, 85% of the Caucasian population is Rh positive. The D^u phenotype is a variant of D antigen and is recognized by performing the antiglobulin test.

About 60% of the traditional D^us, now classified as weak or partial D's may react with Eryclone anti-D (IgM) in slide tests and about 90% may be detected by the tube technique.

Reagent

Eryclone anti-D (IgM) is a ready-to-use reagent, prepared from supernatants of cell cultures with antibody producing B-lymphocytes obtained through EBV transformation and is a blend of monoclonal antibodies of immunoglobulin class IgM. These antibodies are a mixture of several monoclonal antibodies of the same specificity but having the capability of recognizing different epitopes of human red blood cell antigen D (Rho).

Eryclone Anti-D (IgM) does not detect all weak and partial D's. For the confirmation of negative reactions with Eryclone Anti-D (IgM) further testing with an incomplete Anti-D of IgG class is strongly recommended to confirm the presence or absence of weak/partial D's.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and performance.

Reagent Storage and Stability

a. Store the reagent at 2–8°C. Do not freeze.
b. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing D antigen will agglutinate in the presence of antibody directed towards the antigen. Agglutination of red blood cells with Eryclone anti-D (IgM) reagent is a positive test result and indicates the presence of D antigen. No agglutination with the reagent is a negative test result and indicates the absence of D antigen. All negative test results should be further tested for D (presence of weak/partial Ds) by performing the D^u test procedure using an incomplete Anti-D of IgG class, as described later.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Eryclone Anti-D (IgM) reagent is not from human source, hence, contamination due to HBsAg and HIV is practically excluded.
3. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.

Sample Collection and Storage

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2–8° C if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or heparin	:	2 days
Sodium citrate or sodium oxalate	:	14 days
ACD or CPD	:	28 days.

Clotted whole blood should be tested within 14 days.

Additional Material Required for Slide and Tube Tests

Glass slides (50 × 75 mm), test tubes (10 × 75 mm), Pasteur pipettes, isotonic saline, centrifuge, timer, mixing sticks, eryclone anti-human globulin (Coomb's) reagent, Eryclone anti-D (lgG) or RHOFINAL anti-D (lgM + lgG).

Test Procedure

Bring reagent and samples to room temperature before testing.

Slide Test

1. Place one drop of Eryclone anti-D (lgM) reagent on a clean glass slide
2. Pipette one equal drop of whole blood on the slide.
3. Mix well with a mixing stick uniformly over an area of approximately 2.5 cm.
4. Rock the slide gently, back and forth.
5. Observe for agglutination macroscopically at two minutes.

Immediate Spin Tube Test

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Eryclone anti-D (lgM) reagent into a labeled test tube.
3. Pipette into the test tube one drop of the 5% cell suspension and mix well.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
5. Gently resuspend the cell button, observing for agglutination macroscopically.

D^u Test Procedure

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of any incomplete anti-D (IgG class) reagent such as Eryclone anti-D (IgG) into a labeled test tube.
3. Add to the test tube one drop of the 5% cell suspension and mix well. Incubate at 37°C for 15 minutes.
4. Wash the contents of the tube thoroughly, at least three times, with isotonic saline and decant completely after the last wash.
5. Add two drops of Eryclone anti-human globulin reagent and mix well.
6. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
7. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Interpretation of Results

Slide and Tube tests

a. Agglutination is a positive test result and indicates the presence of D antigen. Do not interpret peripheral drying or fibrin strands as agglutination. No agglutination is a negative test result and indicates the absence of D antigen.
b. Cord cells heavily sensitized with anti-D may give a false negative immediate spin test result.

D^u Test Procedure

a. Agglutination indicates the presence of D^u antigen (Presence of weak/partial Ds). No agglutination indicates the absence of D^u antigen (Absence of weak/partial Ds).
b. Mixed field agglutination in the D^u test on red cells from a recently delivered woman may indicate a mixture of maternal RhoD negative and fetal RhoD positive blood.
c. Red cells demonstrating a positive direct antiglobulin test cannot be accurately tested for D^u antigen (Presence of weak/partial Ds).

Remarks

As under centrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrate its own equipment and determine the time required for achieving the desired results. It is strongly recommended that as a routine quality control measures known RhoD positive and RhoD negative red cells be occasionally run, preferably on a daily basis so as to control reagent performance and validate test results. After usage the reagent should be immediately recapped and replaced at 2–8°C storage.

ANTI-D (Rho) (IgG) Monoclonal Blood Typing Antibodies for Slide and Modified Tube Tests

(*Courtesy:* Tulip's Eryclone Range)

Summary

Monoclonal antibodies are derived from hybridoma cell lines, created by fusing mouse antibody producing B-lymphocytes with mouse myeloma cells or are derived from a human B cell line through EBV transformation. Each hybridoma cell line produces homogeneous antibodies of only one immunoglobulin class, which are identical in their chemical structure and immunological activity.

Human red blood cells are classified as Rho(D) positive or Rho(D) negative depending upon the presence or absence of D(Rho) antigen on them. Approximately, 85% of the Caucasian population is Rho(D) positive. The D^u phenotype is a variant of D(Rho) antigen and is recognized by performing the antiglobulin test.

Reagent

Eryclone® anti-D(Rho) (lgG) is a ready-to-use high-protein reagent, prepared from supernatants of cell cultures with

antibody producing B lymphocytes obtained through EBV transformation. These antibodies of the immunoglobulin class IgG are a mixture of several monoclonal antibodies of the same specificity but having the capability of recognizing different epitopes of the human red blood cell antigen D (Rho).

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and performance.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing the D(Rho) antigen will agglutinate in the presence of antibody directed towards the antigen. Agglutination of red blood cells with Eryclone® anti-D(Rho) (lgG) reagent is a positive test result and indicates the presence of D(Rho) antigen. No agglutination with anti-D(Rho) (lgG) reagent is a negative test result and indicates the absence of D(Rho) antigen. All negative test results should be further tested for D^u (Presence of weak/partial Ds) by performing the D^u test procedure as described later.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Eryclone® anti-D (Rho) (IgG) reagent is not from human source, hence, contamination due to HBsAg and HIV is practically excluded.
3. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2–8°C if not tested immediately. Do not use hemolyzed samples.

Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period

EDTA or heparin	: 2 days
Sodium citrate or sodium oxalate	: 14 days
ACD or CPD	: 28 days.

Clotted whole blood should be tested within 14 days.

Additional Material Required for Slide and Tube Tests

Glass slides (50 × 75 mm), test tubes (10 × 75 mm), Pasteur pipettes, isotonic saline, centrifuge, timer, mixing sticks, Eryclone® anti-human globulin (Coomb's) reagent, Eryclone® Rh-hr control.

Test Procedure

It is recommended that a negative control be run simultaneously with each RhoD test sample using Eryclone® Rh-hr control because invalid positive results may be obtained as with all high-protein blood typing reagent, especially with samples having autoantibodies or abnormal serum proteins.

Bring reagent and samples to room temperature before testing.

Slide Test

1. Place one drop of Eryclone® anti-D (Rho) (lgG) reagent on a clean prewarmed glass slide (40–45°C surface temperature).
2. Add one equal drop of whole blood.
3. Mix well with a mixing stick uniformly over an area of approximately 2.5 cm^2.
4. Rock the slide gently, back and forth.
5. Observe for agglutination macroscopically at 2 minutes.

Tube Test

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Eryclone® anti-D (Rho) (lgG) reagent into a labeled test tube.
3. Pipette into the test tube, one drop of the 5% cell suspension and mix well. Incubate at 37°C for 15 minutes.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
5. Gently resuspend the cell button, observing for agglutination macroscopically.

D^u Test Procedure

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of Eryclone® Anti-D (Rho) (lgG) reagent into a labeled test tube.
3. Pipette into the test tube one drop of the 5% cell suspension and mix well. Incubate at 37°C for 15 minutes.
4. Wash the contents of the tube thoroughly, at least three times, with isotonic saline and decant completely after the last wash.

5. Add two drops of Eryclone® anti-human globulin reagent and mix well.
6. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
7. Very gently, resuspend the cell button, observing for agglutination macroscopically.

Interpretation of Results

Slide and Tube Tests

a. Agglutination with reagent and no agglutination with Rh-hr control is a positive test result and indicates the presence of the D(Rho) antigen. Do not interpret peripheral drying or fibrin strands as agglutination. No agglutination with reagent and control is a negative test result and indicates absence of the D(Rho) antigen.
b. Agglutination in Rh-hr (negative) control indicates the presence of autoantibodies or rouleaux formation. In such cases, it is recommended that the determination of Rh factor should be made with a saline reacting anti-D such as RHOFINAL® anti-D (IgM + IgG).
c. Cord cells heavily sensitized with anti-D(Rho) may give a false negative immediate spin test result.

D^u Test Procedure

a. Agglutination with reagent and no agglutination with control indicates the presence of D^u antigen (weak/partial Ds). No agglutination with reagent and control indicates absence of the D^u antigen.
b. Mixed field agglutination in the D^u test on red cells from a recently delivered woman may indicate a mixture of maternal Rho(D) negative and fetal Rho(D) positive blood,
c. Red cells demonstrating a positive direct antiglobulin test cannot be accurately tested for D^u antigen (weak/partial Ds).

Remarks

1. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrates its own equipment and determine the time required for achieving the desired results.
2. It is strongly recommended that as a routine quality control measure known Rho(D) positive and Rho(D) negative red cells be occasionally run, preferably on a daily basis so as to control reagent performance and validation of test results.
3. After usage the reagent should be immediately recapped and replaced to 2–8°C storage.

ANTI-D (RhO) (IgM + IgG) Monoclonal Blood Typing Antibodies for Slide and Tube Tests

(Rhofinal from Tulip)

Summary

Monoclonal antibodies are derived from hybridoma cell lines, created by fusing mouse antibody producing B lymphocytes with mouse myeloma cells or are derived from a human B cell line through EBV transformation. Each hybridoma cell line produces homogeneous antibodies of only one immunoglobulin class, which are identical in their chemical structure and immunological activity. Human red blood cells are classified as Rho(D) positive or Rho(D) negative depending on the presence or absence of Rho(D) antigen on them. Approximately, 85% of the Caucasian population is Rho(D) positive. The D^u phenotype is a traditional definition to describe the weak/partial Ds that can be detected with Rhofinal® anti-D (Rho) (lgM + IgG). About 60% of the D^us(weak/partial D^us) may react with RHOFINAL® anti-D (Rho) (IgM + IgG) in slide tests and about 90% may be detected by the tube technique.

Reagent

RHOFINAL® anti-D (Rho) (lgM + IgG} is ready-to-use reagent, prepared from supernatants of cell cultures with antibody producing B-lymphocytes obtained through EBV transformation and is a blend of monoclonal antibodies of immunoglobulin class IgM and IgG. These antibodies are a mixture of several monoclonal antibodies of the same specificity but having the capability of recognizing different epitopes of the human red blood cell antigen D (Rho).

RHOFINAl® Anti-D (Rho) (IgM + IgG) is a blend of IgM and IgG class of Anti-D (Rho) monoclonals, a characteristic which accords versatility to the reagent. It gives an avid saline reacting slide/tube test reagent the capability of detecting D^u (weak/partial Ds) in the anti-human globulin phase. Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and performance.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human red blood cells possessing D(Rho) antigen will agglutinate in the presence of antibody directed towards the antigen. Agglutination of red blood cells with RHOFINAL® anti-D (Rho) (IgM-IgG) reagent is a positive test result and indicates the presence of D(Rho) antigen. No agglutination with RHOFINAL® anti-D (Rho) (IgM + IgG) reagent is a negative test result and indicates the absence of D(Rho) antigen. All negative test results should be further tested for D^u (weak/partial Ds) by performing the D^u test procedure, as described later.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. RHOFINAL® anti-D (Rho) (lgM + IgG) reagent is not from human source, hence, contamination due to HBsAg and HIV is practically excluded.
3. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Samples should be stored at 2–8°C if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or heparin	:	2 days
Sodium citrate or sodium oxalate	:	14 days
ACD or CPD	:	28 days

Clotted whole blood should be tested within 14 days.

Additional Material Required for Slide and Tube Tests

Glass slides (50 × 75 mm), test tubes (12 × 75 mm), Pasteur pipettes, isotonic saline, centrifuge, timer, mixing sticks, Eryclone® anti-human globulin (Coomb's) reagent.

Test Procedure

Bring reagent and samples to room temperature before testing.

Slide Test

1. Place one drop of RHOFINAL® anti-D (Rho) (lgM + lgG) on a clean glass slide.
2. Pipette one equal drop of whole blood on the slide.
3. Mix well with a mixing stick uniformly over an area of approximately 2.5 cm^2.
4. Rock the slide gently, back and forth.
5. Observe for agglutination macroscopically at 2 minutes.

Tube Test

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of RHOFINAL® anti-D (Rho) (IgM + IgG) reagent into labeled test tube.
3. Pipette into each of the test tube one drop of the 5% cell suspension and mix well.
4. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
5. Gently resuspend the cell button, observing for agglutination macroscopically.

D^u Test procedure

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Place one drop of RHOFINAL® anti-D (Rho) (lgM + lgG) reagent into labeled test tube.
3. Add to the test tube, one drop of the cell suspension and mix well. Incubate at 37°C for 15 minutes.
4. Wash the contents of the tube, at least three times, with isotonic saline and decant completely after the last wash.
5. Add two drops of Eryclone® anti-human globulin reagent and mix well.
6. Centrifuge for 1 minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
7. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Interpretation of Results

Slide and Tube Tests

a. Agglutination is a positive test result and indicates the presence of the D(Rho) antigen. Do not interpret peripheral drying or fibrin strands as agglutination. No agglutination is a negative test result and indicates absence of the D(Rho) antigen.
b. Cord cells heavily sensitized with anti-D(Rho) may give a false negative immediate spin test result.

D^u Test Procedure

a. Agglutination with the reagent and no agglutination with the control indicate presence of the D^u antigen (weak/partial Ds). No agglutination with reagent and control indicates absence of the D^u antigen (weak/partial Ds).

b. Mixed field agglutination in the D^u test on red cells from a recently delivered woman may indicate a mixture of maternal Rho(D) negative and fetal Rho(D) positive blood.
c. Red cells demonstrating a positive direct antiglobulin test cannot be accurately tested for D^u antigen (weak/partial Ds).

Remarks

1. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrates its own equipment and determine the time required for achieving the desired results.
2. It is strongly recommended that as a routine quality control measure known Rho(D) positive and Rho(D) negative red cells be occasionally run, preferably on a daily basis so as to control reagent performance and validation of test results.
3. After usage, the reagent should be immediately recapped and replaced at 2–8°C storage.

Blood Group Testing in Microplates

The microplates consist of 12 × 8 = 96 wells. We can do 12 groups in one plate.

	1	2	3	4	5	6	7	8	9	10	11	12
Anti-A®	O	O	O	O	O	O	O	O	O	O	O	O
Anti-B®	O	O	O	O	O	O	O	O	O	O	O	O
Anti-AB®	O	O	O	O	O	O	O	O	O	O	O	O
Anti-D_1®	O	O	O	O	O	O	O	O	O	O	O	O
Anti-D_2®	O	O	O	O	O	O	O	O	O	O	O	O
A Cell®	O	O	O	O	O	O	O	O	O	O	O	O
B Cell®	O	O	O	O	O	O	O	O	O	O	O	O
O Cell®	O	O	O	O	O	O	O	O	O	O	O	O

In the first 5 wells we do direct blood grouping (cell grouping) and in remaining 3 wells we do serum grouping.

a. For all microtitration techniques, the cell suspension required is 2% (so we prepare 2% cell suspension of patient/donor cell and also 2% suspension of A cell, B cell, O cells (pooled).
b. Antisera used in microtiter plates are also diluted. Roughly we dilute anti-A and anti-B seras as 1:20 dilution and anti-D as 1:10 (Dilution of antisera depends on the titration of anti-A, anti-B and anti-D. We select the best dilution at which the reaction occurs).

Procedure

1. In the first 5 wells put one drop each of diluted (as described above) anti-A, anti-B, anti-AB, anti -D_1, and anti-D_2 (i.e. anti-D of two different companies).
   +
   Add 1 drop of patient/donor red cell suspension (2%).
2. In the next three wells (reverse grouping) put one drop each of A cell, B cell and O cell
   +
   Add one drop of patient serum.
3. Incubate the tubes at RT for 60 minutes
   In emergency cases, centrifuge the plates at 1000 rpm for 3–4 minutes and see for agglutination.

Results: In negative reaction, the red cells trail from the center of the well; and in positive reaction, cells remain in the center or fall in discrete button to the bottom of well.

Direct Anti-human Globulin Test (DAT)

DAT is used to detect in vivo sensitization of red blood cells with immunoglobulin, complement or both. A positive DAT, with or without shortened red blood cell survival, may result from:

- Autoantibodies to intrinsic red blood cell antigens
- Alloantibodies in recipients circulation reacting with antigens on recently transfused donor red blood cells
- Alloantibodies in donor plasma, plasma derivatives or blood fractions, which react with antigens on red blood cells of transfusion recipients
- Alloantibodies in maternal circulation, which cross placenta and sensitize fetal red blood cells (HDN)
- Antibodies directed against certain drugs, which bind to red blood cell membranes (e.g. Penicillin)
- Adsorbed proteins, including immunoglobulins, which attach to abnormal membranes or red blood cells modified by therapy with certain drugs, notably those of cephalosporin group
- Complement components or rarely IgG bound to red blood cells after administration of drugs such as quinidine and phenacetin may induce drug antidrug interaction
- Non-red blood cell immunoglobulins associated with red blood cells in patient with hypergammaglobulinemia or recipients with high dose of intravenous gamma globulin
- In patient with organ transplantation, passenger lymphocytes of donor origin produce antibodies directed against ABO or other antigens on the recipient's cells, causing a positive DAT

- Patients receiving ALG (anti-lymphocyte globulin) or ATG (anti-thymocyte globulin) of animal origin may develop a positive DAT within a few days, apparently related to high titer heterophile antibodies in these products and the presence of corresponding antibodies in animal derived AHG sera.

Major Applications of DAT in Blood Group Serology

Hemolytic Disease of the Newborn (HDN)

In HDN fetal red blood cells in vivo are sensitized with IgG alloantibody of maternal origin thereby demonstrating a positive DAT with cord red blood cells. The most commonly observed HDN is due to Rho(D) incompatibility between mother and fetus.

If the father is Rho(D) positive and the mother is Rho(D) negative and during first pregnancy their progeny inherits Rho(D) positive red blood cell antigens. During parturition, the fetal red blood cells can enter mother's circulation providing antigenic stimulus for the production of anti-D antibodies. These anti-D antibodies normally will not have any effect during the first Rh-incompatible pregnancy unless the mother has anti-D antibodies by previous incompatible blood transfusions.

During subsequent pregnancy, for the same couple, if the fetus is Rho(D) positive again, the anti-D antibodies will be activated along with the presence of anti-D antibodies from the first pregnancy already in the circulation. Since the IgG antibodies cross the placental barrier, these circulating anti-D will sensitize and destroy fetal Rho(D) positive cells. This process is demonstrated by a positive DAT on cord red blood cells (Fig. 11.4).

Transfusion Reactions

A patient will demonstrate positive DAT, if serum contains antibodies against red blood cell antigens of donor red blood cells. Likewise antibody present in donor plasma may also react with recipient red blood cells thereby demonstrating positive DAT.

Other Immune Hemolytic Diseases

A positive DAT may be observed due to acquired hemolytic anemia probably because of autoantibodies directed against individual's own intrinsic red blood cell antigens.

Classification of Autoimmune Hemolytic Anemia

- Warm autoimmune hemolytic anemia (WAIHA)
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, SLE, carcinoma, drug therapy).
- Cold agglutinin syndrome (CAS)
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, *Mycoplasma pneumoniae*, infectious mononucleosis).
- Mixed type autoimmune hemolytic anemia
  - Primary (idiopathic)
  - Secondary (to conditions such as SLE, lymphoma).
- Paroxysmal cold hemoglobinuria (PCH)
  - Primary (idiopathic)
  - Secondary (to conditions such as syphilis, viral infections).
- DAT negative autoimmune hemolytic anemia
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, SLE).

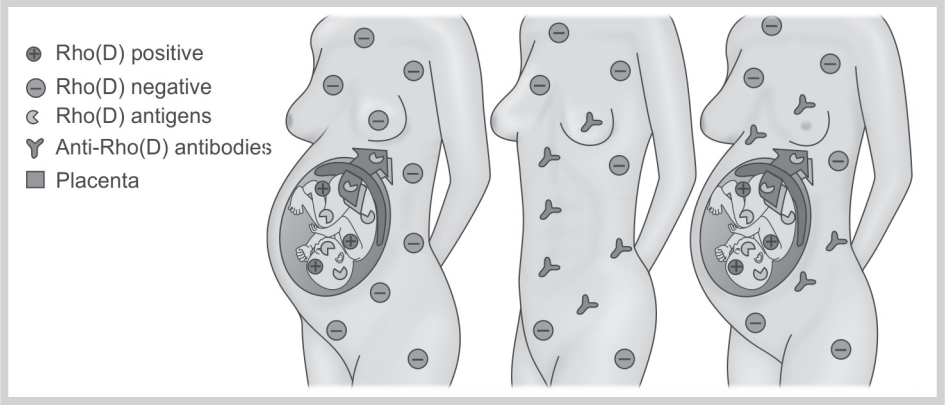

FIG. 11.4: Illustration of HDN

Drug-induced Hemolytic Anemia

Also, certain drugs namely, penicillin, procainamide, cephalosporins may also be associated with immune red blood cell destruction thereby demonstrating a positive DAT (Fig. 11.5).

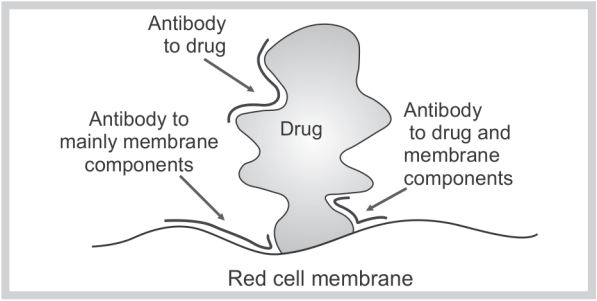

FIG. 11.5: Illustration of drug-induced antibody reactions

Importance of Serological Studies in DAT Positive Results (Table 11.5)

As per blood bankers' technical manual, three investigation approaches are helpful in evaluation of positive DAT:

- Test the DAT positive red blood cells with monospecific anti-human IgG and monospecific anti-human C_3d reagent to characterize type of proteins sensitized with red blood cell membrane,
- Test serum/plasma to detect and identify clinically significant antibodies to red blood cell antigens.
- Test eluate prepared from sensitized red blood cells with a panel of reagent red blood cells to define whether the sensitized protein is immunoglobulin or complement component. Elution frees antibody from sensitized red blood cells and recovers antibody

TABLE 11.5: Probable serological findings with DAT positive—AIHA/drug induced hemolytic anemia

Parameter	*WAIHA*	*CAS*	*Mixed type AIHA*	*PCH*	*Drug-induced AIHA*
DAT positive result	IgG/IgG + C_3/C_3	Mostly C_3	IgG + C_3	Mostly C_3	IgG/IgG + C_3
Immunoglobulin Type	IgG sometimes IgA or IgM rarely alone	IgM	IgG, IgM	IgG	IgG
Eluate	IgG	Non reactive	IgG	Non-reactive	IgG
Serum	• May react by IAT • May hemolyze enzyme treated red cells at 37°C • Mostly agglutinate enzyme treated red cells at 37°C • May agglutinate untreated red cells at 20°C • Rarely agglutinate untreated cells at 37°C	• IgM hem-agglutinating antibody reactive at 4°C usually react at 30°C in albumin	• IgG IAT reactive antibody • IgM hem-agglutinating antibody usually react at 30–37°C in saline, also may react at 4°C saline	• IgG biphasic hemolysin (Donath Landsteiner antibody)	• IgG antibody similar to WAIHA
Specificity	Usually Rh specificity	Usually Anti-I but can be Anti-I rarely Anti-Pr	Usually specificity unclear, can be Anti-I, Anti-I or other cold agglutinin specificities	Anti-P (non-reactive with p and P^k red cells	Specificity often Rh related

in usable form. When only complement is sensitized, eluates are frequently non-reactive.

Indirect Anti-human Globulin Test (IAT)

In IAT procedures, serum or plasma is incubated with red blood cells, washed to remove unbound globulins. Agglutination that occurs after addition of Anti-human globulin reagent indicates reaction between antibody in the serum and antigen present on the red blood cell membrane.

Applications of IAT

IAT determines in vitro sensitization of red blood cells and is used in the following situations:

- Detection of incomplete antibodies to potential donor red blood cells, pregnant women, blood donors
- Identification of antibody specificity using a panel of red blood cells with known antigenic profile
- Determination of red blood cell phenotype using known antisera (e.g. D^u testing)
- Titration of incomplete antibodies.

Probable Sources of Error in Anti-human Globulin Testing

False Negative Results

- Neutralization of anti-human globulin reagent
- Failure to wash cells adequately to remove all serum/plasma. Fill tube at least three-fourth full of saline for each wash
  - If increased serum volumes are used, routine wash may be inadequate. Wash additional times more than three or four wash phases
  - Contamination of Anti-human globulin reagent by extraneous protein. Do not use finger or hand to cover tube. Contaminated droppers or wrong reagent dropper can neutralize entire vial of Anti-human globulin reagent
  - High concentration of IgG paraproteins in test serum (cryoglobulin). Wash additional times more than three or four wash phases
- Interruption in testing
  - Bound IgG may dissociate from red blood cells or leave too little IgG to detect or may neutralize Anti-human globulin reagent. Perform the test immediately
  - Agglutination of IgG coated cells will weaken. Centrifuge and read immediately
- Improper reagent storage
  - Anti-human globulin reagent may lose reactivity if frozen. Reagent may become bacterially contaminated. Store at the recommended storage condition
  - Excess heat or repeated freeze/thaw cycles may cause loss of reactivity of anti-human globulin reagent. Replace the reagent back to the recommended storage condition
- Improper procedure
  - Overcentrifugation may pack cells so tightly that agitation required to resuspend cells breaks up agglutinates. Undercentrifugation may not be optimal for agglutination. The optimum centrifugation speed should be ascertained for each centrifuge
  - Failure to add test serum, enhancement medium or Anti-human globulin reagent may lead to negative test result. Follow the manufacturer's instructions meticulously
  - Too heavy red cell concentration may mask weak agglutination. Too light suspension may be difficult to read
  - Improper/insufficient serum-cell ratio
- Complement
  - Rare antibodies, notably Anti-Jk^a and Anti-Jk^b may only be detected when polyspecific Anti-human globulin reagent is used and active complement is present
- Saline
  - Low pH of saline solution can decrease sensitivity of Anti-human globulin test. Optimal wash solution for most antibodies is pH 7.0–7.2. It has been observed that commercially available infusion saline/saline stored in plastic containers can seriously compromise the sensitivity of anti-human globulin test. Saline stored in plastic containers and further autoclaved leads to leaching of certain chemicals which shifts the pH to the acidic side and impacts the sensitivity of anti-human globulin test. Preferably, use phosphate buffered saline as wash solution or suspending medium
  - Some antibodies may require saline to be at specific temperature to retain antibody on red blood cell. Use 37°C or 4°C saline.

False Positive Results

- Particles or contaminants
  - Dust or dirt in glassware may cause clumping of cells. Fibrin or precipitates in test serum may similarly produce cell clumps that mimic agglutination
- Improper procedure
  - Overcentrifugation may pack cells so tightly that they do not easily disperse and appear positive.

- Centrifugation of test with polyethylene glycol or positively charged polymers prior to washing may create clumps that do not disperse

➢ Cells with positive DAT result
- Cells that are positive by DAT will also be positive in any indirect antiglobulin test. In such cases, antibodies should be eluted from the sensitized cells

➢ Complement
- Complement components, primarily C_4, may bind to cells from clots or from CPDA-1 donor segments during storage at 4°C and occasionally at higher temperature. For DATs, use red blood cells anticoagulated with EDTA, ACD or CPD
- Samples collected in scratched glass tubes can lead to spurious activation of complement
- Complement may attach to cells in specimens collected from infusion lines used to administer dextrose containing solutions. Strongest reactions are seen when large bore needles are used or when sample volume is less than 0.5 mL.

Coomb's Control Cells/Complement Coated Cells

Coomb's control cells should be used routinely in direct and indirect anti-human globulin test. Coomb's control reagent is anti-D IgG sensitized, washed and made up to a 5% suspension. Coomb's control cells are used for:

➢ Procedural validation of tests employing Coomb's reagent. Coomb's control cells are added after performing anti-human globulin test. To a negative result after addition of Coomb's control cells, agglutination indicates that AHG was indeed added and that it has not been neutralized.

➢ Functional validation of Coomb's reagent. The performance of Coomb's reagent can be validated as a quality control measure on routine basis.

Similarly, complement-coated cells can also be prepared in vitro. Thus, complement-coated cells can also be used for functional validation of Coomb's reagent.

Now with commercially available red blood cell stabilizing solution, the Coomb's control cells and complement-coated cells can be prepared in situ and stored in cell stabilizing solution for long-term storage and use.

Indirect Anti-human Globulin Test for the Detection of Red Blood Cell Antibodies

Saline Phase Indirect Anti-human Globulin Test

Specimen

Serum or plasma may be used. Preferably, freshly collected serum should be used.

Reagents
1. Normal saline
2. Polyspecific AHG or monospecific anti-human IgG reagent
3. Coomb's control cells
4. Donor cells/reagent red blood cells.

Procedure
1. To properly labeled test tubes add two drops of serum.
2. Add one drop of reagent red blood cells or donor red blood cells as a 2 to 5% saline suspension to each tube and mix well.
3. Centrifuge for 15 to 20 seconds at approximately 900 to 1000 g. Observe for hemolysis and/or agglutination. Grade and record the results.
4. Incubate at 37°C for 30 to 60 minutes.
5. Centrifuge for 15 to 20 seconds at approximately 900 to 1000 g and observe for hemolysis and/or agglutination. Grade and record the results.
6. Wash the red blood cells three or four times with saline and completely decant after the final wash.
7. Add AHG reagent to the cell button according to the manufacturer's instructions. Mix well.
8. Centrifuge for 15 to 20 seconds at approximately 900 to 1000 g and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coomb's control cells.

Albumin Phase Indirect Anti-human Globulin Test

Specimen

Serum or plasma may be used. Preferably, freshly collected serum should be used.

Reagents
1. Normal saline
2. Bovine albumin (22 or 30%)
3. Polyspecific AHG or monospecific anti-human IgG reagent
4. Coomb's control cells
5. Donor cells/reagent red blood cells.

Procedure
1. To properly labeled test tubes add two drops of serum.
2. Add an equivalent volume of 22 or 30% bovine albumin (unless manufacturer's directions state otherwise).
3. Add one drop of 2 to 5% saline suspended reagent or donor red blood cells to each tube and mix.
4. Incubate at 37°C for 15 to 30 minutes.
5. Centrifuge for 15 to 20 seconds at 900 to 1000 g. Observe for hemolysis and/or agglutination. Grade and record the results.

6. Wash the cells three or four times with saline and completely decant after final wash.
7. Add AHG to cell button according to the manufacturer's instruction. Mix well.
8. Centrifuge and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coomb's control cells.

LISS (Low Ionic Strength Solution) Phase Indirect Anti-human Globulin Test

Specimen
Serum or plasma may be used. Preferably, freshly collected serum should be used.

Reagents
1. Normal saline
2. LISS
3. Polyspecific AHG or monospecific anti-human IgG reagent
4. Coomb's control cells
5. Donor cells/reagent red blood cells.

Procedure
1. Wash reagent or donor red blood cells three times in normal saline and completely decant saline after last wash.
2. Resuspend the cells to a 2–5% suspension in LISS.
3. To properly labeled test tube, add two drops of serum.
4. Add two drops of LISS suspended red blood cell suspension and incubate according to manufacturer's direction. Typically, this is 10 to 15 minutes at 37°C.
5. Centrifuge according to manufacturer's direction. Typically, this is 15 to 30 seconds at 900 to 1000 g and observe for hemolysis and agglutination by gently resuspending the cell button. Grade and record results.
6. Wash the cells three or four times with saline and completely decant after final wash.
7. Add AHG to cell button according to the manufacturer's instruction. Mix well.
8. Centrifuge for 15 to 20 seconds at 900 to 1000 g and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coomb's control cells.

PEG-enhanced Indirect Anti-human Globulin Test

Specimen
Serum or plasma may be used. Preferably, freshly collected serum should be used.

Reagents
1. Normal saline
2. PEG (20% in PBS)
3. Polyspecific AHG or monospecific anti-human IgG reagent
4. Coomb's control cells
5. Donor cells/reagent red blood cells.

Procedure
1. For each sample to be tested, mix 2 drops of test serum, 4 drops of 20% PEG in PBS, and 1 drop of 2–5% red blood cell suspension.
2. Incubate according to manufacturer's directions. Typically, this is 15 minutes at 37°C.
3. Do not centrifuge.
4. Wash the cells four times with saline and completely decant after the final wash.
5. Add AHG to cell button according to the manufacturer's instruction. Mix well.
6. Centrifuge for 15 to 20 seconds at 900 to 1000 g and observe for reaction. Grade and record the results.
7. Confirm the validity of negative tests by adding Coomb's control cells.

LIM (Low Ionic Medium Polybrene) Indirect Anti-human Globulin Test

Specimen
Serum or plasma may be used. Preferably, freshly collected serum should be used.

Reagents
1. Normal saline.
2. Low ionic medium (LIM): To a 500 mL volumetric flask, add 25 g of dextrose and 1 g of Na_2 EDTA $2H_2O$. Fill flask to 500 mL mark with distilled water.
3. Polybrene® (Commercially available).
4. Resuspending medium: 0.2 M trisodium citrate, 5% dextrose; working solution made by mixing 60 mL of 0.2 M trisodium citrate with 40 mL of 5% dextrose.
5. Washing solution (for anti-human globulin testing): 0.01 M trisodium citrate.
6. Polyspecific AHG or monospecific anti-human IgG reagent.
7. Coomb's control cells.
8. Donor cells/reagent red blood cells.

Procedure
1. Prepare 1% suspension of donor or reagent red blood cells in the serum used for testing.
2. Add 1.0 mL of LIM solution. Mix and incubate for 1 minute at room temperature.
3. Add 0.1 mL of 0.05% Polybrene to each tube and mix.
4. Centrifuge according to manufacturer's directions. Typically, this is 10 seconds at 900 to 1000 g and decants the supernatant fluid. Do not resuspend cell button.
5. Add 0.1 mL of resuspending solution. Shake tube gently and observe for persistent agglutination.

If strength of agglutination is weak, examine the test and a known negative control macroscopically. Do not recentrifuge.

6. If desired, the anti-human globulin test may be performed as follows:
   a. Add 0.05 mL of resuspending solution to each tube and mix well.
   b. Wash the cells three times with 0.01 M trisodium citrate solution.
   c. Add two drops of AHG reagent to the cell button and mix.
   d. Centrifuge for 15 seconds at 900 to 1000 g. Read and record the results.
   e. Add Coomb's control cells to each negative tube.

Interpretation of Results for Anti-human Globulin Tests

1. Agglutination/hemolysis after incubation at 37°C constitutes a positive test.
2. The presence of agglutination after addition of AHG reagent constitutes a positive test.
3. Anti-human globulin tests are considered negative when no agglutination is observed after initial centrifugation and positive result with Coomb's control cells. If after addition of Coomb's control cells a negative result is observed, then the test is invalid and must be repeated.
4. For the LIM (Low Ionic Medium-Polybrene) procedure, agglutination that persists after addition of resuspending solution indicates a positive result.

Controls

1. The procedure used for the detection of unexpected antibodies in transfusion testing should be checked daily with weak antibodies.
2. When LIM technique is used, test an unknown serum against reagent red blood cells, an inert serum should be tested against a random cell sample for comparative purposes.

Notes

1. The incubation time and volume and concentration of red cells incubated are those given in literature. In all cases, the manufacturer's package insert should be strictly adhered to.
2. For the PEG procedure:
   a. Omit centrifugation after 37°C incubation, as red blood cells will not resuspend readily.
   b. Use monospecific anti-human IgG rather than polyspecific AHG to avoid unwanted positive reactions due to C_3-binding antibodies.
3. LISS additive and PEG solutions are available from various commercial sources. Manufacturer's instruction should be followed when using these reagent.

Papain—One-stage Enzyme technique/Two-stage Enzyme Technique

Specimen

Serum to be tested. Preferably, freshly collected serum should be used.

Reagent

1. Reagent red blood cells.
2. Polyspecific AHG or monospecific anti-human IgG reagent.
3. Coomb's control cells.
4. Donor cells/reagent red blood cells.

Procedure for One-stage enzyme technique

1. To an appropriately labeled test tube, add two drops of serum.
2. Add two drops of 2–5% saline suspension of reagent red blood cells.
3. Add two drops of papain solution and mix well.
4. Incubate at 37°C for 30 minutes.
5. Centrifuge for 15–20 seconds at 900–1000 g and gently resuspend the cells, observe for agglutination. Grade and record the results.
6. Wash the cells four times with saline and completely decant after the final wash.
7. Add AHG to cell button according to the manufacturer's instruction. Mix well.
8. Centrifuge for 15–20 seconds at 900–1000 g and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coomb's control cells.

Procedure for two-stage enzyme technique

1. Add one drop of washed packed cells and one drop of papain reagent to appropriately labeled tube.
2. Incubate at 37°C for 30 minutes.
3. Wash the papain treated three times with isotonic saline and prepare 2–5% cell suspension.
4. To an appropriately labeled test tube, add one drop of papain-treated red blood cell suspension and two drops of serum under test.
5. Mix well and incubate at 37°C for 30 minutes.
6. Centrifuge for 15–20 seconds at 900–1000 g and gently resuspend the cells, observe for agglutination. Grade and record the results.
7. Wash the cells four times with saline and completely decant after the final wash.
8. Add AHG to cell button according to the manufacturer's instruction. Mix well.
9. Centrifuge for 15–20 seconds at 900–1000 g and observe for reaction. Grade and record the results.

10. Confirm the validity of negative tests by adding Coomb's control cells.

Antibody Titration Studies

Antibody Titration for Characterizing Type of Antibody in Serum

Specimen

Serum (antibody) to be titrated.

Reagents

1. Red blood cells that express the antigen(s) corresponding to the antibody specificity(ies), in a 2–5% saline suspension. Uniformity of cell suspensions is very important to ensure comparability of results.
2. Normal saline (Dilutions may be made with 6% albumin if desired).

Procedure

The master dilution technique for titration studies is as follows:

1. Label ten test tubes according to the serum dilution (e.g. 1 in 1,1 in 2, etc.).
2. Deliver one volume of saline to all test tubes except the first.
3. Add an equal volume of serum to each of the first two tubes (undiluted and 1 in 2).
4. Using a clean pipette, mix the contents of the 1 in 2 dilution several times, and transfer one volume into the next tube (1 in 4 dilution).
5. Continue the same process for all dilutions, using a clean pipette to mix and transfer each dilution. Remove one volume of diluted serum from the final tube and save it for use if further dilutions are required.
6. Label ten 10 × 75 mm or 12 × 75 mm tubes for the appropriate dilutions.
7. Using separate pipettes for each dilution, transfer two drops of each diluted serum into the appropriately labeled tubes, and add one drop of red blood cell suspension.
8. Mix well and test by serologic technique appropriate to the antibody.
9. Examine test results macroscopically, grade and record the reactions. The prozone phenomenon may cause reactions to be weaker in the more concentrated serum preparations than in higher dilutions; to avoid misinterpretation of results, it may be preferable to examine first the tube containing the most dilute serum and proceed through the more concentrated samples to the undiluted specimen.

Interpretation

1. Observe the highest dilution that produces 1 + macroscopic agglutination. The titer is the reciprocal of the dilution. If there is agglutination in the tube containing the most dilute serum, the endpoint has not been reached, and additional dilutions should be prepared and tested.
2. In comparative studies, a significant difference in titer is three or more dilutions. Variations in technique and inherent biologic variability can cause duplicate tests to give results that differ by one dilution in either direction.
3. Titer values alone can be misleading, without additional evaluation of strength of agglutination.

The observed strength of agglutination can be assigned a number and the sum of these numbers for all tubes in a titration study represents the score. The arbitrarily assigned threshold for significance in comparing scores is a difference of 10 or more between different test samples (Table 11.6).

TABLE 11.6: Examples of antibodies tilers, endpoint and scores

	Reciprocal of serum dilution											
	1	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	*Titer*	*Score*
Strength:	3+	3+	3+	2+	2+	2+	1+	±	±	0	64 (256)	
Sample 1												
Score:	10	10	10	8	8	8	5	3	2	0		64
Strength:	4+	4+	4+	3+	3+	2+	2+	1+	±	0	128 (256)	
Sample 2												
Score:	12	12	12	10	10	8	8	5	3	0	80	
Strength:	1+	1+	1+	1+	±	±	±	±	±	0	8 (256)	
Sample 3												
Score:	5	5	5	5	3	3	3	2	2	0	33	

Notes

Titration is a semiquantitative technique and technical variables greatly affect the results. Hence, care should be taken to achieve the most uniform possible practices.

1. Careful pipetting is essential. Pipettes with disposable tips that can be changed after each dilution is recommended.
2. Optimal time and temperature of incubation, time and force of centrifugation must be used consistently.
3. The age, phenotype and concentration of test red blood cells influence the results. When the titers of several antibody containing sera are to be compared, all should be tested against red blood cells (preferably freshly collected) from the same donor. If this is not possible, the tests should use a pool of reagent red blood cells from donors of the same phenotype. When a single serum is to be tested against different red blood cell samples, all samples should be collected and preserved in the same manner, and diluted to the same concentration before use.
4. Completely reproducible results are virtually impossible to achieve. Comparisons are valid only when specimens are tested concurrently. In prenatal testing of sequential serum samples to detect changing antibody activity, samples should be frozen for comparison with subsequent samples. Each new sample should be tested in parallel with the immediately preceding sample. In tests with a single serum against different red blood cell samples, material from the master dilution must be used for all tests.
5. Measurements are more accurate with large volumes than with small volumes, a master dilution technique gives more reliable results than individual dilutions for a single test. The volume needed for all planned tests should be calculated and an adequate quantity of each dilution prepared.

Antibody Titration Studies for Early Detection of Hemolytic Disease of the Newborn

Specimen

Serum for titration (containing potentially significant unexpected antibodies to red blood cell antigens, 1 mL). If possible, test the current sample in parallel with the most recent previously submitted (preceding) sample from the current pregnancy.

Materials

1. Anti-human IgG reagent.
2. Dilute bovine albumin (approximately 6% w/v), optional: 22% (w/v) bovine albumin, 1 mL; isotonic saline, 3 mL.
3. Micropipettes or equivalent: 0.1–0.5 mL delivery, with disposable tips.
4. Red blood cells: Group O reagent red blood cells with double dose expression of antigen to which the serum contains antibody (use R_2R_2 RBCs when titrating anti-D); wash three times and dilute to a 2% red blood cell suspension with isotonic saline.

Quality Control

1. Test the preceding sample in parallel with the current sample.
2. Prepare dilutions using separate pipette for each tube. Failure to do so will result in falsely high titers due to carry-over.
3. Confirm all negative reactions with Coomb's control cells.

Procedure

1. Using 0.5 mL volumes, prepare serial dilutions of serum in saline or 6% albumin. The initial tube should contain undiluted serum and the doubling dilution range should be from 1 in 2 to 1 in 2048 (total of 12 tubes).
2. Place 0.1 mL of each dilution into appropriately labeled 10 or 12 × 75 mm test tubes.
3. Add 0.1 mL of red blood cell suspension to each dilution.
4. Gently agitate the contents of each tube; incubate at 37°C for 1 hour.
5. Wash the tubes four times with saline; completely decant the final wash supernatant.
6. To the cell buttons thus obtained, add Anti-human IgG according to the manufacturer's direction.
7. Centrifuge as for hemagglutination tests.
8. Examine the results macroscopically; grade and record the reactions.
9. Add one drop of Coomb's control cells to all negative tests; recentrifuge and examine the tests macroscopically for mixed field agglutination; repeat antibody detection tests when tests with Coomb's control cells are nonreactive.

Results

The titer is reported as the reciprocal of the highest dilution of serum at which 1 + agglutination is observed. A titer greater than or equal to 16 is considered significant and may warrant monitoring for HDN by cordocentesis, high resolution ultrasound, or examination of the amniotic fluid for bilirubin pigmentation.

Notes

1. Titration studies should be performed upon initial detection of the antibody; save an aliquot of the serum

(frozen at -20°C or colder) for comparative studies with the next submitted sample.
2. When the titer is less than 16 and the antibody specificity has been associated with HDN, it is recommended that repeat titration studies be performed every 2–4 weeks, beginning at 18 weeks of gestation; save an aliquot of the serum (frozen at -20°C or colder) for comparative studies with the next submitted sample.
3. When the decision has been made to monitor the pregnancy by an invasive procedure such as amniocentesis, no further titrations are warranted.
4. Each institution should develop a policy to ensure some degree of uniformity in reporting and interpreting antibody titers.
5. For antibodies to low incidence antigens, consider using paternal red blood cells.
6. Do not use enhancement techniques (albumin, PEG, LISS) or enzyme treated red blood cells, because elevated titers may be obtained.
7. LISS should not be used as diluent in titration studies; non-specific uptake of globulins may occur in serum-LISS dilutions.
8. Failure to obtain the correct results may be caused by incorrect technique, notably; failure to use separate pipette tips for each dilution or failure to mix thawed frozen serum.

Use of Sulfhydryl Reagents to Distinguish between IgM and IgG Antibodies

Specimen
Two mL of serum to be tested.

Reagents
1. Phosphate buffered saline at pH 7.3.
2. About 0.01 M dithiothreitol (DTT) prepared by dissolving 0.154 g of DTT in 100 mL of pH 7.3 PBS store at 2–8°C.

Procedure
1. Dispense 1 mL of serum into each of two test tubes.
2. To one tube, labeled as control, add 1 mL of pH 7.3 PBS.
3. To the other tube, labeled as test, add 1 mL of 0.01 M DTT.
4. Mix and incubate at 37°C for 30–60 minutes.
5. Test the antibody activity in each sample by titration against red blood cells of appropriate phenotype (Table 11.7).

Notes
1. Sulfhydryl reagent used at low concentration may weaken antigens of Kell system. For investigation of antibodies in Kell system, it may be necessary to use alkylation with iodoacetic acid, followed by dialysis.
2. Gelling of serum or plasma sample may be observed during treatment with DTT. This can occur if the DTT has been prepared incorrectly, and has a concentration above 0.01 M. Gelling may also occur if serum and DTT are incubated too long. An aliquot of the sample undergoing treatment can be tested after 30 minutes of incubation, if the activity thought to be due to IgM has disappeared, there is no need to incubate further. Gelled samples cannot be tested for antibody activity because overtreatment with DTT causes denaturation of all serum proteins.

Elution Techniques

Citric Acid Elution Method

Specimen
Packed DAT positive red blood cells washed six times with saline.

Reagents
1. Elution solution: Citric acid (monohydrate), 1.3 g, KH_2PO_4 0.65 g saline to 100 mL, store at 4°C.

TABLE 11.7: Effect of dithiothreitol on blood group antibodies

	Dilution					
Test sample	1/2	1/4	1/8	1/16	1/32	Interpretation
Serum + DTT	3+	2+	2+	1+	0	IgG
Serum + PBS	3+	2+	2+	1+	0	
Serum + DTT	0	0	0	0	0	IgM
Serum + PBS	3+	2+	2+	1+	0	
Serum + DTT	2+	1+	0	0	0	IgG + 1gM*
Serum + PBS	3+	2+	2+	1+	0	

* May also indicate only partial inactivation of IgM

2. Neutralizing solution: Na_3PO_4, 13.0 g; distilled water to 100 mL; store at 4°C.
3. Supernatant saline from final wash of the red blood cells to be tested.

Procedure

1. Chill all reagents to 4°C in ice bath before use.
2. Place 1 mL of packed red blood cells in a 13 × 100 mm test tube.
3. Add 1 mL of eluting solution and note the time.
4. Stopper the tube and mix by inversion for 90 seconds.
5. Remove the stopper and promptly centrifuge the tube at 900–1000 g for 45 seconds.
6. Transfer supernatant fluid to a clean test tube and add 5–6 drops of neutralizing solution; save red blood cells for use in adsorption studies if needed.
7. Check pH; adjust it, if necessary, to pH 7.0 by adding more neutralizing solution.
8. Centrifuge at 900–1000 g for 2–3 minutes to remove precipitate that forms after neutralization. Harvest the supernatant eluate and test it in parallel with supernatant saline from final wash.

Notes

1. Once the red blood cells have been rendered DAT-negative, they may be tested for the presence of blood group antigens, except those of the Kell blood group system. Expression of antigens in the Kell system is markedly weakened after citric acid treatment.
2. Citric acid modified red blood cells may also be treated with protease and used in autologous adsorption studies.

Cold Acid Elution

Specimen

Packed DAT positive red blood cells washed six times with saline.

Reagents

1. Glycine-HCl (0.1 M, pH 3.0), prepared by dissolving 3.75 g of glycine and 2.922 g of sodium chloride in 500 mL of distilled water. Adjust pH to 3.0 with 12N HCl. Store at 4°C.
2. Phosphate buffer (0.8 M, pH 8.2), prepared by dissolving 109.6 g of Na_2HPO_4 and 3.8 g of KH_2PO_4 in approximately 600 mL of distilled water. Adjust pH, if necessary, with either 1N NaOH or 1N HCl. Dilute to a final volume of 1 titer with distilled water. Store at 4°C.
3. Normal saline, at 4°C.
4. Supernatant saline from final wash of red blood cells to be tested.

Procedure

1. Place the red blood cells in 13 × 100 mm test tube and chill them in an ice bath for 5 minutes before adding glycine-HCl.
2. Add 1 mL of chilled saline and 2 mL of chilled glycine-HCl to 1 mL of washed red blood cells.
3. Mix and incubate the tube in an ice bath for 1 minute.
4. Quickly centrifuge the tube at 900–1000 g for 2–3 minutes.
5. Transfer the supernatant eluate into a clean test tube, and add 0.1 mL of pH 8.2 phosphate buffer for each 1 mL of eluate.
6. Mix and centrifuge at 900–1000 g for 2–3 minutes.
7. Transfer the supernatant eluate into a clean test tube, and add test in parallel with the supernatant saline from the final wash.

Notes

1. Keep glycine in ice bath during use, to maintain correct pH.
2. Phosphate buffer will crystallize during storage at 4°C. Redissolve it at 37°C before use.
3. Addition of phosphate buffer restores neutrality to the acidic eluate. Unneutralized acidity may cause hemolysis of the reagent red blood cells used in testing the eluate. The addition of 22% bovine albumin (one part to four parts of eluate) may reduce such hemolysis.

Glycine-HCl/EDTA Elution

Specimen

Packed DAT positive red blood cells washed six times with saline.

Reagents

1. Disodium EDTA (10% w/v): $Na_2EDTA2\ H_2O$,10 g, distilled water 100 mL.
2. Glycine-HCl (0.1 M at pH 1.5): Glycine 3.754 g; NaCl 2.922 g; distilled water 500 mL; adjust to pH 1.5 with 12 N HCl; store at 4°C.
3. TRIS base: Hydroxymethyl aminomethane, 12.1 g; distilled water 100 mL.
4. Supernatant saline from final wash of the red blood cells to be tested.

Procedure

1. Mix 4 mL of glycine-HCl and 1 mL of EDTA in 16 × 100 mm test tube.
2. Immediately add 1 mL of washed red blood cells and mix well.
3. Incubate at room temperature for 1–2 minutes.

4. Centrifuge the tube at 900–1000 g for 2–3 minutes.
5. Transfer the supernatant eluate into a clean test tube and adjust to pH 7.5 with 1 M TRIS base.
6. Mix and centrifuge at 900–1000 g for 2–3 minutes.
7. Transfer the supernatant eluate into a clean test tube, and test it in parallel with the supernatant saline from the final wash.

Notes

1. Once the red blood cells have been rendered DAT-negative, they may be tested for the presence of blood group antigens, except those in the Kell system. Treatment with glycine-HCl/EDTA denatures Kell system antigens.
2. Red blood cells modified with glycine-HCl/EDTA may be treated with protease and used in autologous adsorption studies.

Heat Elution

Specimen

Packed DAT positive red blood cells washed six times with saline.

Reagents

1. Six percent bovine albumin, prepared by diluting 22% or 30% bovine albumin with saline.
2. Supernatant saline from final wash of the red blood cells to be tested.

Procedure

1. Mix equal volumes of washed packed cells and 6% bovine albumin in 13 × 100 mm test tube.
2. Place the tube at 56°C for 10 minutes. Agitate the tube periodically during the incubation period.
3. Centrifuge the tube at 900–1000 g for 2–3 minutes, preferably in a heated centrifuge.
4. Immediately transfer the supernatant eluate into a clean test tube, and test in parallel with supernatant saline from final wash.

Donath-Landsteiner Test

Specimen

Serum separated from freshly collected blood sample maintained at 37°C.

Reagents

1. Freshly collected normal serum, to use as a source of complement.
2. 50% suspension of washed group O red blood cells that express the P antigen.

Procedure

1. Label three sets of three 10 × 75 mm test tubes as follows: A_1-A_2-A_3; B_1-B_2-B_3; C_1-C_2-C_3.
2. To tubes 1 and 2 of each set, add 10 volumes of the patient's serum.
3. To tubes 2 and 3 of each set, add 10 volumes of fresh normal serum.
4. To all tubes, add one volume of 50% suspension of washed P-positive red blood cells and mix well.
5. Place the three 'A' tubes in a bath of melting ice for 30 minutes, and then at 37°C for 1 hour.
6. Place the three 'B' tubes in a bath of melting ice, and keep them in melting ice for 90 minutes.
7. Place the three 'C' tubes at 37°C, and keep them at 37°C for 90 minutes.
8. Centrifuge all tubes, and examine the supernatant fluid for hemolysis.

Interpretation

The Donath-Landsteiner test is considered positive when the patient serum, with or without added complement, causes hemolysis in the tubes that were incubated first in melting ice and then at 37°C (i.e. tubes A_1 and A_2), and there is no hemolysis in any of the tubes maintained throughout at 37°C or in melting ice. The A_3, B_3 and C_3 tubes serve as a control for complement activity and should not manifest hemolysis.

Notes

1. The biphasic nature of the hemolysin associated with PCH requires that serum be incubated with cells at cold temperature and then at 37°C.
2. Active complement is essential for demonstration of the antibody. Because patient with PCH may have low levels of serum complement, fresh normal serum should be included in the reaction medium as a source of complement.
3. To avoid loss of antibody by cold autoadsorption before testing, the patient's blood should be allowed to clot at 37°C, and the serum separated from the clot at this temperature.

Chequerboard Titration for Quality Control of Anti-IgG Potency in Polyspecific AHG Reagent and Evaluation of Complement Potency with Complement-coated Cells

Reagents and Materials Required for Chequerboard Titration

1. Anti-D IgG reagent with albumin titer 256–512.
2. Polyspecific AHG reagent
3. Freshly collected O RhoD positive cells
4. Normal saline
5. 12 ×100 mm and 12 × 75 mm test tubes
6. Pipettes 1 mL and 5 mL

7. Table centrifuge
8. Timer
9. Water bath or laboratory incubator.

Reagent Preparation Procedure

Preparation of 3% cell suspension

1. Collect 2 mL of freshly drawn venous blood in a clean 12 × 100 mm test tube with suitable anticoagulant.
2. Centrifuge at 3000 rpm for 2–3 minutes to form a cell button.
3. Discard the supernatant.
4. Resuspend the cell button in 5 mL of normal saline.
5. Centrifuge at 3000 rpm for 2–3 minutes.
6. Repeat the washing of cells (steps 4 and 5) twice more so that the cells are washed three times.
7. After the final centrifugation, remove the supernatant without disturbing the cell button.
8. Take 0.75 mL of packed cells and resuspend them in 24.25 mL of normal saline to get a 3% cell suspension.

Dilutions of Anti-D (IgG) Reagent

1. Take a set of ten, 12 × 100 mm test tubes and number them from 1 to 10.
2. Add 2 mL of normal saline to each of the tubes from tube number 2 to 10.
3. Add 2 mL each of anti-D (IgG) reagent to tube number 1 and 2.
4. Mix the content of tube number 2 and transfer 2 mL of diluted reagent to tube number 3 with the help of pipette.
5. Continue this serial dilution till tube number 10.
6. Discard 2 mL of diluted reagent from tube number 10.

Cell Sensitization

1. To each of the above dilutions of anti-D (IgG), add 2 mL of well mixed freshly prepared 3% cell suspension.
2. Mix well all the tubes and cover them with aluminum foil.
3. Incubate the tubes at 37°C for 30 minutes, with periodic mixing.
4. Centrifuge the tubes at 3000 rpm for 2–3 minutes.
5. Remove the supernatant and resuspend the cell button in 5 mL of normal saline.
6. Centrifuge at 3000 rpm for 2–3 minutes.
7. Repeat the washing (Step 4 and 5) at least four times.
8. Resuspend the cell button from each tube in 2 mL of normal saline to get a 3% suspension of sensitized cells.

Note

Thorough washing of sensitized cells (after incubation) is very important as even slight traces of free anti-D IgG can lead to false negative results.

Dilutions of Anti-human Globulin Reagent

1. Take a set of six, 12 × 100 mm test tubes and number them from 1 to 6.
2. Add 2.5 mL of normal saline to each of the tubes from tube number 2 to 6.
3. Add 2.5 mL of polyspecific AHG reagent to tube number 1 and 2.
4. Mix the content of tube number 2 and transfer 2.5 mL of the diluted reagent to tube number 3.
5. Continue this serial dilution till tube number 6.
6. Discard 2.5 mL of diluted reagent from tube number 6.

Preparation of Complement-Coated Cells

Reagents and material required:

1. LISS solution
2. Buffered saline
3. O group red blood cells 50% suspension
4. Inert O group serum
5. Test tubes 12 × 100 mm
6. Table centrifuge
7. Water bath or laboratory incubator.

Preparation of 50% Cell Suspension of O Group Red Blood Cells

1. Collect 1 mL of freshly drawn venous blood in a clean 12 × 100 mm test tube containing suitable anticoagulant.
2. Centrifuge the tube at 3000 rpm for 2–3 minutes to form cell button.
3. Discard the supernatant.
4. Resuspend the cell button in 5 mL of buffered saline.
5. Centrifuge at 3000 rpm for 2–3 minutes.
6. Repeat the washing of cells (steps 4 and 5) twice more so that the cells are washed three times.
7. After the final centrifugation, remove the supernatant without disturbing the cell button.
8. Add 1 mL of buffered saline to the packed red blood cells to get a 50% O group red cell suspension.

Collection of Inert O Group Serum

1. Collect 2 mL of freshly drawn venous blood in a clean 12 × 100 mm test tube.
2. Immediately centrifuge at 3000 rpm for 2–3 minutes.
3. Collect 0.5 mL of serum in a clean test tube.

Sensitization of O Group Red Blood Cells

1. Place 8.5 mL of LISS into a 20–25 mL container.
2. Add 0.5 mL of fresh inert O group serum to it.
3. Mix well and add 1 mL of 50% O group red cell suspension.
4. Mix thoroughly and incubate at 37°C for 30 minutes with occasional further mixing.

5. Centrifuge at 3,000 rpm for 2–3 minutes to form a cell button.
6. Discard the supernatant and resuspend the cell button in 20 mL buffered saline.
7. Centrifuge at 3,000 rpm for 2–3 minutes.
8. Repeat the washing of cells (steps 6 and 7) three more times so that cells are washed four times.
9. After the final centrifugation, remove the supernatant without disturbing the cell button.
10. Add 14.5 mL of buffered saline to packed red blood cells to obtain 2–3% suspension of complement-coated cells.

Chequerboard Titration (Table 11.8)

1. Take a set of sixty, 12 × 75 mm test tubes, number and arrange them as shown below in the Table 11.8:
2. Add 0.2 mL each of N (neat) AHG in the respective tubes.
3. Similarly add dilutions of AHG in their respective tubes (horizontal rows).
4. Referring to Table 11.9, add 0.2 mL each of 2% suspension of sensitized cells with anti-D (IgG) dilutions in their respective tubes (vertical rows).
5. Mix well all the tubes.
6. Centrifuge the tubes at 3000 rpm for 20 seconds.
7. Gently dislodge the cell button and observe for agglutination.
8. Chart the results as given in Table 11.8.
   i. Add 0.2 mL neat AHG reagent.
   ii. Similarly add dilutions of AHG in respective tubes.
   iii. Referring to Table 11.9, add 0.2 mL each of 2% suspension of complement-coated cells to all the tubes containing AHG reagent with dilutions.
   iv Mix well all the tubes.
   v. Centrifuge the tubes at 3000 rpm for 20 seconds.
   vi. Gently dislodge the cell button and observe for agglutination.

Preparation of Coomb's Control Cells

Reagents and materials required:

1. Anti-D (IgG)
2. Freshly collected O Rho(D) positive cells
3. Normal saline
4. Test tubes 12 × 100 mm
5. Pipettes 1 mL and 5 mL
6. Table centrifuge, timer
7. Water bath or laboratory incubator.

Procedure

Since commercially available anti-D (IgG) reagent have albumin titer of 256–512, diluting anti-D (IgG) reagent 1:40 to 1:50 in normal saline is enough to achieve sensitization with O Rho(D) positive cells:

1. Take equal volume of 1:40 to 1:50 diluted anti-D (IgG) in a 12 × 75 mm test tube and 3% cell suspension of O Rho(D) positive cells.
2. Mix well and incubate at 37°C for 30 minutes. Periodic mixing during 30 minutes interval ensures thorough sensitization.
3. Remove the supernatant and resuspend the cell button in 5 mL of normal saline.
4. Centrifuge at 3000 rpm for 2–3 minutes.
5. Repeat the washing (steps 3 and 4) at least four times.
6. Resuspend the cell button in normal saline to obtain a 3% suspension of Coomb's control cells.

ANTI-HUMAN IgG MONOSPECIFIC COOMB'S REAGENT FOR DIRECT AND INDIRECT ANTIGLOBULIN TEST

(*Courtesy:* Tulip's Erybank Range)

Summary

Generally, antibodies involved in transfusion reactions are of two types namely, the complete and incomplete, whereas the complete antibodies agglutinate red cells

TABLE 11.8: Chequerboard titration

Dilution of AHG					3% Rho (D) positive senstitized cells				
N	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
N									
1:2									
1:4									
1:8									
1:16									
1:32									

TABLE 11.9: Complement potency titration

Dilution of AHG	2% cell suspension of complement coated Cells
N	
1:2	
1:4	
1:8	
1:16	
1:32	

in saline medium, the incomplete type of antibodies sensitizes red cells without agglutination.

In the direct antiglobulin tests, Anti-human globulin reagent is used to detect antibodies adsorbed to the red blood cells in vivo. After direct antiglobulin testing with a polyspecific reagent reveals globulins, anti-human IgG monospecific Coombs reagent is used to characterize the coating proteins.

In the indirect antiglobulin tests, anti-human globulin reagent is used to detect antibodies adsorbed to red blood cells in vitro. Anti-human IgG monospecific Coombs reagent is used in indirect antiglobulin testing to distinguish patterns of reactivity in a single serum containing complement-binding and non complement-binding antibodies.

Anti-human IgG monospecific Coombs reagent is useful for antibody detection, antibody identification and umbilical cord red blood testing.

Reagent

Erybank® anti-human IgG monospecific Coomb's reagent is a ready-to-use reagent containing antibodies reactive with human gamma globulins. Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and titer.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Normal human red blood cells, in presence of antibody directed towards the antigen they possess, may fail to agglutinate and become sensitized. This may be due to the particular nature of antigen and antibody involved. Erybank® Anti-human IgG monospecific Coomb's reagent would react with red cells sensitized with gamma globulins and cause agglutination of red blood cells.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.
4. Erybank® anti-human IgG monospecific Coomb's reagent is not from human source, hence, contamination due to HBsAg and HIV is practically excluded.

Sample Collection and Storage

No special preparation of patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples.

For Direct Antiglobulin Test

Blood drawn into EDTA is preferred but oxalated, citrated or clotted whole blood may be used. The blood sample

Reactions of Common Erythrocyte Antibodies

Antibody	*Saline*	*Albumin*	*AHG*	*Enzyme*	*In Vitro*	*Optimal C^0*		
For	*Medium*	*Medium*	*Test*	*Test*	*Hemolysis*	*4*	*24*	*37*
H, I	M	S	F	S	S	M	S	F
A, B, A, B	M	F	F	M	S	M	S	F
Lu^a	M	S	F	F	N	M	S	M
Lu^b	S	S	M	F	N	R	S	M
M, N	M	S	S	F	N	M	M	F
P_1	M	S	S	S	F	M	S	F
PP_1P^k	M	M	M	M	M	S	M	M
Le^a, Le^b	M	S	S	M	S	M	S	F
S, s	S	S	M	S	N	F	S	M
K, k, Js^a Js^b	F	S	M	F	N	F	S	M
C, D, E, c, e,	S	S	M	M	N	F	S	M
Fy^a, Fy^b	F	F	M	F	N	N	F	M
Jk^a, Jk^b	F	S	M	M	F	N	S	M

*M = Most (> 20%), S = Some (5–20%), F = Few (1–5%), R = Rare (< 1%), N = Not reported.

should be tested as soon as possible after collection and should not be stored.

For Indirect Antiglobulin Test

Serum not more than 48 hours old should be used for testing purpose.

Additional Material Required

For Direct Antiglobulin Test

Test tubes (12 × 75 mm), Pasteur pipettes, centrifuge, isotonic saline, Coomb's control cells, optical aid.

For Indirect Antiglobulin Test

Test tubes (12 × 75 mm), Pasteur pipettes, centrifuge, incubator (37°C), isotonic saline, Erybank® bovine serum albumin, reagent red blood cells for antibody detection and antibody identification, Coomb's control cells, optical aid.

Procedure

Bring reagent to room temperature before testing.

Direct Antiglobulin Test

1. Prepare a 5% suspension of red blood cells to be tested in isotonic saline.
2. Pipette one drop of the cell suspension into a test tube.
3. Fill the tube with fresh isotonic saline and centrifuge for 30 seconds at 3400 rpm (1000 g).
4. Decant and repeat this washing at least thrice.
5. Add two drops of Erybank® anti-human IgG monospecific Coomb's reagent and mix well.
6. Centrifuge for one minute at 1000 rpm (125 g) or for 20 seconds at 3400 rpm (1000 g).
7. Very gently, resuspend the cell button observing for agglutination macroscopically.
8. To all negative results, add one drop of Coomb's control cells and observe for agglutination.

Indirect Antiglobulin Test for Antibody Identification

1. Prepare 5% suspension of reagent red blood cells to be tested in isotonic saline.
2. Pipette two drops of serum to be tested in an appropriately labeled test tube.
3. Pipette one drop of 5% reagent red blood cell suspension and mix well.
4. If required, add two drops of Erybank® bovine serum albumin reagent and mix well and incubate at 37°C for 15 minutes.
5. If enhancement medium is not being used, incubate the tube at 37°C for 30 minutes.
6. After incubation, wash the cells thoroughly with isotonic saline for minimum three times. Decant completely after last wash.
7. Add two drops of Erybank® anti-human IgG monospecific Coomb's reagent into the test tube containing the sedimented cells and mix well.
8. Centrifuge for one minute at 1000 rpm (125 g) or 20 seconds at 3400 rpm (1000 g).
9. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Interpretation of Results

Direct Antiglobulin Test

Agglutination of red blood cells is a positive result and indicates presence of human IgG on the red blood cells.

No agglutination is a negative test result and indicates absence of human IgG on red blood cells.

Indirect Antiglobulin Test

Agglutination of red blood cells is a positive result and indicates presence of antibody against the antigen in the serum under test.

No agglutination of red blood cells is a negative result and indicates absence of antibody against the antigen in the serum under test.

Remarks

1. To all negative test results, after the antiglobulin test, one drop of Coomb's control cells should be added. If Coomb's control cells do not agglutinate then the test must be repeated.
2. Red blood cells showing a positive direct antiglobulin test cannot be used for the indirect antiglobulin test.
3. It is recommended that anti-IgG activity of anti-human IgG monospecific Coomb's reagent be tested from time to time preferably on a daily basis using Coomb's control cells as a positive control.
4. All glassware used in the test should be scrupulously clean, dry and free from contamination with human serum.
5. Contaminated bovine serum albumin, saline or glassware may inactivate anti-human IgG monospecific Coomb's reagent.
6. Use of various drugs and certain diseases (such as megaloblastic anemia) are known to be associated with a positive direct antiglobulin test.
7. Cord cells obtained from a newborn exhibiting hemolytic disease of the newborn, especially due to ABO incompatibility may give false negative results.
8. Erybank® anti-human IgG monospecific Coomb's reagent is free from anti-T activity.
9. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrate its own equipment and determine the time required for achieving the desired results.

ANTIHUMAN GLOBULIN REAGENT FOR DIRECT AND INDIRECT ANTIGLOBULIN TESTS

(Eryclone® from Tulip)

Summary

Generally antibodies involved in transfusion reactions are of two types, namely, the complete and the incomplete, whereas the complete antibodies agglutinate red cells in saline medium, the incomplete type of antibody sensitizes red cells without agglutination. Usually, IgM class of antibodies and IgG_1 and IgG_3 type of IgG antibodies fix complement. Cell lysis, in vivo is mediated through the complement system and the complement component C_3b is further acted upon to produce C_3d.

In the direct antiglobulin tests, anti-human globulin reagent is used to detect antibodies adsorbed to the red blood cells in vivo. In the indirect antiglobulin tests, Anti-human globulin reagent is used to detect antibodies adsorbed to the red blood cells in vitro. Anti-human globulin reagent is useful for compatibility testing, antibody detection, antibody identification, umbilical cord red blood testing and detection of the D^u variant of the human red blood cell antigen D(Rho).

Reagents

Eryclone® anti-human globulin is a balanced ready-to-use blend of highly purified immunoglobulins. It contains Anti-human IgG antibodies and antibodies reactive with human complement components C_3b and C_3d. These anti-complement antibodies are IgM class monoclonals and they impart the necessary sensitivity to the reagent. Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, avidity and titer.

Reagent Storage and Stability

a. Store the reagent at 2–8°C. Do not freeze.
b. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Normal human red blood cells, in presence of antibody directed towards the antigen they possess, may fail to agglutinate and become sensitized. This may be due to the particular nature of the antigen and antibody involved. Eryclone® anti-human globulin reagent would react with red cells sensitized with gammaglobulins or components of human complement involved and cause agglutination of the red blood cells.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. Extreme turbidity may indicate microbial contamination or denaturation of protein due to thermal damage. Such reagent should be discarded.
4. Eryclone® reagent are not from human sources, hence, contamination due to HBsAg and HIV is practically excluded.

Sample Collection and Storage

No special preparation of the patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples.

For Direct Antiglobulin Test

Blood drawn into EDTA is preferred but oxalated, titrated or dotted whole blood may be used. The blood sample should be tested as soon as possible after collection and should not be stored.

For Indirect Antiglobulin Test

Serum, not more than 48 hours old, should be used. Donor units may be tested up to the end of their dating.

Preparation of Coomb's Control Cells

1. Dilute Eryclone® anti-D (IgG)/Erybank® anti-D (polyclonal) reagent 1:50 in isotonic saline.
2. Prepare a 5% suspension of group 'O' RhoD positive cells in isotonic saline.
3. Mix equal volumes of diluted anti-D reagent (as in 1 above) and 5% suspension of 'O' RhoD positive cells (as in 2 above) and incubate at 37°C for 15 minutes.
4. Decant and wash thoroughly with isotonic saline at least thrice.
5. Resuspend in isotonic saline to make a 5% suspension of coombs control cells.

Additional Material Required

For direct antiglobulin test: Test tubes (10 × 75 mm), Pasteur pipettes, centrifuge, isotonic saline, coomb's control cells, optical aid.

For indirect antiglobulin test and compatibility test: Test tubes (10 × 75 mm), Pasteur pipettes, Erybank® bovine serum albumin, centrifuge, incubator (37°C), isotonic saline, coomb's control cells, optical aid.

Procedure

Bring reagent to room temperature before testing.

Direct Antiglobulin Test

1. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
2. Pipette one drop of the cell suspension into a test tube.
3. Fill the tube with fresh isotonic saline and centrifuge for 30 seconds at 3400 rpm (1000 g).
4. Decant and repeat this washing at least thrice.
5. Add two drops of Eryclone® anti human globulin reagent and mix well.
6. Centrifuge for one minute at 1000 rpm (125 g) or for 20 seconds at 3400 rpm (1000 g).
7. Very gently, resuspend the cell button observing for agglutination macroscopically.
8. To all negative antiglobulin tests add one drop of Coomb's control cells and observe for agglutination.

Indirect Antiglobulin Test

Major cross-match procedure.

Initial Phase

1. Label two test tubes as A (for albumin) and B (for saline), depending upon the number of donors to be cross-matched, as many pairs of such labeled tubes would be required.
2. Prepare a 5% suspension of the red cells to be tested in isotonic saline.
3. Pipette two drops of recipient serum in both the labeled test tubes.
4. Pipette one drop of donor red cells in both the labeled test tubes and mix well.
5. Only to the albumin tube (A), add two drops of Erybank® bovine serum albumin reagent and mix well.
6. Centrifuge both the tubes for one minute at 1000 rpm (125 g) or for 20 seconds at 3400 rpm (1000 g).
7. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.
8. Proceed to incubation phase.

Incubation Phase

1. Incubate the saline tube at room temperature and the albumin tube at 37°C for 15 minutes.
2. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.
3. Proceed to the antiglobulin phase.

Antiglobulin Phase

1. Only the albumin tubes (A) are tested in the anti-globulin phase.
2. Wash the mixture of red blood cells and serum thoroughly with isotonic saline for minimum of three times. Decant completely after the last wash.
3. Place two drops of Eryclone® anti-human globulin reagent into the test tubes containing the sedimented cells and mix well.
4. Centrifuge for one minute at 1000 rpm (125 g) or for 20 seconds at 3400 rpm (1000 g).
5. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Interpretation of Results

Direct Antiglobulin Phase

Agglutination of the red blood cells is a positive test result and indicates the presence of human IgG or components of complement on the red blood cells.

No agglutination is a negative test result and indicates the absence of human IgG or components of complement on the red blood cells.

Indirect Antiglobulin Phase

In all phases of the compatibility test, if no agglutination or hemolysis is observed, then the patient and the donor may be considered compatible. If hemolysis or agglutination at any point till the completion of the antiglobulin phase is observed, the patient and the donor are considered incompatible.

Remarks

1. If plasma is used in the indirect antiglobulin test, the complement-dependent antibodies may not be detected due to the absence of calcium.
2. To all negative test results, after the antiglobulin test phase, one drop of Coomb's control cells should be added. If Coomb's control cells do not agglutinate then the compatibility test must be repeated.
3. In the indirect antiglobulin test procedure an auto control tube (individual's cells in his own serum) should be run.
4. Red blood cells showing a positive direct antiglobulin test cannot be used for the indirect antiglobulin test.
5. It is recommended that anti-IgG activity of the anti-human globulin reagent be tested from time to time preferably on a daily basis using Coomb's control cells as a positive control.
6. All glassware used in the test should be scrupulously clean dry and free from contamination with human serum.
7. Contaminated bovine serum albumin, saline or glassware may inactivate anti-human globulin reagent.
8. Use of various drugs and certain diseases (such as megaloblastic anemia) are known to be associated with a positive direct antiglobulin test.

9. Cord cells obtained from a newborn exhibiting hemolytic disease of the newborn, especially due to ABO incompatibility may give false negative results.
10. Eryclone® Anti-human globulin reagent does not contain anti-C_4 and is free from anti-T activity.
11. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrate its own equipment and determine the time required for achieving the desired results.

PREPARING COOMB'S CONTROL CELLS Agtrol®

(*Courtesy:* Tulip, Starter Pack)

Summary

Anti-human globulin reagent is used in blood group serology for performing compatibility testing, antibody screening, antibody detection and detection of D^u red cell type. Usage of Coomb's control cells is advocated for functional validation of anti-human globulin reagent and procedural validation of tests employing anti-human globulin reagent.

Reagent

Agtrol starter pack for preparing Coomb's control cells contains.

1. Ready-to-use, standardized-prediluted anti-D (IgG) monoclonal antibody reagent.
2. Red blood cell preserving solution for serological applications.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its performance characteristics.

Reagent Storage and Stability

Store the reagent at 2–8°C. Do not freeze.

The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Human 'O' Rho(D) positive cells in presence of Agtrol prediluted monoclonal reagent do not agglutinate but are sensitized with IgG antibodies. After processing, these sensitized red blood cells are resuspended in red blood cell preserving solution for long-term storage and use. When anti-human globulin reagent is added to these sensitized cells the incomplete anti-D (IgG) antibodies are agglutinated by the anti-human IgG component. The agglutination reaction validates the serological activity of the anti-human globulin reagent and confirms that the anti-human globulin reagent was added in the test procedure.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Agtrol anti-D reagent is not from human source, hence, contamination due to HBsAg and HIV is practically excluded.
3. Agtrol anti-D reagent and red cell preserving solution contains 0.1% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. Extreme turbidity in both Agtrol anti-D IgG and red blood cell preserving solution reagent may indicate microbial contamination. Such reagent must be discarded.

Sample Collection and Storage

No special preparation is required prior to sample collection by approved techniques. Samples should be stored at 2–8°C if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

- EDTA or heparin: 2 days
- Sodium citrate or sodium oxalate: 14 days
- ACD or CPD: 28 days
- Clotted whole blood should be used within 14 days.

Additional Collection and Storage

Test tubes (12 × 75 mm), Pasteur pipettes, isotonic saline/isotonic buffered saline (available from Tulip:Osmosol), anti-human globulin reagent (Available from Tulip: Eryclone® anti-human globulin reagent), Rho(D) positive red blood cells, incubator at 37°C, laboratory centrifuge, optical aid.

Procedure

Bring all the reagents to room temperature (25–30°C) before testing.

Preparation and Validation of Coomb's Control Cells

Preparation of 5% Coomb's control cell suspension:

1. Collect fresh O Rho(D) positive red blood cells preferably with citrate as an anticoagulant.
2. Wash 1 mL of freshly collected O Rho(D) positive red blood cells with isotonic saline at least three times.
3. After the third wash, thoroughly decant the supernatant. To the cell button, add 5 mL of Agtrol anti-D (IgG) reagent and gently resuspend the red blood cells.
4. Incubate the mixture at 37°C for 15 minutes.
5. After incubation, wash the sensitized red blood cells thoroughly at least 4 to 5 times with isotonic saline.

6. Decant the supernatant thoroughly after the last wash. Resuspend the cell button gently with about 1–2 mL of Agtrol red blood cell preserving solution. The complete resuspended red cells should be added back to the balance Agtrol red blood cell preserving solution in the dropper vial. A stabilized suspension of 5% Coomb's control cells is thus, obtained. Label appropriately with the date of preparation.
7. Store the Coomb's control cells at 2–8°C. Use within 4 weeks of preparation.

Validation of Prepared 5% Coomb's Control Cell Suspension

1. Add one drop of Coomb's control cells into a test tube.
2. Add two drops of anti-human globulin reagent and mix well.
3. Centrifuge for 1 minute at 1000 rpm or 20 seconds at 3400 rpm.
4. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Use of Coomb's Control cells

Validation of Anti-human Globulin Reagent

1. Add one drop of Coomb's control cells into a test tube.
2. Add two drops of anti-human globulin reagent and mix well.
3. Centrifuge for 1 minute at 1000 rpm or 20 seconds at 3400 rpm.
4. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Confirmation of Negative Antiglobulin Test Reactions

1. Add one drop of Coomb's control cells to the samples negative during direct or indirect antiglobulin test.
2. Centrifuge for 1 minute at 1000 rpm or 20 seconds at 3400 rpm.
3. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Interpretation and Results

Agglutination reaction indicates that the anti-human globulin reagent is functional and the test is valid. No agglutination indicates that the anti-human globulin reagent does not have sufficient activity and the test is invalid.

Remarks

1. As undercentrifugation or overcentrifugation could lead to erroneous results, it is recommended that each laboratory calibrates its own equipment and determine the time required for achieving the desired results.
2. Erroneous results may also occur due to improper red blood cell concentration, improper temperature while performing the test.
3. Store the Coomb's control cells at 2–8°C with cap tightly closed.
4. Do not contaminate the prepared Coomb's control cell suspension as it may subsequently effect the stability.
5. Glassware used to retrieve the Agtrol reagent and Coomb's control cell suspension should be scrupulously clean and sterile.

LOW IONIC SALT SOLUTION FOR SEROLOGICAL APPLICATIONS

(Tuliss from Tulip)

Summary

The antigen-antibody interaction in blood group serology is dependent on antigen density, concentration of antibody, pH, ionic concentration of reaction medium and temperature. Reducing the ionic concentration of the reaction medium especially enhances the uptake of weak antibodies by the red blood cell antigens. Usage of low ionic salt solution is helpful in detection of weak antibodies during cross-match techniques, antibody screening and antibody identification.

Reagent

Tuliss is a buffered low ionic salt solution of appropriate sodium chloride molarity useful in serological applications such as antibody detection and cross-match techniques.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

In blood group serology, the ionic concentration of reaction medium is largely dependent on the concentration of sodium and chloride ion contributed by isotonic saline. When optimum concentration of antibody is present, antigen-antibody interaction occurs even though the sodium and chloride ions are present in sufficient quantity. But when weak antibodies are present, sodium and chloride ions may interfere with binding of antibody to the antigens present on the red blood cell membrane. By lowering the ionic concentration of salt, the ionic strength is reduced which increases the rate of antibody uptake by red blood cells.

Note

1. In vitro diagnostic reagent for laboratory and professional use only.
2. The reagent contains 0.1% sodium azide as a preservative. Avoid contamination with skin and mucosa. On disposal flush with large quantity of water.
3. Do not freeze or expose the reagent to elevated temperatures. After usage immediately replace the reagent vial back to 2–8°C.
4. Marked turbidity may indicate reagent deterioration or contamination, such reagent should not be used. Do not use the reagent beyond expiry date.

Sample Collection and Storage

No special preparation is required prior to sample collection by approved techniques. Samples should be stored at 2–8°C if not tested immediately. Do not use hemolyzed samples. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period:

EDTA or heparin	:	2 days
Sodium citrate or sodium oxalate	:	14 days
ACD or CPD	:	28 days

Clotted whole blood should be used within 14 days.

Additional Material Required

Test tubes (12 × 75 mm), Pasteur pipettes, laboratory centrifuge, incubator (37°C), isotonic saline/isotonic buffered saline (Available from Tulip:Osmosol), donor red blood cells and recipient serum for cross-match, reagent red blood cells for antibody detection, anti-human globulin reagent for cross-match and antibody detection (Available from Tulip: Eryclone® anti-human globulin reagent), optical aid.

Procedure

Bring all the reagent to room temperature (25–30°C) before testing.

Indirect Antiglobulin Test for Cross Match

Initial Phase

1. Wash donor red blood cells three times in isotonic saline. Decant the supernatant completely after last wash.
2. Finally wash the donor blood red cells with Tuliss. A final wash with Tuliss is recommended to reduce the effect of residual isotonic saline on the final ionic concentration of the test medium.
3. Prepare a 2–3% donor red blood cells suspension in Tuliss.
4. To an appropriately labeled test tube, add two drops of recipient serum.
5. Add two drops of Tuliss suspended donor red blood cells.
6. Centrifuge the tube at 1000 rpm for 30 seconds.
7. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.

Incubation Phase

1. Incubate the tube containing the mixture of donor red blood cells and recipient serum at 37°C for 10 minutes.
2. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.
3. Proceed to the antiglobulin phase.

Antiglobulin Phase

1. Wash the mixture of donor red blood cells and recipient serum thoroughly with isotonic saline minimum for three times. Decant completely after the last wash.
2. Place two drops of anti-human globulin reagent into the test tube and mix well.
3. Centrifuge at 1000 rpm for 30 seconds.
4. Very gently, resuspend the cell button and observe for agglutination macroscopically.

For Antibody Detection

Initial Phase

1. Wash red blood cells three times in isotonic saline. Decant the supernatant completely after last wash.
2. Finally, wash the reagent red blood cells with Tuliss. A final wash with Tuliss is recommended to reduce the effect of residual isotonic saline on the final ionic concentration of the test medium.
3. Prepare a 2–3% reagent red blood cell suspension in Tuliss.
4. To an appropriately labeled test tube, add two drops of serum to be tested.
5. Add two drops of Tuliss suspended reagent red blood cells.
6. Centrifuge the test tube at 1000 rpm for 30 seconds.
7. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.

Incubation Phase

1. Incubate the tube containing the mixture of donor red blood cells and recipient serum at 37°C for 10 minutes
2. First observe for hemolysis. Resuspend the cell button and observe for agglutination macroscopically.
3. Proceed to the antiglobulin phase.

Antiglobulin Phase

1. Wash the mixture of reagent red blood cells and serum thoroughly with isotonic saline minimum for three times. Decant completely after the last wash.
2. Place two drops of anti-human globulin reagent into the test tube and mix well.
3. Centrifuge at 1000 rpm for 30 seconds.
4. Very gently, resuspend the cell button and observe for agglutination macroscopically.

Interpretation of Results

Cross-match

In all phases of the compatibility test, if no agglutination or hemolysis is observed, then the patient and donor may be considered to be compatible. If hemolysis or agglutination at any point till the completion of the antiglobulin phase is observed, the patient and donor are considered to be incompatible.

Antibody Detection

Agglutination or hemolysis indicates that the antibody has reacted with the corresponding red blood cell antigen. No agglutination or hemolysis indicates the absence of corresponding antibody.

Remarks

1. As undercentrifugation or over-centrifugation could lead to erroneous results, it is recommended that each laboratory calibrate its own equipment and determine the time required for achieving the desired results.
2. Erroneous results may also occur due to improper red blood cell concentration, improper incubation time or temperature while performing the test.
3. The ionic strength of the test system is dependent on the amount of serum used. Alteration of the ionic strength of Tuliss procedure by addition of excess human serum will increase the ionic strength and decrease the sensitivity of the test system.
4. The performance of Tuliss reagent should be periodically evaluated with a known LISS enhanced antibody and the corresponding antigen for positive result and red cell lacking the corresponding antigen for negative result.
5. To all negative test results after the antiglobulin test phase, one drop of Coomb's control cells should be added. If Coomb's control cells do not agglutinate, then the test must be repeated.
6. Low ionic strength media have been used to enhance many antigen-antibody reactions. However, not all antibodies are reactive in a LISS test system. Some weakly reactive IgM antibodies of ABO system may not be detected in the system employing low ionic strength media.

STABILIZED, ACTIVATED PAPAIN ENZYME SOLUTION FOR SEROLOGICAL APPLICATIONS

(Liquipap from Tulip)

Summary

Enzyme treatment enhances the reactivity of red blood cells with certain antibodies of Rh, Kidd, Lewis and P systems. Certain clinically significant antibodies of Rh and Kidd systems can be detected only with enzyme treated cells. Traditionally, papain needs to be prepared fresh for use and long-term storage at 20°C is recommended. This leads to frequent reagent preparation, lot to lot variation of and strict quality control to assess adequate and correct performance. Stabilized papain solution overcomes this limitation. Thus, an activated, stabilized papain enzyme solution is useful in detecting clinically significant antibodies for specific serological studies. Proteolytic activity of papain destroys blood group antigens notably M, N, S, Fy^a and Fy^b, a property which may be useful for the identification and separation of mixed antibodies.

Reagent

Liquipap is a stabilized ready-to-use papain reagent useful for serological applications such as antibody screening, antibody detection and cross-match techniques.

Reagent Storage

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

The sialic acid molecules present in the red cell membrane impart a net negative charge to the surface of the red cell. Due to the negative charge a repulsive force exists between two red blood cells, which is termed as the 'zeta potential'. Proteolytic enzymes, such as papain, reduce the red blood cell surface charge by cleaving the sialic acid molecules from the polysaccharide chains on the red blood cell membrane. Also, the enzyme treatment causes spicule formation on the red cell thereby exposing the red blood cell antigens on the surface. This dual action of reduction in the 'zeta potential' and exposure of the red blood cell antigens on the surface enhances the agglutination reaction.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains 0.1% sodium azide as a preservative. Avoid contamination with skin and mucosa. On disposal flush with large quantities of water.
3. Do not freeze or expose the reagent to elevated temperatures. After usage, immediately replace the reagent vial back to 2–8°C.
4. Marked turbidity may indicate reagent deterioration or contamination, such reagent should not be used. Do not use the reagent beyond expiry date.

Sample Collection and Preparation

No special preparation is required prior to sample collection by approved techniques. Serum samples may be stored at 2–8°C up to 3 hours if not tested immediately. Do not use hemolyzed samples.

Red blood cells used for detecting antibodies should preferably be fresh.

Additional Material Required

- Test tubes (10 × 75 mm), Pasteur pipettes, laboratory centrifuge, incubator (37°C), isotonic saline/isotonic buffered saline (Available from Tulip: Osmosol), donor red blood cells and recipient serum for cross-match, reagent red blood cells for antibody detection, optical aid.

Procedure

Bring all the reagent to room temperature (25–30°C) before testing.

One-stage Test

A. For cross-match

1. Wash the donor red blood cells to be tested at least three times in isotonic saline.
2. Prepare 2 to 3% red blood cell (donor) suspension in isotonic saline.
3. To an appropriately labeled test tube, add two drops of recipient serum to be tested and two drops of donor red blood cell suspension. Mix the contents thoroughly but gently.
4. Immediately add two drops of Liquipap reagent.
5. Incubate at 37°C for 15 to 30 minutes.
6. Centrifuge at 1000 rpm for 2 minutes,
7. Gently resuspend and observe for agglutination and/or hemolysis macroscopically and microscopically.

B. For antibody detection

1. Wash the reagent red blood cells to be tested at least three times in isotonic saline.
2. Prepare 2 to 3% reagent red blood cell suspension to be tested in isotonic saline.
3. To an appropriately labeled test tube, add two drops of serum to be tested and two drops of reagent red blood cell suspension under test.
4. Mix the contents and immediately add two drops of Liquipap reagent .
5. Incubate at 37°C for 15 to 30 minutes.
6. Centrifuge at 1000 rpm for 2 minutes.
7. Gently resuspend and observe for agglutination and/or hemolysis macroscopically and microscopically.

Alternatively, a two-stage test using Liquipap reagent can also be performed as follows:

Two Stage Test

A. For cross-match:

1. Wash the donor red blood cells three times in isotonic saline.
2. To an appropriately labeled test tube, add one drop of washed packed cells (donor) and one drop of Liquipap reagent.
3. Incubate the test tube at 37°C for 15 to 30 minutes.
4. Wash the Liquipap-treated donor red blood cells at least three times with isotonic saline.
5. Prepare Liquipap treated 2 to 3% red blood cell suspension of donor in isotonic saline.
6. To an appropriately labeled test tube, add one drop of Liquipap treated 2 to 3% donor red blood cell suspension.
7. Add two drops of recipient serum to be tested.
8. Mix well and incubate at 37°C for 30 minutes.
9. Centrifuge at 1000 rpm for 2 minutes.
10. Gently resuspend and observe for agglutination and/or hemolysis macroscopically and microscopically.

B. For antibody detection:

1. Wash the reagent red blood cells to be tested three times in isotonic saline.
2. To an appropriately labeled test tube, add one drop of washed packed reagent red blood cells under test and one drop of Liquipap reagent.
3. Incubate the test tube at 37°C for 15 to 30 minutes.
4. Wash the Liquipap treated reagent red blood cells under test at least three times with isotonic saline.
5. Prepare Liquipap treated 2 to 3% reagent red blood cell suspension under test in isotonic saline.
6. To an appropriately labeled test tube, add one drop of Liquipap treated 2 to 3% reagent red blood cell suspension under test.
7. Add two drops of serum to be tested.
8. Mix well and incubate at 37°C for 30 minutes.
9. Centrifuge at 1000 rpm for 2 minutes.
10. Gently resuspend and observe for agglutination and/or hemolysis macroscopically and microscopically.

Interpretation of Results

Agglutination and/or hemolysis indicate an antibody directed against the antigen present on the red blood cell under test.

No agglutination and/or hemolysis indicate absence of enzyme reactive antibodies directed against the antigen present on the red blood cell under test.

Remarks

1. As undercentrifugation or over-centrifugation could lead to erroneous results, it is recommended that each

laboratory calibrate its own equipment and determine the time required for achieving the desired results.
2. Erroneous results may also occur due to improper red blood cell concentration, improper incubation time or temperature while performing the test.
3. All enzyme preparations are subject to some loss of potency, it is therefore, recommended to check the reagent performance with known negative control (neutral AB serum) and positive control (Coomb's control cells) on a regular basis.
4. The ability of papain to denature IgG molecule renders the one-stage technique less sensitive though it is a convenient method for use in cross-match techniques.
5. Papain is not suited for the detection of Anti-M, N-S-Duffy since the corresponding antigens are destroyed during the proteolytic action of papain enzyme.
6. Liquipap reagent is a colorless to pale yellow clear solution. Repeated exposure to elevated temperatures may impart a dark color to the reagent. In such cases, the reagent performance must be assessed closely before use.
7. Usage of isotonic buffered saline while performing the test ensures in maintaining the optimum pH of the reaction milieu for antigen antibody reaction.
8. Alternatively, LISS (Low ionic strength solution) can be used while performing the test. LISS lowers the ionic concentration of the reaction milieu thereby potentiating the rate of antibody uptake by the antigen present on the red blood cell membrane.
9. It is recommended to run a control with each assay series.

BLOOD TRANSFUSION

The procedures described earlier in this chapter are done mainly for providing appropriate blood for transfusion, i.e. the donor's blood should be compatible in all ways with the recipient's blood.

Blood Donors

Most individuals can give blood without any ill effects, as a matter of fact, without any symptoms at all. The donors may be volunteers or paid donors. Blood transfusion should always be a safe, harmless procedure, therefore, the donors must be screened. Two basic principles govern the transfusion services:

1. The donor should not be harmed in any way.
2. The recipient should be equally safe, i.e. the transfusion should be absolutely safe for the recipient too.

Donor Screening

1. *Identification:* Identify the donor name, address and other pertinent information. No donor should donate twice in 3 months' period. Preferably, the donors should be between the ages 18 and 60 years.
2. *History:* The questions should be guided about the present state of health; recent illness, if any; operations, if any; if the donor has ever been transfused; (for woman) if she ever gave birth to children who developed jaundice shortly after birth; if they have had malaria, jaundice, syphilis, tuberculosis; if they have heart disease, diabetes, etc. Donors may give wrong information on two accounts: (i) They want to donate blood for want of money. (ii) They do not want to donate blood for they are afraid of doing so. Hence, a battery of tests to be done becomes necessary.
3. *Physical examination:*
   - Temperature reading, (exclude those having fever)
   - Blood pressure should be normal or only slightly raised. Disqualify those who have moderate to marked hypertension
   - Pulse should be normal without any irregularities
   - Auscultate the chest for any respiratory or cardiac disorder. Discard individuals with respiratory/cardiac problem
   - Pregnant women should not donate if they are anemic
   - Finding of any venereal disease should outrightly disqualify the donor until he receives adequate treatment and gets rid of his problem.
4. *Hemoglobin*: The donors should not be anemic. It is best to disqualify anybody having Hb < 12 g% but keeping in view our health, dietetic, and economic structure up to 10 g% can, however, be accepted, weighing the urgency of the demand for blood. As given in the hematology section specific gravity by copper sulfate method is adequate for screening donors' Hb.
5. *Malarial parasite*: Malarial blood can cause transfusion malaria if it is transfused. Screen all donors by making thick and thin smears of their blood. In some chronic malaria patient, no parasites may be seen on the peripheral smear examination but a unit of their blood would pass on enough parasites to the recipient.
6. *Microfilaria*: Screening for microfilaria should be done both by making a wet, coverslip mount of a drop of fresh blood (looking for the shipping movement of the microfilaria) and also on the direct and stained films used for searching malarial parasites. All microfilaria-harboring donors are rejected.

7. *Icteric index*: This is the easiest method of measuring and quantitating the amount of pigment (mostly bilirubin) in the blood. Serum hepatitis is one of the most dreaded complications of transfusion. If a person has had jaundice during the last 5 years or is having it—he should be disqualified as a donor. History might be difficult to obtain and hence, liver function tests may be necessary where there is even slightest of doubt. The simplest and quickest to perform is icteric index, a measurement of the level of bilirubin, expressed in units based upon a comparison of the color of the serum and the color of a standard dilute solution of potassium dichromate. If the Icterus index is > 6 units, the donor should not be allowed to donate.
   A major complication of transfusing blood from a hepatitis B/C patient or carrier is the *transmission* of the disease to the recipient. Now ELISA, ICT techniques are available.
8. *Serology*: VDRL/Kahn's tests should be performed on blood of all donors. The blood positive for this test may pass on live spirochetes to the recipient. Weighing the need of blood even such a positive sample may be transfused and the recipient given a course of penicillin. In no case should any drug be put into the transfusion bottle. It is known that *Treponema pallidum* cannot survive for more than 48–72 hours with the condition of storage of anticoagulated blood in a refrigerator at 4ºC. Within that time they are all dead. Hence, blood which has been stored as mentioned above cannot transmit syphilis since there would be no viable organisms remaining. A new syphilis patient's blood may be VDRL/Kahn's negative even though his blood would contain the spirochetes, hence, some believe in storing all blood for 48–72 hours before allowing it to be transfused. It is a good practice to indicate to the doctor who has requisitioned the blood, date and time of procuring that unit of blood.
9. *Grouping and typing of donor*: This has already been dealt with at great length earlier in this chapter.
10. *Antibody Screening*: Ideally an indirect Coombs' test should be done on every donor to screen for atypical, incomplete antibodies.
11. HIV/AIDS screening is a Must.

(Information regarding kits—refer to Serology chapter).

Drawing of Blood

1. Have the donor lie down.
2. Under aseptic conditions, do a venepuncture with a fairly large needle on the donor set-15 or 16 gauge needle, for gravity flow collection method. The bottle is kept below the level of arm and has an airway so that air may escape while the bottle gets filled with blood.
3. Where vacuum bottles are available a smaller gauge needle can be used. Do not puncture such a bottle.
4. Plastic bags containing the preservative fluid are already collapsed and hence, need no airway.
5. A unit of blood may mean different volumes at different centers. A standard unit is 420 mL of blood and 80 mL of ACD or CPD solution, i.e. a total 500 mL.
6. While the blood is being drawn swirl the bottle gently to allow the ACD to act.
7. Flow of blood is usually hastened by: (i) having a blood pressure cuff on the arm above the needle, inflated to a pressure of 40–50 mm Hg and (ii) asking the patient to gently flex the fingers or clench and unclench his fist to assist the flow of the blood into the vein from which it is being taken.
8. When the full amount of donation has been collected, draw the needle from the bottle and put 5 to 10 mL samples into 2 or 3 sterile, dry tubes, each with the same number as that of the large blood transfusion bottle.
9. Now the needle from the vein and the blood pressure cuff can be removed.
10. Immediately apply pressure with cotton at the area, preferably with the arm held straight up in an extended position. Have the donor hold it for minimum 3 to 5 minutes.
11. Close the bottle immediately after sterilizing the rubber cover.
12. Once closed do not open and enter the bottle until the requisitioning doctor inserts the giving set. Be sure the bottle is completely and correctly labeled with number, name, group, type, date, etc. Double check this.
13. Do not let the donor get up immediately. Let the donor's body adjust to the loss of this blood. Offering a nourishing drink and snacks to the donor is indeed a very good way of saying thanks to the donor. Encourage him to drink little extra water for a few hours after the donation.
14. The preservative have been mentioned in the foregoing pages.
15. Store the donated blood at 4–6ºC (37–42ºF) in a refrigerator that should solely be used for keeping the blood transfusion bottles only. If this blood is kept constantly at the required temperature—a duration of 21 days may be allowed to lapse between collection and transfusion of the blood. If storage conditions are less ideal, then it is best to shorten the storage time to about 14 days (after this duration it should not be transfused).

ADVERSE DONOR REACTIONS

Problems with Blood Flow

Occasionally, venipuncture is unsuccessful or the vein may develop spasm after venipuncture so that blood flow is not maintained

If this happens:

1. Do not try to probe around in the vein, as this can result in a hematoma and discomfort for the donor.
2. Remove the needle and discard the pack as it will be contaminated.
3. Never resite the needle in the same arm.
4. Reassure the donor, giving a full explanation for the unsuccessful venipuncture in order to retain their confidence, and apologize.

If the donor consents, a further venipuncture on the other arm may be attempted, if a suitable vein is located. No more than a total of 350/450 mL of blood should be withdrawn from both sides.

If there is a failure to maintain a blood flow during the collection, the person who has performed the venipuncture should be informed immediately. Slowing of the flow rate may be due to:

- Reduced cuff pressure: Check that cuff pressure has been maintained
- Occlusion of the lumen of the needle by the vein wall: Rotating the needle may help
- Positioning of the lumen of the needle on a valve within the vein: Try to re-establish the flow by withdrawing the needle gently or even by slight rotation of the needle.

Before doing any of these things:

1. Explain that there is a problem with the blood flow and ask whether the donor is experiencing any discomfort.
2. Remove the swab and check that there is no hematoma present.
3. If there are no other apparent problems, proceed with adjusting the needle.
4. Avoid excessive manipulation of the needle or squeezing the donor tubing as small clots may form which will then be released into the circulation.

A failure to reestablish a blood flow will result in a partial collection. This should be marked on the donor's record form and the donor should be given an explanation and apology, if the collection is too slow, the donation should be discontinued. This should be recorded on the donor's record form.

Hematoma

Hematoma can be prevented by good venipuncture technique and application of adequate pressure following donation.

If a hematoma is noted:

1. Stop the donation.
2. Apply firm pressure until the venipuncture site stops oozing blood.
3. Apply an anti-inflammatory cream in a circular motion over the area and cover it with a small; plaster or swab dressing, then apply a pressure bandage.
4. Reassure the donor, explaining what has happened and the reason for the bruise, and then apologize.
5. Ask the donor to keep the plaster on for 24 hours and the bandage on for 2 to 4 hours. If they feel that it is too tight and stopping their circulation, it should be loosened.
6. Tell the donor that they can use their arm normally, but should not lift any heavy objects. Also, tell them that they can take painkillers for moderate discomfort, but that if the area becomes unduly painful, they should contact the transfusion center or their own doctor.
7. Record details of the hematoma on the donor's record form.

Accidental Puncture of the Artery

This is an uncommon complication of blood donation, and one should be able to recognize it immediately by a very fast flow of bright red blood.

If accidental puncture of the artery occurs:

1. Discontinue the donation immediately and apply hard pressure to the puncture site immediately after the withdrawal of the needle. Raise the limb above heart level.
2. Maintain pressure for a minimum of 15 minutes.
3. When the bleeding has stopped, apply a pressure bandage and tell the donor to keep this on for 4 to 6 hours.
4. Reassure the donor, giving a full explanation of what has happened, and apologize.
5. Record the appropriate information on the donor's record form.
6. Do not allow the donor to leave until they are feeling well and after the most senior member of the donor clinic staff has discharged them.
7. If you suspect that tissue bleeding may still be continuing, refer the donor to the nearest hospital or health center. If the donor lives near the donor clinic, ask him to come back for assessment the following day.

Mild, Moderate or Severe Reactions

Most people can tolerate the withdrawal of 350 or 450 mL of blood without any ill effects. Others experience reactions ranging in severity from a feeling of uneasiness' to

obvious shock-like symptoms, fainting or even generalized convulsions. These reactions can occur at any time—during the donor selection process, during donation, in the resting or refreshment area or even hours following a donation.

There is a psychological element to most reactions, so a friendly, cheerful atmosphere at the session can often reduce donor anxiety and perhaps prevent any adverse reactions. Donor reactions do sometimes occur, however, and can be categorized as follows:

Mild: Vasovagal symptoms without loss of consciousness.

Moderate : A progression of symptoms associated with a mild donor reaction resulting in unconsciousness.

Severe : Any of the above, accompanied by convulsions (uncommon).

Mild Donor Reactions

The signs of mild donor reactions include:

- Anxiety
- Increased respiration
- Rapid pulse
- Pallor and mild sweating
- Dizziness/continuous yawning
- Nausea/vomiting.

When mild donor reactions occur:

1. Discontinue the donation.
2. Raise both of the donor's legs and lower the head to improve the blood supply. If a donor is vomiting, turn him on one side to avoid accidental inhaling of vomitus.
3. Loosen or remove tight clothing.
4. Keep the donor cool by opening windows or switching on a fan.
5. Have a suitable receptacle available at the bedside in case the donor vomits.
6. Allow a sufficient rest period.
7. Offer a cold drink.
8. Once the donor has recovered, assist him from the bed to the refreshment area where another cold drink should be given.
9. Reassure and talk to the donor throughout all these stages. Explain that this type of reaction is common and does not mean that they are now physically 'unwell'.
10. Record the reaction on the donor's record form.
11. Advise the donor that, if symptoms persist, they should report to the blood bank or consult a doctor.
12. Ensure that the donor is fully recovered before leaving the session and has been seen by a trained member of staff.

Moderate Donor Reactions:

The signs of moderate donor reactions include:

- Loss of consciousness (fainting)
- Repeated periods of unconsciousness
- A slow pulse which may be difficult to feel because of poor volume
- Shallow respirations.

When moderate donor reactions occur:

1. Discontinue the donation.
2. Raise both of the donor's legs and lower the head.
3. Ensure that a medical officer or a senior nurse examines the donor.
4. Loosen or remove tight clothing.
5. Keep the donor cool by opening windows or switching on a fan.
6. Have a suitable receptacle available at the bedside in case the donor vomits.
7. Check the pulse rate regularly. The appearance of the donor and the pulse rate are a good guide to the donor's condition.
8. If possible, remove the donor to another room for privacy and to prevent other donors from seeing what is happening.
9. If there is no other room available, put screens around the donor.
10. Ensure that someone remains with the donor.
11. Reassure and talk to the donor throughout all these stages. It may be necessary to advise the donor not to donate in future.
12. Record the reaction on the donor's record form.
13. Ensure that the donor rests for some time and is fully recovered before leaving.
14. Advise the donor that if symptoms persist, they should contact their doctor of the nearest hospital.
15. Ensure that the donor is discharged by a senior member of staff.
16. Where feasible, arrange transport home for the donor.

Severe Donor Reactions

A faint may be accompanied by convulsions. Convulsions may be preceded by all the signs and symptoms of a vasovagal attack or they may occur without warning. Convulsions vary in severity from loss of consciousness accompanied by a slight twitching of extremities to a grand mal: type seizure with incontinence of urine or feces. A medical officer or trained nurse must be called immediately.

Faints are common but convulsions are very uncommon. If the correct procedure for donor screening has been

carried out through the medical history and health check convulsions should not occur. Most convulsions stop within a few minutes, but they are often very upsetting for other donors so staff not actively involved in looking after a convulsing donor should distract and reassure other donors.

When Generalized convulsions occur:

1. Turn the donor to a lateral position to maintain a clear airway.
2. Gently restrain the donor to prevent and injury.
3. Put screens around the donor to maintain privacy.
4. Check the pulse rate frequently.
5. Ensure that the donor is examined by a medical officer.
6. Loosen tight clothing.
7. Keep the donor cool by opening windows or switching on a fan.
8. If a convulsion lasts longer than 5 minutes, this is a medical emergency and a medical officer must be in attendance. Valium may be given intravenously under the direction of the medical officer. IM Valium is ineffective in these circumstances.
9. Reassure the donor and explain what has happened.
10. Tactfully advise the donor not to donate blood again.
11. Record the incident:
    - On the donor's record form.
    - In the blood bank incident book.
12. Recheck the donor's medical history and record of the predonation health check to identify whether there were any indications that a convulsion might occur.
13. Advise the donor that they should contact their doctor or the nearest hospital.
14. Ensure that the donor rests for some time and is fully recovered before leaving the session.
15. Ensure that the donor is discharged by a medical officer or a very senior member of staff.
16. Inform the donor's own doctor about the incident.
17. Arrange transport home for the donor when fully recovered and ensure that they are escorted or arrange for their transfer to hospital.

Hyperventilation

Hyperventilation is a rapid overbreathing which lowers the carbon dioxide content of blood. In turn, this leads to muscle spasms. Talking to the donor to reassure him and relieve anxiety should prevent hyperventilation.

If hyperventilation occurs:

1. Instruct the donor to breathe quietly and slowly, but not deeply.
2. If this fails to relieve muscle spasms, instruct the donor to rebreathe expired air into a paper bag.
3. Explain what is happening and reassure the donor.

Accidents

There may be a risk of injury to the head or body if a donor faints and falls

When head injuries or other injuries requiring medical attention occur:

1. Always ensure that the donor is examined by a medical officer or senior nurse.
2. If there is any doubt about the donor's condition, arrange for his transfer to hospital with a doctor or qualified nurse as escort.
3. Record the incident:
    - On the donor's record form.
    - On an accident form (this should also be filled in if a member of staff is involved in an accident)
    - In the blood bank incident book.

If the Injury is of a minor nature:

1. Ensure that the donor rests for some time and is fully recovered before leaving, unless transfer to hospital has been arranged.
2. Ensure that the donor is discharged by a senior member of staff who should decide whether the donor's own doctor should be informed.
3. Advise the donor that if he feels unwell, he should contact his doctor or the nearest hospital.

Compatibility Testing

The Cross-match

The final green signal for allowing blood transfusion is compatibility between the donor's and recipient's blood. This is done in vitro in the laboratory/blood bank. A compatibility test is mandatory before all transfusions.

Cross-matching is done in two parts: (i) the major cross-match and (ii) the minor cross-match. In the former, the donor's cells are mixed with the patient's serum and in latter the patient's cells are mixed with the donor's serum (Fig. 11.6).

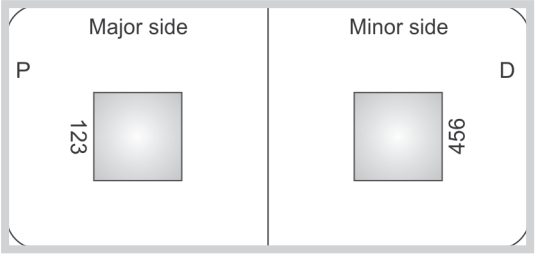

FIG. 11.6: Cross-matching

Saline Cross-match

Saline suspension of the cells is mixed with serum. This is done at room temperature and only complete, saline active, 'cold' antibodies will be detected.

The Open Slide Method

1. Label one tube with patient's name or number (number is preferable as there may be many patients with the same name).
2. Label another tube with the donor's name or number—one for each bottle of blood to be cross-matched.
3. Half-fill each tube with saline.
4. Label one slide for each cross-match. Draw a line in the middle to separate the two sides. Put the patient's number on the left side and mark it 'P' on that side. This is the major side which uses the patient's (P) serum. Put the donor's number on the right side and identify it further by marking it 'D'. This is the minor side, which uses the donor's (D) serum. Draw two circles one on each side with a glass marking pencil (Fig. 11.7).
5. Make 5% red cell suspension in each of the labeled tubes—patient's and the donor's bloods. (Get the donor cells and serum from one of the 'pilot' tubes filled at the end of the donation of blood). Do not enter the bottle of ACD blood for this purpose unless absolutely necessary. If you do enter the bottle, you have almost inevitably introduced bacteria—even if you have been careful—and that blood should be used immediately or not at all.
6. To the patient's (P), major side of the slide, add one drop of patient's serum and one drop of the donor's cell suspension
7. To the donor's (D), minor side of the slide, add one drop of donor's serum and one drop of the patient's cell suspension.
8. Mix by gently rotating the slide and incubate at room temperature for 10–15 minutes. (Drying will make the results difficult to interpret. Drying can be retarded if the slides are kept in a petri dish).
9. Examine both macroscopically and microscopically for agglutination (by gently rotating and tipping the slide).

This is a simple and a rapid method. However, incompatibilities due to weak saline agglutinins and all incomplete immune antibodies will not be detected. The period of incubation should not be reduced or else agglutination may not occur. Almost all ABO group incompatibilities will be detected by this method. Rh incompatibilities will never be detected.

Saline Tube Method

1. Label one tube for the patient's 2–5% saline cell suspension. Label one tube for each donor's saline cell suspension. Label two tubes for each cross-match—one marked P with patient's number and donor's number, and one marked D with donor's number and patient's number.
2. Add saline to the cell suspension tubes and make the cell suspensions.
3. To the patient's tubes (P. major side), add one drop of patient's serum and one drop of donor cell suspension.
4. To the donor's tubes (D. minor side) add one drop of donor's serum and one drop of patient cell suspension.
5. Mix the cells and sera by gently tapping the tubes.
6. Let stand at room temperature for 30 minutes.
7. Examine for agglutination both macroscopically and microscopically.

This method needs a longer incubation period but has the advantage of detecting weak reactions more often. However, it still will only detect complete agglutinins—not immune antibodies of the Rh type.

Immediate-spin and thermal incubation modification:

1–5. As in the previous method.

6. After 2–3 minutes at room temperature, centrifuge at 500 to 1000 rpm for 2 minutes.
7. Remove and examine macroscopically, holding the bottom of the tube over a concave mirror (a microscope mirror is good enough) so that you can observe the button of cells at the bottom when you tap the tube gently to resuspend the cells. If the cells, show agglutination, check them microscopically by placing a drop on a slide. This indicates incompatibility and the cross-match result can be recorded. If the cells resuspend without any clumps, then proceed to the next step.
8. Incubate the tube for 60 minutes at 37°C.
9. Examine the cells for agglutination, both, macroscopically and microscopically. Centrifugation brings the cells into close proximity and hastens agglutination where it is going to occur—the immediate spin. Incubation at 37°C may detect some complete warm antibodies active in saline but missed in room temperature incubations.

Albumin Tube Method

1. Set up the tubes as for saline method.
2. Incubate the cells and serum for 60 minutes at 37°C—thermal saline incubation.

3. Allow a drop of albumin to run down the inside of each cross-matching tube so that it forms a layer between the cells and serum (human/bovine albumin, 22–30%).
4. Incubate at 37°C for an additional 30 minutes.
5. Dislodge the cells gently and look for agglutination microscopically. This method will pick up some incompatibilities not detected in saline due to incomplete, immune antibodies, such as Rh antibodies.

Coomb's Cross-matching

1. Wash the patient's and donor's cells three times in saline. Then make 5–10% suspension of each in saline.
2. To labeled patient's tubes (P. major side) add 2 drops of patient's serum and 2–4 drops of donor cell suspension.
3. To labeled donor's tube (D. minor side) add 2 drops of donor's serum and 2–3 drops of patient cell suspension.
4. Incubate tubes at 37°C for one hour.
5. Centrifuge, decant the serum and wash the cells thrice in saline.
6. Add Coombs' serum to each tube and after letting stand for 5 minutes at room temperature, centrifuge at 500–1000 rpm for 2 minutes.
7. Examine the cells macroscopically and microscopically.

The Coombs' serum in the cross-match as in the indirect Combs' test, will detect the presence of incomplete, immune antibodies such as Rh antibodies and show any Rh incompatibility. Remember that not all Rh negative persons have anti-Rh (D) antibodies—in fact only very few do—so that not all cross-matches of Rh-negative recipients with Rh-positive donors—or vice versa—will be incompatible. Transfusion of the blood may be perfectly safe. However, the Rh-positive transfused cells may sensitize the recipient so that he or she develops anti-Rh antibodies. On subsequent cross-matches (after 2–3 weeks), an incompatibility would be demonstrated. Thus, where Rh-negative individuals have been previously transfused, it is important to do a more sensitive cross-match, since there is a good chance that the previous transfusion may have been with Rh-positive blood.

It is obvious that cross-matching of bloods of the same groups generally gives a compatible result (major and minor). Cross-matching of O group blood with any other group gives a compatible major side (hence, O group people are called universal donors) but always gives an incompatible minor side. Any group of blood may be given to AB group of patient with a compatible major cross-match (universal recipients) but again there is an incompatible minor cross-match.

Given on next page are the diagrammed results of the cross-matching of the different groups of blood (+ = agglutination, — = no agglutination).

Blood which shows a major incompatibility should never be transfused (in the patient's body the 'major side' would be the full plasma volume—about 2,500 mL of antibodies and the transfused donor cells—200–250 mL). The minor cross-match is important but not as important as the major side, (in the patient's body, the minor side would be the donor plasma volume 250–300 mL—of antibodies and the large number of his own cells—about 2,500 mL. The donor plasma becomes diluted in the much larger volume of the patient's plasma, and thus, the donor antibodies become diluted and dispersed so that although a few cells may be coated or agglutinate, there is no major reaction). In an emergency, therefore, it is possible to use blood which is minor incompatible (but major compatible) without leading to transfusion reaction. Sometimes however, one can get into difficulty by transfusing blood which shows a minor incompatibility as given below.

High titer of donor antibodies: If the titer (amount) of antibodies in the donor blood is very high, then even if there is dilution of the donor's plasma in the patient's plasma, the antibodies may still be present in significant numbers to cause agglutination, hemolysis and result in a reaction. O donors with high titer are said to be dangerous universal donors.

Method of Cross-matching Universal Donor Blood

1. Do major cross-match as described (Fig. 11.7).
2. Make a 1:100 dilution of donor serum in saline.
3. Use this diluted serum for a minor cross-match.
4. If there is no agglutination on minor side using the 1:100 diluted serum, then this a low titer (< 1:100) plasma and can safely be transfused as long as too many bottles of such blood are not needed.
5. If there is agglutination on the minor side, it means that this is high titer (> 1:100) plasma and should better not be used especially if more than one unit is required (for AB patient, it is possible to use A, B, or O donor blood. Whichever is given, it should be cross matched in this way).

Factors Leading to False Results

1. Auto-agglutination
2. Cold antibodies
3. Bacterial contamination
4. Drying.

If Still an Unexpected Incompatibility is Obtained

1. Test for autoagglutination.

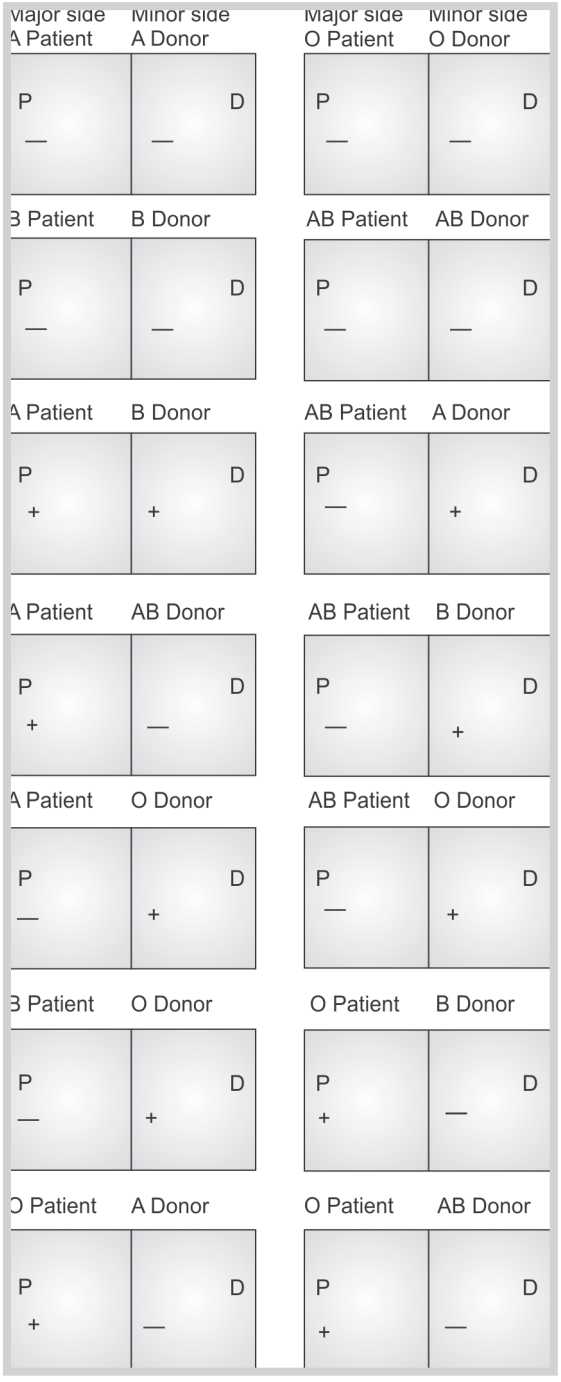

FIG. 11.7: Method of cross-matching universal donor blood

2. Test for cold antibodies.
3. Regroup the patient and donor cells.
4. Back-type (serum type) the patient and donor serum.
5. Repeat the cross-match by tube technique.
6. Check the records to see if patient's present grouping is correct.
7. Ask for a fresh patient specimen, if necessary.

Choice of Material for Transfusion

1. Shock due to hemorrhage or decreased blood volume: Whole blood fresh, stored or preserved.
2. Shock resulting from trauma, burns, or infection: Plasma liquid, frozen or dried; plasma substitutes.
3. Chronic anemia: Whole blood or resuspended red blood cells.
4. Leukopenia: Large amounts of fresh whole blood. Leukocyte concentrates.
5. Prothrombin factor deficiency not corrected by vitamin K: Fresh whole blood.
6. Hemophilia: Factor VIII (AHF) cryoprecipitate concentrate. Factor IX complex concentrate. Whole blood or plasma administered within 6 hours of collection.
7. Thrombocytopenia: Platelet transfusion with fresh (< 3 days old) platelets.

Blood and its Products

Whole blood: In most instances whole blood is requisitioned by the concerned physician/surgeon, except in certain circumstances when a special preparation may be asked for.

Fresh Blood

Stored bank blood contains nearly all of the substances required in a usual transfusion therapy. A few labile substances are lost.

1. *Platelets*: These stick to the rubber and glass surfaces and also die soon or become non-functional. If platelets are needed, fresh blood is necessary.
2. *WBC's*: These die fairly rapidly. On occasions, where the patient is suffering from severe leukopenia with infection, a transfusion of fresh blood provides viable leukocytes which help to fight the infection but which do not raise the WBC count.
3. *Factor VIII*: Anti-hemophilic globulin. Patients with hemophilia should be transfused with fresh blood if they need transfusion to help stop bleeding, since AHG disappears rapidly from stored blood.
4. *Other labile factors:* Can be provided by giving frozen or dried plasma.

Packed Cells

Sedimented cells: The bottle kept in the refrigerator shows at bottom settled cells and above it the plasma. In chronic anemia one may want to transfuse just the packed cells. This can be prepared by aspirating the plasma into a separate sterile bottle, leaving the cells in original bottle.

Label the plasma bottle with the date, the bottle number and the group and type of the blood from which it is obtained, aspirate a small amount of plasma into a 'pilot' tube for later cross-matches.

The packed cells should be transfused within 4 hours. Aspirate plasma only when someone comes to collect it, this ensures that the packed cells would be used immediately.

Plasma

The ACD plasma aspirated from the sedimented cells may be stored for months at 4–6°C and is safe for transfusion. If the plasma becomes cloudy or shows floating granules (bacterial colonies)—suck little bit of it from the top, do a hanging drop and Gram stain of the same—discard if it is found contaminated. Minor cross-matching should be done with the patient's red cells and the donor's plasma.

Plasma may be used as:

1. *Liquid plasma:* It should contain 5% dextrose to prevent precipitation of fibrin at room temperature. The prothrombin titer diminishes rapidly after 72 hours. Liquid plasma may be kept at room temperature for 3 years. Hepatitis virus is attenuated or destroyed in plasma that has remained at room temperature for 6 months.
2. *Frozen plasma:* Plasma frozen within 72 hours after blood is drawn may be stored indefinitely at –20°C (–4°F). Reliquefy at 37°C (98.6°F) in a water bath and use promptly. Frozen plasma retains its full content of labile constituents (prothrombin, complement, antibodies). Hepatitis virus is also preserved, frozen plasma should therefore not be used unless no other substitute for blood is available.
3. *Dried plasma*: Plasma dried after freezing within 72 hours after blood is drawn is stable at room temperature for 5 years if kept in an airtight container. Reliquefy with 0.1% solution of citric acid and administer within 1 hour. Dried plasma retains its full content of labile constituents. Hepatitis virus is preserved too.

Blood Transfusion Complications

Complications Appearing Early

1. Hemolytic reaction:
   - Immediate or
   - Delayed.
2. Reactions due to infected blood.
3. Allergic reactions to white cells, platelets or proteins.
4. Pyrogenic reactions (febrile reactions)
   - Pyrogens may come from the bottle, tubing, or the blood itself. Precaution should be taken that equipment, solutions, etc. used should be absolutely sterile
   - Bacterial contamination too would lead to pyrogenic reaction.
5. Circulatory overload.
6. Air embolism.
7. Thrombophlebitis.
8. Hyperkalemia.
9. Citrate toxicity.
10. Clotting abnormalities (after massive transfusion).

Complications Appearing Late

1. Disease transmission, e.g.
   - Hepatitis B/C
   - Syphilis
   - Malaria
   - Cytomegalovirus
   - AIDS.
2. Transfusional iron-overload.
3. Immune sensitization.

Investigations in a Case of Transfusion Reaction

The occurrence of a transfusion reaction should be immediately reported to the blood supplying laboratory or bank.

The reporting authority should send:

1. A post-transfusion blood sample.
2. A post-transfusion urine sample.
3. A pre-transfusion blood sample.
4. If the blood has been discontinued, the bottle and the tubing intact should also be sent.

The Laboratory or the Bank Providing the Blood should Already have

1. The patient's original cross-match specimen (these should be preserved for at least 48 hours after dispatching the blood or its products).
2. The donor's pilot tube/bottle (also to be preserved for 48 hours as mentioned above).
3. All the laboratory/bank records.

Proceed as Mentioned Below

1. Inspect the post-transfusion urine sample for the presence of hemolysis—hemoglobinuria. Centrifuge the specimen to see if the red color stays in the supernatant (hemoglobinuria) or goes down with the sediment (hematuria).

2. Centrifuge the post-transfusion blood specimen. Inspect the serum for the presence of hemolysis. If hemolysis has been established report it to the concerned physician/surgeon so that he may treat the patient as a case of hemolytic transfusion reaction.
   To Establish the Cause of Hemolysis:
3. Regroup and retype the original donor pilot blood sample, the original patient's cross-match sample, the blood in the blood bottle or tubing.
4. Group and type the new patient's pretransfusion blood sample (if available—and post-transfusion specimen).
5. Recross-match—if available use combined saline and Coombs' cross-matches—the donor blood with the original patient specimen.
6. Cross-match the donor blood with the new patient specimen.
7. Make hanging drop preparation and Gram stain of blood in tubing or blood bottle looking for bacteria. Culture the blood in the bottle or tubing.
8. Schumm's test—a spectroscopic examination of plasma for the bands which are typical of methemalbumin—found when there has been intravascular hemolysis.

Interpretation of Results

1. If there was no evidence of hemolysis in the blood and no free hemoglobin in the urine, the patient has not had a hemolytic reaction, but a pyrogenic/allergic reaction.
2. If there is evidence of hemolysis in the blood and hemolysis in the urine (hemoglobinuria), a hemolytic transfusion reaction has occurred.
3. If the recross-matching shows incompatibility, then the first cross-match was done or recorded in error.
4. If the recross-match with the original patient specimen is compatible, but the cross-match with the new patient specimen is incompatible, the mistake lies in mistaken identification of the patient, either when the first sample for cross-match was done, or at the time of giving the transfusion.
5. If the saline cross-matches are compatible but the Coombs' cross-matches are incompatible then the problem lies with an immune antibody incompatibility—the most common being the Rh incompatibility.

Laboratory Diagnosis of Hemolytic Disease of the Newborn

Laboratory findings at birth:

1. *Cord blood:*
   - Variable anemia (Hb < 18 g%)
   - Reticulocytosis
   - Hyperbilirubinemia
   - Positive direct Coombs' test
   - Baby is Rh positive.
2. *The mother:*
   - Is Rh negative, and
   - Has a high plasma titer of anti-D.

TROUBLE SHOOTING

General Instructions for Blood Grouping

Sample Preparation

- Depending upon whether serum or plasma is to be used as sample for testing, blood may be collected with or without an anticoagulant.

  Serum versus Plasma

  For blood grouping tests, serum is preferred to plasma for the following reasons:
  1. Plasma samples may clot when incubated at 37°C.
  2. The detection of some antibodies depends upon complement activation and anticoagulants such as citrate of EDTA prevent complement activation by chelating calcium.
- Containers for blood collection and processing should be clean and dry, free of detergents; acids and alkalies, ideally made of plastic or siliconized glass tubes.

Sample Processing

- Need to wash red cells (Tube test)

  Red cell suspension used in blood grouping should be washed free of their own plasma. If this is not done clots will form when the red cell suspension, which contains fibrinogen, is mixed with serum, which contains residual thrombin.

  Other reasons for washing red cells are as follows:
  1. Plasma tends to cause rouleaux formation, which interferes with the interpretation of agglutination tests.

     Rouleaux or pseudoagglutination is a phenomenon characterized by a person's serum causing his own and other red cells to line up in formations which resemble stacks of coins. This is easily mistaken for true agglutination.

     Causes of rouleaux:
     a. Concentration of serum.
     b. Increase of plasma proteins.
     c. The transfusion of macromolecular medium, e.g. dextran.
     d. Inverted albumin/globulin ration as in chronic nephritis, Kala-azar, and multiple myeloma.
     e. Infections with an increased red cell sedimentation rate.

2. Plasma contains anticoagulants, which are anti-complementary and may thus interfere with the detection of complement binding antibodies.
3. Preservative substances added to red cell suspension, e.g. lactose or neomycin, are occasionally responsible for agglutination due to the presence of a corresponding antibody in the patient's plasma; most of the antibodies concerned do not react with red cells washed in saline.
4. Plasma contains blood group substances corresponding to those on the red cells and these substances may inhibit the antibody in the test serum.
5. Plasma may contain so-called albumin autoagglutinins and may then cause false-positive reactions when whole blood is added to a serum-albumin mixture.

Sample Storage

- Effect of storage on red cell antigens
  1. Red cells stored as clotted blood lose their antigenic activity more rapidly than when stored with citrate anticoagulant.
  2. Similarly, when blood is collected into plastic bags, if the donor line is not emptied immediately after collection and then refilled with blood mixed with anticoagulant, the clotted blood in tubing is an unreliable source of red cells for cross matching tests
  3. Red cells stored as clotted blood may give false-positive reactions in the antiglobulin test due to uptake of complement components during storage at 4°C.
  4. Reagent red cells may be stored as whole blood with CPD or ACD but more usually they are stored as washed cells in a preservative solution. A modified Alsever's solution, with added inosine and with antibiotics is commonly used permitting satisfactory storage for at least 35 days at 4°C.
- Clotted whole blood should be tested within 14 days
- Anticoagulated blood using various anticoagulants should be tested within specific time limits as follows:
  - EDTA or heparin: 2 days
  - Sodium citrate or sodium oxalate: 14 days
  - ACD or CPD: 28 days.

Equipment

- Prior to testing, check whether pipettes and other instruments used are well calibrated and in proper working condition.

Reagents

- The blood grouping reagents contain 0.1% sodium azide as preservative. Avoid contact of the reagent with skin and mucosa. On disposal flush with large quantities of water
- Adhere to appropriate storage conditions of the reagent as mentioned in the respective package inserts.

Common Causes of False Negative and False Positive Results in ABO Testing

False Negative Results

- Reagent or test serum not added to a tube
- Hemolysis not identified as a positive reaction
- Inappropriate ratio of serum (or reagent) to red cells
- Tests not centrifuged sufficiently
- Tests incubated at temperatures above 20–24°C
- Incorrect interpretation of test results.

False Positive Results

- Overcentrifugation of tubes
- Use of contaminated reagents, red cells or saline
- Use of dirty glassware
- Incorrect interpretation of results.

Interpretation of Agglutination Reactions

To avoid false readings and to standardize recording of results, tests should be read with the aid of the microscope (Table 11.10).

TABLE 11.10: Interpretation of agglutination reactions

++++	One complete mass of agglutination, readily visible on the slide before microscopic examination
+++	Large separate masses of agglutination, readily visible on the slide before microscopic examination
++	Smaller agglutinates, still readily visible on the slide before examination under the microscope
+	A granular appearance, just visible on the slide before examination under the microscope. The microscope reveals big clumps of agglutinates of more than 20 cells
(+)	Smaller clumps (12–20) cells only detectable by use of microscope
GW	Good weak—clumps of 8–12 cells only detectable microscopically
W	Weak—Weak reactions with uniform distribution of small clumps of 4–6 cells
?	Sticky—uneven distribution of cells, 2 or 3 sticking together, more noticeable when the cells are "rolling" on the slide
0 or -	All cells free and evenly distributed. The 0 sign is preferred since—can easily be altered to +
MF/NF	Mixed filed or negative field—agglutinates in a field of free cells, e.g. the type of reaction observed with mixed bloods

ABO Grouping

Problem: False positive results

Possible causes		*Solutions*
1.	Bacterial contamination (some bacteria such as *Staphylococcus aureus* will agglutinate all red cell samples thereby giving false positive results)	Check the reagents for turbidity. Extreme turbidity may indicate bacterial contamination. Such contaminated reagents should not be used for testing
2.	The antisera may be contaminated with another antibody. There may have been accidental mixing of antisera; this could be due to the interchanging of caps. For example, if either anti-A, anti-B or anti-A, B is contaminated with anti-D as anti-D reagent is colorless and contamination is not visually observed in such a case the anti A or anti B or anti A, B reagent will give false positive results with all Rh positive blood groups	Check the color of the cap on the antisera bottle. Also check the antisera with the blood of a person whose blood group is known. If anti-A, anti-B or anti-A, B vial is contaminated with anti D, then the anti-A, anti-B or anti-A, B should be checked with Rh negative blood sample, if it gives no agglutination thereby confirming anti-D contamination or else it could be some other contamination
3.	Particles of dust, debris, chemicals or detergents on the slide or in the tube giving non-specific agglutination	Clean and dry glassware should be used while carrying out the test
4.	Peripheral drying or fibrin strands were mistaken for agglutination in case of slide test	The test should not be carried out directly under the fan. The vials should be capped immediately after use. The results of the test should be read at 2 minutes not beyond as drying may be interpreted as positive result. Except in case of anti-A_1 lectin, the agglutination should be observed at one minute
5.	Excessive centrifugation in case of tube test	Ensure that centrifugation is carried out for either one minute at 1000 rpm or 20 seconds at 3400 rpm. Centrifugation should be adequate to produce a cell button with a clear supernatant but without packing the cells so tightly that they are difficult to dislodge. Each laboratory must interval its equipment at regular interval

Problem: False negative results

Possible causes		*Solutions*
1.	Storage of antisera at higher/lower temperatures than specified	Reagents should be stored at 2–8°C when not in use. Thermal damage due to faulty storage may result in a loss of reactivity. Check the turbidity of the reagents; extreme turbidity may also be due to thermal damage. Antibody activity decreases at lower temperatures. Do not freeze the reagents
2.	Blood sample stored for too long	Check the period of time for which the blood sample has been sotred. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period (when stored at 2–8°C) • EDTA or heparin—2 days • Sodium oxalate or sodium citrate—14 days • ACD or CPD—28 days • Clotted whole blood—14 days
3.	Use of hemolyzed samples	Check the samples before use. Do not use hemolyzed samples
4.	Prozoning (zone of antibody excess) or postzoning (zone of antigen excess)	The antigen and the antibody should be present in optimal concentrations of the agglutination to be seen properly. Check the sample volume and the reagent volume used. Both the sample and reagent volume should be equal in slide test and a 5% suspension of cells should be used in tube test. Ensure that there are no air bubbles while dispensing samples and reagents
5.	Outdated or contaminated reagent	Check the expiry date of the reagent. Also check the reagent for contamination. Extreme turbidity may be due to microbial contamination

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Possible causes		*Solutions*
6.	Blocking effect when the cells are coated, e.g. in hemolytic disease of the newborn (HDN), acquired immune hemolytic anemia (IHA)	Check the history of the patient for the presence of disorders in which case the cells may be coated in vivo. This can be established by doing a DAT on washed cells
7.	Weak antigens	In the tube test, every tube with negative reaction should be centrifuged and the results should be read again after 5 minutes, so that weak antigens are not overlooked
8.	Under centrifugation	Centrifugation should be carried out for 1 minute at 1000 rpm or for 20 seconds at 3400 rpm as per recommended procedures to give enough time for antigen-antibody to bind
9.	Vigorous shaking for resuspension of cells after centrifugation	Resuspend the cells slowly and gently after centrifugation. Each laboratory must calibrate its equipment at regular intervals
10.	In case of A_1 lectin, A antigen is not fully expressed on the red cells of newborns below one year of age	Check the age of the patient. If below 1 year repeat typing after child reaches 1 year of age
Problem: Hemolysis or red blood cells		
Possible causes		*Solutions*
1.	Use of wet slides and tubes	Wet glassware can cause hemolysis of RBC's. Ensure that only dry glassware is used for testing
Problem: Weak agglutination		
Possible causes		*Solutions*
1.	Preleukemic states and acute myeloblastic leukemia	Check the clinical history of the patient
2.	Acquired loss of antigens occur in healthy elderly individuals	Check the age of the patient
Problem: Mixed field agglutination in ABO grouping or discrepancy observed between the red cell group and the reverse group		
Possible causes		*Solutions*
1.	Transfusion of blood of a different group. (e.g. group O to group A or more significantly group A to group O)	It is extremely important to verify the cause of mixed field agglutination. An investigation of the patient's history may help to verify the cause of mixed field agglutination
2.	Chimera (this is a condition where an individual possesses more than one population of red cells)	This may be caused by exchange of blood cells during early fetal life of nonidentical twins or may be artificially induced by compatible but not same blood group. Check the history of the patient
3.	Blood group antigens altered by bacterial enzymes. Some group A individuals with intestinal obstruction, carcinoma of the colon or rectum and other disorders of the lower intestine acquire a B-like antigen	Check the history of the patient for the presence of any of these conditions and perform reverse grouping in case of acquired B to confirm the blood group
Problem: Delayed agglutination		
Possible causes		*Solutions*
1.	Subgroups of A, other than A_1 such as A_2, A_3, A_x, etc. have weakly expressed A antigenic sites on the red cells, hence these red cells give weak reaction with anti-A reagent as compared to A_1 cells	Check these cells with Anti-A_1 lectin, these cells should not react with anti-A_1 lectin Graded reaction helps the clinician to differentiate between strong (A_1) and other weaker subgroups of A group
2.	Reagents used immediately after removing from the refrigerator	The reagent vial must be brought to room temperature prior to starting the test
3.	The antigen and antibody are not present in optimal concentrations	The sample volume and the reagent volume dispensed should be as per the instructions given in the protocol

Rh Typing

Problem: False positive results

Possible causes		*Solutions*
1.	Bacterial contamination (some bacteria will agglutinate all red cell samples thereby giving false positive results)	Check the reagents for turbidity. Extreme turbidity may indicate bacterial contamination. Such contaminated reagents should not be used for testing
2.	The antisera may be contaminated with another antibody. There may have been accidental mixing of antisera; this could be due to interchanging of caps	Check the color of the cap on the antisera bottle. Also check the antisera with the blood of a person whose blood group is known
3.	Particles of dust, debris, chemicals or detergents on the slide or in the tube giving nonspecific agglutination	Clean and dry glassware should be used while carrying out the test
4.	Peripheral drying or fibrin strands were mistaken for agglutination in case of slide test	The test should not be carried out directly under the fan. The results should be read within 2 minutes. The vial should be capped immediately after use
5.	Excessive centrifugation in case of tube test	Ensure that centrifugation is carried out for either one-minute at 1000 rpm or 20 seconds at 3400 rpm. Centrifugation should be adequate to produce a cell button with a clear supernatant but without packing the cells so tightly that they are difficult to dislodge. Each laboratory must calibrate its equipment at regular intervals
6.	Rouleaux formation in case of tube test may be mistaken for agglutination. Rouleaux formation is said to occur when the red cells appear like a stack of coins. Rouleaux formation may be caused by the following	Rouleaux formation can be distinguished from agglutination by adding 2 drops of saline to the reaction mixture; if the clumping of cells dissolves then it indicates rouleaux formation Check the history of the patient for the conditions like multiple myeloma. The suspension of the cells prepared should be as per the instructions in the package insert

Problem: False negative results

Possible causes		*Solutions*
1.	Storage of antiseras at higher/lower temperatures than specified	Reagents should be stored at 2–8°C when non in use. Thermal damage due to faulty storage may result in a loss of reactivity. Check the turbidity of the reagents; extreme turbidity may be due to thermal damage. Do not freeze the reagents
2.	Blood sample stored for too long than recommended	Check the period of time for which the blood sample has been stored. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period (when stored at 2–8°C) • EDTA or heparin—2 days • Sodium oxalate or sodium citrate—14 days • ACD or CPD—28 days • Clotted whole blood—14 days
3.	Use of hemolyzed samples	Check the samples before use. Do not sue hemolyzed samples.
4.	Prozoning (zone of antibody excess) or postzoning (zone of antigen excess)	The antigen and the antibody should be present in optimal concentrations of the agglutination to be seen properly. Check the sample volume and the reagent volume used. Both the sample and reagent volume should be equal in slide test and a 5% suspension of cells should be used in tube test. Ensure that there are no air bubbles while dispensing samples and reagents
5.	Outdated or contaminated reagent	Check the expiry date of the reagent. Also check the reagent for contamination. Extreme turbidity may be due to microbial contamination
6.	Blocking effect when the cells are coated, e.g. in hemolytic disease of the newborn (HDN), acquired immune hemolytic anemia (IHA)	Check the history of the patient of the presence of disorders in which the cells may be coated

Contd...

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Possible causes		*Solutions*
7.	Weak antigens	In the tube test, every tube with negative reaction should be centrifuged and results should be read again after 5 minutes, so that weak antigens are not overlooked
8.	Under centrifugation	Centrifugation should be carried out for 1 minute at 1000 rpm or for 20 seconds at 3400 rpm
9.	Vigorous shaking for resuspension of cells after centrifugation	Resuspend the cells slowly and gently after centrifugation. Each laboratory must calibrate its equipment at regular intervals
Problem: Hemolysis of red blood cells		
Possible causes		*Solutions*
1.	Use of wet slides and tubes	Wet glassware can cause hemolysis of RBC's. Ensure that only dry glassware is used for testing
Problem: Delayed or weak agglutination		
Possible causes		*Solutions*
1.	Reagents used immediately after removing from the refrigerator	The reagent vial must be brought to room temperature prior to starting the test. Anti-D (IgG) type reacts at 37°C hence at low temperatures it may not react properly
2.	The antigen and antibody are not present in optimal concentrations	The sample volume and the reagent volume dispensed should be as per the instructions given in the protocol
3.	In case of weak D or partial D cells in slide test	Should be confirmed by tube test or Coombs test

General Instructions for Anti-human Globulin (Coombs Reagent)

Sources of Error in Antiglobulin Testing—Coombs Cells

False Negative Results

- *Neutralization of anti-human globulin (AHG) Reagent*
  1. Failure to wash cells adequately to remove all serum/plasma. Fill tube at least ¾ full of saline for each wash. Check dispense volume of automated washers.
  2. If increased serum volumes are used, routine wash may be inadequate. Wash additional times or remove serum prior to washing.
  3. Contamination of AHG by extraneous protein. Do not use finger or hand to cover tube. Contaminated droppers or wrong reagent dropper can neutralize entire bottle of AHG.
  4. High concentration of IgG paraproteins in test serum; protein may remain even after multiple washes.
- *Interruption in testing*
  1. Bound IgG may dissociate from red cells and either leave too little IgG to detect or may neutralize AHG reagent.
  2. Agglutination of IgG-coated cells will weaken. Centrifuge and read immediately.
- *Improper reagent storage*
  1. AHG reagent may lose reactivity if frozen. Reagent may become bacterially contaminated.
  2. Excess heat or repeated freezing/thawing may cause loss of reactivity of test serum.
  3. Reagent red cells may lose antigen strength on storage. Other subtle cell changes may cause loss of reactivity.
- *Improper procedures*
  1. Overcentrifugation may pack cells so tightly that agitation required to resuspend cells breaks up agglutinates. Undercentrifugation may not be optimal for agglutination.
  2. Failure to add test serum, enhancement medium or AHG may cause negative test.
  3. Too heavy a red cell concentration may mask weak agglutination. Too light suspension may be difficult to read.
  4. Improper/insufficient serum: Cell ratios.
- *Complement*
  1. Rare antibodies may only be detected when poly-specific AHG is used and active complement is present.
- *Saline*
  1. Low pH of saline solution can decrease sensitivity. Optimal saline wash solution for most antibodies is pH 7.0 to 7.2.

2. Some antibodies may require saline to be at specific temperature to retain antibody on cell. Use 37 or 4°C saline.

False Positive Results

- *Cells agglutinated prior to washing*
  1. If potent agglutinins are present, agglutinates may not disperse during washing. Observe cells prior to addition of anti-human globulin (AHG) or use control tube substituting saline for AHG; reactivity prior to addition of AHG or in saline control invalidates AHG reading.
- *Particles or contaminants*
  1. Dust or dirt in glassware may cause clumping (not agglutination) of red cells. Fibrin or precipitates in test serum may similarly produce cell clumps that mimic agglutination.
- *Improper Procedures*
  1. Overcentrifugation may pack cells so tightly that they do not easily disperse and appear positive.
  2. Centrifugation of test with polyethylene glycol or positively charged polymers prior to washing may create clumps that do not disperse.
- *Cells have positive direct antiglobulin test (DAT)*
  1. Cells that are positive by DAT will also be positive in any indirect antiglobulin test.
- *Complement*
  1. Complement components, primarily C_4 may bind to cells from clots or from CPDA-1 donor segments during storage at 4°C and occasionally at higher temperatures. For DATs, use red cells anticoagulated with EDTA, ACD or CPD.
  2. Samples collected in tubes containing silicone gel may have spurious complement attachment.
  3. Complement may attach to cells in specimens collected from infusion lines used to administer dextrose-containing solutions. Strongest reactions are seen when large-bore needles are used to when sample volume is less than 5 μl.

Anti-human Globulin (AHG or Coombs Reagent)

Problem: False positive results

Possible causes		*Solutions*
1.	Presence of colloidal silica, which is absorbed by the red cells when saline is stored in glass bottles	Ensure that all glassware used is clean and dry and properly stored saline is being used for the test. Use freshly prepared normal saline
2.	Red cells may be agglutinated before the washing is carried out	Check properly for agglutination before proceeding to the antiglobulin phase
3.	Overcentrifugation causes tight packing of the cells that cannot be dispersed easily and is mistaken for a positive control	Ensure that centrifugation is carried out at the proper speed for the appropriate time as per the instructions given in the package insert. Each laboratory must calibrate its equipment at regular intervals
4.	Absorption of normal cold antibody and complement onto the cells during the refrigeration of clotted blood sample can give false positive results. Anticoagulants have anti-complement activity. Refrigerated ACD blood also gives false positive results at times	The blood sample should be tested as soon as possible after collection and should not be stored. For the indirect antiglobulin test, serum not more than 48 hours old should not be used
5.	Use of various drugs and certain disease conditions such as megaloblastic anemia are known to be associated with positive direct antiglobulin test	Check the patient's history for disease conditions like megaloblastic anemia
6.	In diseases such as pernicious anemia and multiple myelomatosis, autoagglutination takes place (all erythrocytes are agglutinated non-specifically)	Check the patient's history for the presence of such disorders
7.	False positive Coombs test is seen in blood with high reticulocyte count	Check the history of the patient for high reticulocyte count

Problem: False Negative Results

Possible causes		*Solutions*
1.	Insufficient washing of the sample may lead to neutralization of anti-human globulin by the globulin fraction of the serum	Washing must be carried out thoroughly and for the number of times as mentioned in the pack insert to ensure complete removal of free IgG from the sample
2.	Serum residues remaining in the poorly washed glassware can cause neutralization of anti-human globulin	Only clean and dry glassware must be used for testing
3.	Anti-human globulin reagent not working	To all negative test results, after the antiglobulin phase Coombs control cells should be added. If the Coombs control cells do not agglutinate then the test should be repeated using fresh anti-human globulin
4.	Contaminated bovine serum albumin may inactivate antihuman globulin	Ensure that the bovine serum albumin, saline and glassware are free from contamination
5.	Anti-human globulin may be neutralized with human globulin	Check the Anti-IgG of the human globulin reagent using Coomb's control cells
6.	Interrupted or delayed testing. Too much time has elapsed between washing the erythrocytes and adding anti-human globulin reagent, so that antibodies have eluted. The Coombs serum has in fact reacted with the antibody but agglutination is not visible, since the antibodies are no longer bound to the erythrocytes	The washing should be undertaken as quickly as possible to minimize the elution of antibody from the cells Addition of the anti-human globulin followed by centrifugation and reading of results should be in immediate succession
7.	If plasma is used in the indirect antiglobulin test, the complement dependant antibodies may not be detected due to the absence of calcium	Do not use plasma. Serum not more than 48 hours old should be used for the indirect antiglobulin test
8.	Improper centrifugation	Under centrifugation leads to false negative results. Resuspension of centrifuged cells vigorously breaks up weak agglutination leading to false negative results. Centrifugation should be carried out at the appropriate speed for an amount of time as given in package insert
9.	If the red cells are few the reaction is difficult to read	Ensure that 5% suspension of red cells is used in the tests as per the instructions given in the package insert
10.	Omission of anti-human globulin in the test by mistake	Ensure that all the reagents are added properly as per the instructions given in the pack insert. Eryclone AHG offers the advantage of being color coded so that it helps in identification
11.	The incubation temperature was not the optimal one for the antibodies. It was either too high or too low, so that the antibody coating of the erythrocytes did not occur	Incubate at the optimal temperatures as per the instructions given in the pack insert
12.	Only one drop of anti-human globulin reagent may have been used for the D^u test. If one drop is used then residual anti-D from previous incubation or excess wash solution may neutralize/dilute the reagent affecting its reactivity and giving rise to false negative reactions	Ensure that two drop of anti-human globulin reagent are used for the D^u test
13.	Anti-human globulin reagent has deteriorated	Confirm the results using Coomb's control cells
14.	Cord cells sensitized heavily with anti-D yield a false positive result in direct antiglobulin test	Check the cells for sensitization before testing

Anti-A_1 Lectin

Problem: False positive results

Possible causes		*Solutions*
1.	Bacterial contamination (some bacteria will agglutinate all red cell samples thereby giving false positive results)	Check the reagents for turbidity. Extreme turbidity may indicate bacterial contamination. Such contaminated reagents should not be used for testing
2.	Particles of dust, debris, chemicals or detergents on the slide or in the tube giving non-specific agglutination	Clean and dry glassware should be used while carrying out the test
3.	Peripheral drying or fibrin strands were mistaken for agglutination in case of slide test	The test should not be carried out directly under the fan. The vials should be capped immediately after use. The results of the test should be read at one minute and not beyond as drying may be interpreted as positive result
4.	Excessive centrifugation in case of tube test	Ensure that centrifugation is carried out for either one-minute at 1000 rpm or 20 seconds at 3400 rpm. Centrifugation should be adequate to produce a cell button with a clear supernatant but without packing the cells so tightly that they are difficult to dislodge. Each laboratory must alibrate its equipment at regular intervals
Problem: Hemolysis of red blood cells		
Possible causes		*Solutions*
1.	Use of wet slides and tubes	Wet glassware can cause hemolysis of RBC's. Ensure that only dry glassware is used for testing
Problem: False negative results		
Possible causes		*Solutions*
1.	Whole blood red cells used directly for the slide test	10% red cell suspension should be used for the slide test as mentioned in the pack insert.
2.	Storage of antiseras at higher/lower temperatures than specified	Reagents should be stored at 2–8°C when not in use Thermal damage due to faulty storage may result in a loss of reactivity Antibody activity decreases at lower temperatures Do not freeze the reagents
3.	Blood sample stored for too long	Check the period of time for which the blood sample has been stored Anticoagulated blood using various anticoagulants should be tested within the mentioned time period (when stored at 2–8°C) • EDTA or heparin—2 days • Sodium oxalate or sodium citrate—14 days • ACD or CPD—28 days • Clotted whole blood—14 days
4.	Use of hemolyzed samples	Check the samples before use. Do not use hemolyzed samples
5.	Sample used for newborns below one year of age	A_1 antigen is not fully expressed on the red blood cells of newborns below one year of age
6.	Prozoning (zone of antibody excess) or postzoning (zone of antigen excess)	The antigen and the antibody should be present in optimal concentrations of the agglutination to be seen properly. Check the sample volume and the reagent volume used. Both the sample and reagent volume should be equal in slide test and a 5% suspension of cells should be used in tube test. Ensure that there are no air bubbles while dispensing samples and reagents

Anti-H Lectin

Problem: False positive results

Possible causes		*Solutions*
1.	Bacterial contamination (some bacteria will agglutinate all red cell samples thereby giving false positive results)	Check the reagents for turbidity. Extreme turbidity may indicate bacterial contamination. Such contaminated reagents should not be used for testing
2.	Particles of dust, debris, chemicals or detergents on the slide or in the tube giving non-specific agglutination	Clean and dry glassware should be used while carrying out the test
3.	Peripheral drying or fibrin strands were mistaken for agglutination in case of slide test	The test should not be carried out directly under the fan. The vials should be capped immediately after use. The results of the test should be read at 2 minutes not beyond as drying may be interpreted as positive result
4.	Excessive centrifugation in case of tube test	Ensure that centrifugation is carried out for either one-minute at 1000 rpm or 20 seconds at 3400 rpm. Centrifugation should be adequate to produce a cell button with a clear supernatant but without packing the cells so tightly that they are difficult to dislodge Each laboratory must calibrate it equipment at regular intervals
Problem: Hemolysis of red blood cells		
	Possible causes	*Solutions*
1.	Use of wet slides and tubes	Wet glassware can cause hemolysis of RBC's. Ensure that only dry glassware is used for testing
Problem: False negative results		
Possible cause		*Solution*
1.	Storage of antiseras at higher/lower temperatures than specified	Reagents should be stored at 2–8°C when not in use Thermal damage due to faulty storage may result in a loss of reactivity Antibody activity decreases at lower temperatures Do not freeze the reagents
2.	Blood sample stored for too long	Check the period of time for which the blood sample has been stored Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period (when stored at 2–8°C) • EDTA or heparin—2 days • Sodium oxalate or sodium citrate—14 days • ACD or CPD—28 days • Clotted whole blood—14 days
3.	Use of hemolysed samples	Check the samples before use. Do not use hemolyzed samples
4.	Prozoning (zone of antibody excess) or postzoning (zone of antigen excess)	The antigen and the antibody should be present in optimal concentrations of the agglutination to be seen properly Check the sample volume and the reagent volume used. Both the sample and reagent volume should be equal in slide test and a 5% suspension of cells should be used in tube test. Ensure that there are no air bubbles while dispensing samples and reagents

CHAPTER 12

Cerebrospinal and Other Body Fluids

CEREBROSPINAL FLUID

Cerebrospinal fluid (CSF) is formed primarily in ventricular choroid plexuses by a combination of both, active process and ultracentrifugation. Concentrations of sodium, chloride, magnesium and glutamine are greater in CSF than in plasma, while concentrations of glucose, potassium, calcium, cholesterol, uric acid, iron, thyroxine and zinc are lower in CSF.

Normal Values for Lumbar CSF in Adults

Pressure	70–150 mm of water column (patient lying on side)
Volume	90–150 mL
Specific gravity	1.006–1.008
Total solids	0.85–1.70 g%
Cells	0–8 lymphocytes/cu mm Neutrophils and erythrocytes absent
Protein	20–50 mg%
Of this	
albumin is	50–70%
α_1 globulin is	3–9%
α_2 globulin is	4–10%
β globulin is	10–18%
γ globulin is	3–9%
fibrinogen is	Absent
Sodium	144–154 mEq/L
Potassium	2.0–3.5 mEq/L
Chloride	118–132 mEq/L
pH	7.3–7.4
Creatinine	0.5–1.2 mg%
Cholesterol	0.2–0.6 mg%
Glucose	50–80 mg%
Glutamine	6–16 mg%
Iron	1–2 mg%
Thyroxine	0.1–0.2 mg%
Urea	6–16 mg%
Uric acid	0.5–4.5 mg%

Lumbar Puncture

Lumbar puncture needle is a long needle with a stylette inside. Lumbar puncture is usually performed at L_3-L_4 or lower to avoid damage to the spinal cord. In small children the conus medullaris extends lower than in adults, so puncture should be performed at L_4-L_5 or lower.

Indications

1. Detection and diagnosis of suspected meningitis, subarachnoid hemorrhage, encephalitis, central nervous system (CNS) syphilis, spinal cord tumor or multiple sclerosis.
2. Differential diagnosis of cerebral infarction *vs* intracerebral hemorrhage (almost 80% of latter show blood or xanthochromia).
3. Introduction of anesthetics, radiographic contrast media or drugs.
4. Treatment of elevated CSF pressure in selected patients with benign intracranial hypertension.
5. Removal of exudate or blood from subarachnoid space.

The procedure should be done with a stylette inside to avoid implantation of skin, which may form dermoid cyst in the spinal canal. A manometer and three-way stopcock should be attached to the needle, so that initial pressure can be accurately measured and CSF removed under control.

Complications of Lumbar Puncture

1. Production of cerebellar pressure cone in patients with increased intracranial pressure.
2. With spinal cord tumor, progression of paresis to paralysis may follow lumbar puncture.
3. Introduction of infection by:
   - Passing the needle through superficial or deep sepsis in the lumbar region.
   - Improperly sterilized equipment.
   - Poor technique.
   - Development of dermoid cyst if lumbar puncture is performed without the stylette.
   - Postpuncture headache resulting from leakage of CSF (incidence can be decreased by using a small bore needle and keeping the patient horizontal for 24 hours).
4. In infants death due to asphyxiation caused by restraint or tracheal obstruction from pushing the head forward.

Elective lumbar puncture should be performed in the morning rather than late afternoon or evening.

CSF Rhinorrhea and Otorrhea

Fluid coming through ear or nose may be CSF. Confirmation can be done by inserting a glucose-oxidase strip into nose or ear for 5 minutes: a 2+ or 3+ reaction indicates glucose present, is considered evidence of CSF rhinorrhea or otorrhea (30% false positives). Normal concentration of glucose in nasal secretions is 10 to 25 mg%. If the test is inconclusive, cotton may be placed in the nasopharynx and radioactive iodinated serum albumin (RISA) injected into the lumbar subarachnoid space, the cotton is left in place for 12 hours and then counted for gamma radiation.

CSF Pressure

If the opening pressure exceeds 180, reassure the patient and straighten the leg, back and neck and to make sure there is no breath holding or abdominal or jugular compression. If the pressure then falls to normal, it is probable that the initial elevation was artefactual. CSF pressure is directly related to pressure in the jugular and vertebral veins, which communicate with the intracranial dural sinuses and spinal dura. Hence, CSF pressure is decreased with circulatory collapse and increased with congestive heart failure, obstruction of the superior vena cava, straining, breath holding, or pressure against the abdomen (e.g. obese patients in lying position).

Pathologically Increased Pressure is Usually Due to:

- Inflammation of the meninges
- A space-occupying lesion (SOL), such as a tumor, abscess, cerebral-edema or intra-cerebral hemorrhage.

If the Initial Pressure Exceeds 200 mm. CSF, not more than 1–2 mL of fluid should be removed, a 25 to 50% fall in pressure after removing 1–2 mL suggests cerebellar herniation or spinal cord compression above the puncture site. STOP removing CSF. Observe the patient for several hours. Usually, three 2 mL samples are taken in sterile tubes and labeled sequentially.

If Initial Pressure is Normal and there is Clinical Suspicion of Subarachnoid Block, Queckenstedt's Test may be done: Normally if both jugular veins are manually compressed, CSF pressure rapidly returns to normal when compression ceases. With sinus thrombosis, obstruction at the foramen magnum, or a mass lesion in the spinal canal, the rise of CSF pressure may be decreased/delayed—this is a positive Queckenstedt's test. In such cases, normal variations in pressure due to respiration will be diminished/absent but straining or abdominal compression should result in increased CSF pressure (due to vertebral vein congestion) if the needle is placed correctly.

Almost 80% of patients with cord compression have a positive Queckenstedt's test. Lesions responsible for cord compression include:

- Herniated intervertebral disc
- Vertebral fracture
- Extradural abscess
- Adhesions due to pachymeningitis
- Neoplasms (primary or metastatic) involving vertebrae, meninges, or spinal cord.

Gross Examination

Normal CSF is crystal clear.

Evaluate color by holding the CSF tube beside a distilled water tube against a clean white paper. If a pale yellow or pink color is noted—centrifuge the sample at high speed for 5 minutes and examine the supernatant visually. Xanthochromia (pale pink to pale orange or yellow color in supernatant) is usually graded from 1 to 4 and may be due to:

- CSF protein > 100 mg%
- Traumatic tap with lysis of erythrocytes due to detergent in needle or sample tube
- Bilirubinemia (both conjugated bilirubin in adults and unconjugated bilirubin in neonates may pass the blood-CSF barrier)
- Intracerebral or subarachnoid hemorrhage
- Contamination of CSF by iodine/merthiolate used to disinfect the skin
- Carotenemia
- Melanin in CSF due to meningeal melanosarcoma.

Two to twelve hours after a subarachnoid hemorrhage, pale orange xanthochromia appears in CSF in 90% cases. Yellow xanthochromia due to conversion of hemoglobin to bilirubin within 2 to 4 days. The orange xanthochromia of oxyhemoglobin usually disappears in 4 to 8 days, while yellow xanthochromia due to bilirubin typically persists for 12–40 days. Gross blood due to subarachnoid hemorrhage may disappear within 24 hours, but generally persists for 7–14 days.

Turbidity in CSF may result from large numbers of leukocytes or bacteria and varies from slight opalescence typical in tuberculous meningitis to the grossly purulent appearance in some cases of pyogenic meningitis. Turbidity is usually graded from 0 (crystal clear) to 4+ (newsprint cannot be seen though the tube).

Clotting in CSF may be seen Grossly in:
- Traumatic tap
- Markedly elevated CSF protein or
- Moderately elevated CSF protein in association with tuberculous meningitis (cobweb coagulum).

Cell Counts

Diluting the Fluid

1. Draw Unna's polychrome methylene blue to the '1' mark in a RBC pipette and fill pipette to '101' mark with spinal fluid. This colors white cells blue and red cells yellow.
2. Turbid fluid: When many cells are present (as in turbid or purulent fluid), better counts are obtained with a WBC pipette and WBC diluting fluid.
3. Bloody fluid: When significant numbers of red cells are present in the fluid, the possiblity of traumatic bleeding should be considered. Fresh RBCs are intact with a smooth round margin. Older cells have crenated appearance.

Count

Count 9 large squares in the counting chamber for both RBCs and WBCs—the total multiplied by 1.1 gives the number of cells per cubic mm.

Differential count: Centrifuge the CSF and make smears from the sediment. Stain and count as for a blood smear.

Various Types of Cells in CSF

Large number of polymorphs → pyogenic meningitis due to:
- *Neisseria meningitidis*
- *Haemophilus influenzae*
- Pneumococci
- Streptococci
- Staphylococci
- Coliforms.
- Sometimes in viral meningitis or aseptic meningeal reaction
- Rarely in intracerebral hematoma, fungal meningitis, RISA injection, or following lumbar puncture with detergent-contaminated needles.

Mixed Reaction (Neutrophils, Lymphocytes and Monocytes) Occurs in:
- Subacute bacterial meningitis
- Tuberculous meningitis
- Mycotic meningitis
- Viral meningoencephalitis.

Monocytic and/or Lymphocytic Reaction is Seen in:
- Viral meningoencephalitis
- Multiple sclerosis
- Tuberculous
- Fungal
- Syphilitic

} meningitis

Blasts may be seen in leukemic cell infiltrates in the meninges.

Abnormal malignant/benign cells in certain CNS neoplasms.

Globulin Test

These are valueless if the spinal fluid is bloody.

1. *Pandy's test*: Place 1–2 mL of a saturated solution of phenol in a small test tube and add 1 drop of spinal fluid. Cloudiness against a black background indicates increased amounts of globulin. Report as 0, +, ++, +++ or ++++.
2. *Ross Jones test*: Carefully layer 0.5 mL clear spinal fluid over 1 mL of saturated solution of ammonium sulfate. A thin white ring appearing at the juncture of the liquids, which disappears on mixing, indicates a 1+ reaction. Heavy cloudiness persisting after mixing is a 4+ reaction.

Total proteins (Quantitative method of Dennis and Ayer): Place 1.2 mL of clear spinal fluid, 0.8 mL of distilled water, and 2 mL of 5% sulfosalicylic acid in a small test tube and mix by inversion. Let stand for 5 minutes, then read in a colorimeter against a known standard protein suspension (make known suspension by mixing 2 mL of a standard protein solution with 2 mL of 5% sulfosalicylic acid). If the unknown is too heavy with protein, dilute and compare. Consider dilution factor in the calculation.

$$\frac{\text{Transmission standard}}{\text{Transmission unknown}} \times 50 = \text{mg of protein/100 mL of fluid}$$

Nowadays microprotein colorimetric biochemistry kits are available for quicker and accurate analysis.

CSF electrophoresis shows prodominantly albumin.

In adults, levels of 60–75 mg% are considered slightly increased, levels of 75–150 mg% moderately increased and beyond 150 mg% are markedly increased.

Increase in CSF protein may occur with any lesion causing injury to cerebral tissue or blood-brain barrier; viral, tuberculous, mycotic, syphilitic or bacterial meningoencephalitis, polyneuritis, intracerebral hemorrhage, degenerative disease, or aseptic meningeal reaction. Brain tumors cause variable increases, depending upon their location—gliomas deep in the pons or cerebrum may be associated with normal levels, while acoustic neuromas in tumors of the corpus callosum usually cause marked increase in CSF protein. Other causes include diabetic neuropathy, myxedema, heavy metal intoxication, isopropanol intoxication, hypercalcemia and diphenylhydantoin (Dilantin) intoxication. Multiple sclerosis causes minimal increase in CSF protein; whereas, cerebral thrombosis, subdural hematoma and aseptic and viral meningitis usually are associated with normal CSF protein.

Conditions that Elevate CSF Protein

Mild Elevation, to 300 mg%

Viral meningitis, neurosyphilis, subdural hematoma, cerebral thrombosis, brain tumor, multiple sclerosis (rarely > 100 mg%).

Electrophoretic Evidence of IgG

Multiple sclerosis, subacute sclerosing panencephalitis, neurosyphilis.

Moderate or Pronounced Elevation

Acute bacterial meningitis, tuberculous meningitis, spinal cord tumor, cerebral hemorrhage, intracranial tumor, Guillain-Barré syndrome (ascending polyneuritis).

The term albuminocytologic dissociation refers to increased CSF protein with normal or near normal CSF cell count—classically seen in Guillain-Barré syndrome, but it may also occur in subarachnoid block, brain tumor, multiple sclerosis, cerebral thrombosis and various types of polyneuritis.

Froin's syndrome refers to CSF changes, which may occur with subarachnoid block at or below the foramen magnum—markedly increased total protein (often > 500 mg%), xanthochromia (owing to increased protein) and spontaneous clotting.

Protein Electrophoresis of CSF

Proteins larger than 9S (sedimentation coefficient) do not diffuse rapidly into the CSF, unless there is injury to the blood-CSF barrier.

Monoclonal increase can be shown in some myeloma cases. Multiple sclerosis causes increased IgG in CSF.

Besides multiple sclerosis, diffuse or discrete pathologic gamma bands can be seen in:

- Subacute sclerosing leukoencephalitis
- Advanced neurosyphilis
- Primary lateral sclerosis
- Viral encephalitis
- Fungal meningitis
- Monoclonal gammopathy
- Myelopathy due to vitamin B_{12} deficiency.

Lange's Colloidal Gold Test

Colloidal Gold Test

Though obsolete, it can be of great use if electrophoresis cannot be done. It is an empirical way of evaluating CSF protein fractions.

In the Lange's method, progressive dilutions of CSF are added to 10 test tubes containing colloidal gold solution (Fig. 12.1). Precipitation causes the brilliant red colloidal gold color (0) to change to:♥

Reddish blue	(1+)
Purple	(2+)
Deep blue	(3+)
Pale blue	(4+)
Colorless	(5+)

The highest CSF concentration is reported on the left with progressively decreasing concentrations to the right. Normal fluids cause either no reaction or slight precipitation in the middle dilutions, e.g. 0001210000.

A first zone curve is found in about 50% of patients with multiple sclerosis as well as in general paresis of insane. It may also be seen in encephalitis, postinfections, encephalomyelopathy, sarcoidosis, hemorrhage, aseptic meningeal reaction, polyneuritis, and meningeal carcinoma. A typical series would be 5554210000 (Fig. 12.1).

A midzone curve or endzone curve is nonspecific and may be found in any CSF with high protein content.

Glucose

Normally, CSF glucose is about 60–80% of corresponding blood levels, or 50–80 mg%. Usually CSF glucose less than 40 mg% is considered decreased. Possible causes include:

- Systemic hypoglycemia
- Bacterial/tuberculous/fungal meningitis
- Meningeal carcinomatosis/leukemia infiltration
- Sarcoidosis involving CNS
- Subarachnoid hemorrhage
- Viral meningitis including mumps meningoencephalitis.

Conditions that Effect the CSF Glucose

No significant change.

Viral meningitis, neurosyphilis, brain or cord tumor, cerebral thrombosis, multiple sclerosis, polyneuritis.

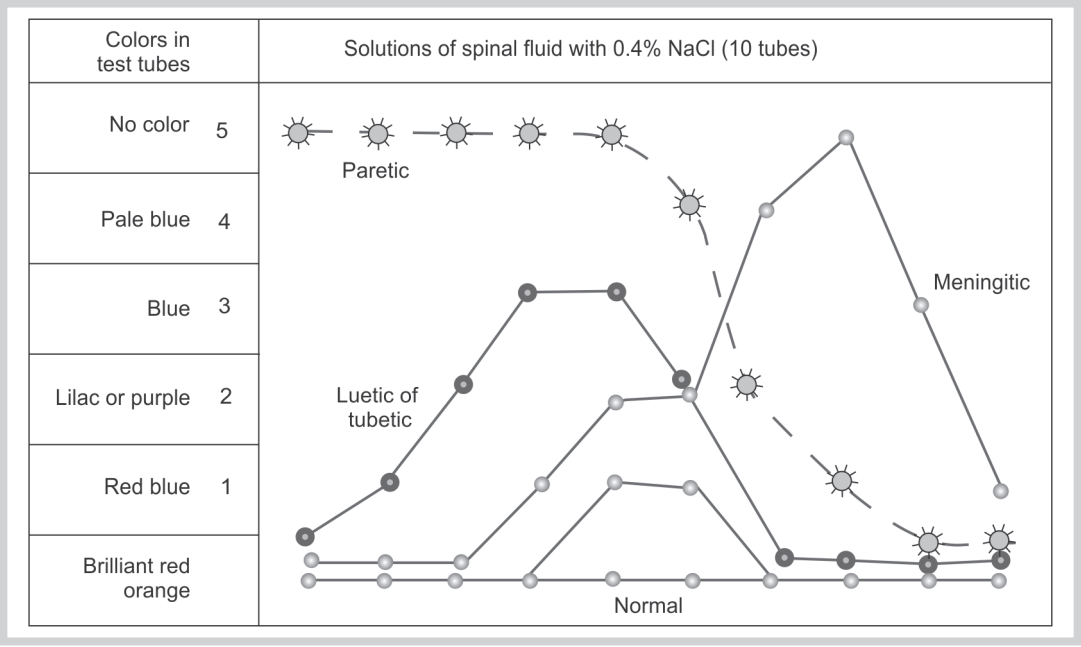


FIG. 12.1: Lange's colloidal gold test

Moderate reduction
CNS leukemia, meningeal carcinomatosis, subarachnoid hemorrhage, partially treated bacterial or fungal meningitis (CSF lactate will be high).

Marked reduction
Bacterial/tuberculous/fungal meningitis.

Enzymes

LDH
Lactate dehydrogenase is increased in CSF in:
- Bacterial, Viral } meningitis
- Subarachnoid hemorrhage
- Primary/secondary malignancy of brain/cord.

SGOT
Aspartate transferase (AT) is raised in CSF in:
- Primary/secondary malignancy of brain/cord
- Bacterial meningitis
- Intracerebral hemorrhage
- Subarachnoid hemorrhage.

CPK
Raised CSF-CPK (creatine phosphokinase) is found in:
- Brain infarct
- Multiple sclerosis
- Brain tumors
- Demyelinating disease, and
- Polyneuropathies.

Bacteriologic Examination

Smears and cultures for bacteria should be made of all fluids when indicated.

1. **Smears:** Make smears directly if fluid is very turbid, otherwise from sediment of centrifuged CSF. All smears should be stained with Gram's stain. If no characteristic bacteria are found, do an acid-fast stain and search for mycobacteria (AFB) stain should also be done if a pellicle forms on standing. An India ink preparation is required for *Cryptococcus*. Immunofluorescent stains can be used for *Haemophilus influenzae* and some other organisms.
2. **Cultures:** Cloudy fluid should be streaked on chocolate agar, Sabouraud's agar, and agar plates and inoculated into blood broth and thioglycollate medium. All media are incubated at 37°C, some in candle jars (for CO_2 atmosphere). Sediment of centrifuged fluid should be cultured on special media for tubercle bacilli and fungi and inoculated into guinea pigs. Mice should be inoculated intraperitoneally if coccidioidomycosis is suspected.
3. **Virus isolation:** This is possible only in very sophisticated laboratories and is helpful in aseptic meningitis and arthropod-borne encephalitis.

Serologic Tests

One can do:
- VDRL using CSF in syphilis.

TABLE 12.1: CSF in differential diagnosis

Disease	*Initial pressure mm H_2O column*	*Appearance*	*Cells/cu mm*	*Protein mg%*	*Glucose mg%*	*Colloidal gold*	*Remarks*
Normal	70–150	Crystal clear	0–8, lympho's	20–50	50–80	0000110000	In fasting afebrile individuals
Acute purulent meningitis	↑	Opalescent to purulent clot	500–20,000 mostly poly's	50–1000+	0–45	Variable	Organism in sediment or clot, culture positive
Tuberculous meningitis	↑	Opalescent fibrin web, pellicle	10–500 mostly lympho's	45–500+	0–45	Variable	Sugar and chloride values falling progressively
Early, acute syphilitic meningitis	↑	Clear to turbid, occasional clot	25–2000 mostly lympho's	45–400+	15–75	Ist/midzone curve	Often +ve serologic test in CSF and blood
Late CNS syphilis	↑	Normal	Normal or ↑	Normal or ↑	Normal	Depending on activity	Often +ve serologic test in CSF
Aseptic meningeal reaction (brain or extradural abscess, thrombosis, etc.)	Usually normal	Clear or turbid, often xanthochromic	↑	Normal or ↑	Normal	Variable	CSF culture negative
Acute poliomyelitis	Usually normal	Usually clear and colorless	↑	↑	45–100	Normal or midzone	
Viral encephalitis (arthropod borne)	Normal or ↑	Normal	0–100, mostly	Normal or increased	45–100	Variable	Proved by serologic tests
Viral meningo-encephalitis	Normal or ↑	Normal	0–2000 + mostly lympho's	Normal or ↑	Normal	Variable	Proved by virus isolation and serologic tests
Postinfectious encephalitis	Usually ↑	Normal	Slightly ↑	Normal or increased	Increased	Variable	
Traumatic (bloody) tap	Normal	Bloody	Many fresh RBCs	↑	Normal	Normal	Most blood in Ist tube, least blood in last tube
Cerebral hemorrhage: ventricular, subarachnoid	Slightly ↑	Bloody, supernatant yellow	Many RBC's crenated or fresh	↑	Variable	Normal	Blood present in all specimens equally
Subdural hematoma	Usually ↑	Clear/yellow	Normal	Normal or ↑	Variable	Normal	
Brain tumor	Usually ↑	Clear/xan-thochromic	Normal or increased	Usually ↑	Normal or increased	Variable	If papilledema is present lumbar puncture is contraindicated
Spinal cord tumor (Subarachnoid block)	Normal or low	Often xan-thochromic	Normal or ↑	Usually ↑	Normal or increased	Variable	Little fluid obtained
Multiple sclerosis	Low	Normal	Normal or increased	Normal or increased	Normal	Normal, Ist or midzone	50% cases have normal CSF
Uremia	Usually	Normal	Normal or ↑	Normal or ↑	Normal or ↑	Variable	CSF NPN is high
Diabetic coma	Low	Normal	Normal or ↑	Normal	Increased	Normal	May have spasticity, weakness, convulsions

TABLE 12.2: Grades of syphilitic spinal fluid

Investigation	*Grade I*	*Grade II*	*Grade III*
Serology	—	±	++
Number of WBCs/cu mm	5–25	25–100	70–100
Protein increase	+	++	+++
Colloidal gold curve	0000000000	0023454310	5555554310

- Latex agglutination and complement fixation tests in cryptococcal meningitis.

SYNOVIAL FLUID (SF)

Normally, about 1 mL of SF is present in each large joint: knee, ankle, hip, elbow, wrist, and shoulder (Table 12.3).

Clinical Indications for Aspiration

- Arthritis of unknown etiology, manifested by effusion.
- Possible infectious arthritis, with or without effusion, to obtain material for culture.
- Effusions of known etiology, to relieve pain or to allow mobility.

Aspiration must be done under absolute aseptic conditions. Since effusion often exists when aspiration is indicated, 10–20 mL of fluid may usually be obtained. The specimen is collected in 3 to 4 sterile tubes.

1. Plain tube for gross examination, evaluation of viscosity, and mucin clot test.
2. EDTA tube for cell counts and microscopic study.
3. A sterile, plain or preferably heparinized tube (precludes clot formation) for microbiologic study.
4. Appropriate tube(s) for serologic or chemical examinations: Plain tube for serologic tests or enzyme assays, heparinized tube for total protein, oxalate fluoride tube for glucose.

Viscosity

When normal fluid drips from a syringe, a tenacious 'string' at least 4 cm long forms with each drop. This provides an estimate of whether viscosity is normal, decreased (string less than 4 cm in length), or markedly decreased (string less than 1 cm in length).

Another method for evaluating viscosity is to see how far a drop of fluid can be stretched between the thumb and index finger before breaking: fluids with very low viscosity will behave like water. Decreased viscosity reflects decreased hyaluronate in the synovial fluid.

TABLE 12.3: Presence of synovial fluid

Synovial fluid	*Findings*	*SI units parameter*
Appearance	Clear or colorless to pale yellow	
Crystals	Absent	
Glucose		
Transudate	≤ 10 mg/dL lower than blood glucose (whole blood adult normal 60–89 mg/dL, child norm 51–85 mg/dL)	
Exudate	Lower than whole blood levels	
Lactate dehydrogenase		
Transudate	≤ Client's serum LD (serum adult normal 45–90 U/L, child normal 60–170 U/L)	
Exudate	> Client's serum LD	
pH	7.4	
Specific gravity		
Transudate	< 1.016	< 1.016
Exudate	> 1.016	> 1.016
Total protein		
Transudate	1–3 g/dL	10–30 g/L
Exudate	> 3 g/dL	> 30 g/L
Volume	< 4 mL	
Viscosity	High	
White blood cells		
Transudate	< 100/mm^3	< 100 × 10^9/L
Exudate	> 1000/mm^3	> 1000 × 10^9/L

Mucin Clot Test (Ropes' Test)

This is done by adding 1 mL of synovial fluid to 20 mL of 5% (v/v) acetic acid in a small breaker. Normally, a compact

large clot will form, surrounded by clear solution, this is graded as 'good'. If a soft clot forms in a turbid solution, this is graded as fair. A friable clot with cloudy surrounding fluid is graded as 'poor' or 'fragile'. No clot formation, with flakes in a cloudy suspension, is graded as 'very poor'. Good clots do not break up when agitated, while poor clots break up into small shreds. This procedure actually is an estimate of synovial hyaluronate and not mucin, which is absent in joint fluid.

Microscopic Examination

Total and differential counts as for CSF. But the usual leukocyte diluent with 1% glacial acetic acid precipitates synovial fluid hyaluronate and is unsatisfactory, instead methylene blue in saline can be used. If the fluid is very turbid, use saline dilution or digestion with hyaluronidase (2 mL SF incubated with 150 IU hyaluronidase for 1 hour at 37°C) may be helpful. Differential count can be done from EDTA sample (sediment) that has been centrifuged, a film made and stained as for peripheral blood.

LE cells are frequently seen in stained SF from patients with systemic lupus erythematosus (SLE). Sometimes, they can be seen in cases of rheumatoid arthritis. Large phagocytes containing neutrophils may be found in SF and are called 'Reiter cells', they are nonspecific and may be present in effusions of varying etiology. RA cells or 'Ragocytes' are neutrophils containing 0.5 μ to 1.5 μ inclusions better seen with phase contrast microscopy. They are seen in 94% cases of rheumatoid joint fluids but are nonspecific for they can also be found in septic arthritis, gout, etc.

Both wet preparation (a drop of SF put on a slide and coverslipped) and stained films should be studied for crystals, using polarized light to detect monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD). MSU crystals appear birefringent and needle or rod shaped; while CPPD crystals will appear birefringent and rhomboid or rod shaped. MSU crystals are found in acute/chronic gout joints. CPPD crystals are found in pseudogout or chondrocalcinosis.

Immunologic Studies

Seronegative rheumatoid arthritis may have a positive joint fluid, but this is not very specific. Decreased synovial fluid complement (under 30% of serum level) occurs in rheumatoid arthritis and SLE (Table 12.4).

PLEURAL FLUID

The pleural surfaces are normally moistened by 1 to 10 mL of fluid derived by ultrafiltration of plasma. Normal protein concentration of this fluid is 1–2 g% with no fibrinogen (Table 12.5).

TABLE 12.4: Synovial analysis in arthritis

	Appearance	*Viscosity*	*White cells*	*Mucin clot*	*Protein total*	*(Avg-g%) Globulin*	*Remarks*
Normal	Straw-colored,	High	200–600/25% poly's	Good	1.36	0.05	
	clear, cloudy						
Traumatic	Yellow to bloody	High	± 2000/30% poly's	Good	4.27		
Osteoarthritis	Yellow, clear	High	± 1000/20% poly's	Good	3.08	0.75	Cartilage fibrils
Rheumatic	Yellow, slightly cloudy	Low	± 10,000/50% poly's	Good	3.74	1.07	
Systemic lupus	Straw-colored,	High	± 5000/10% poly's	Good			
erythematosus	slightly cloudy						
Gout	Yellow to milky cloudy	Low	± 12,000/60% poly's	Fragile	4.18	1.54	Urate crystals
Tuberculous	Yellow, cloudy	Low	± 25,000	Fragile	5.3	2.0	Tubercle
arthritis			50–60% poly's				bacilli
Septic arthritis	Grayish or bloody,	Low	± 80,000/90% poly's	Fragile	5.64	2.45	Bacteria
	turbid						
Rheumatoid	Yellow to greenish,	Low	± 15,000	Fragile	4.74	1.79	Rheumatoid
arthritis	cloudy		65% ± poly's				factor

TABLE 12.5: Presence of pleural fluid

Pleural fluid	*Observation*	*SI units parameter*
Appearance	Clear, slightly amber	
Cholesterol		
Transudate	< 60 mg/dL	< 1. 55 mmol/L
Exsudate	> 60 mg/dL	> 1. 55 mmol/L
Glucose		
Transudate	Approximates whole blood levels (whole blood adult normal 60–89 mg/dL, child normal 51–85 mg/dL)	
Exudate	Lower than whole blood levels	
Lactate dehydrogenase		
Transudate	< Client's serum LD (serum adult norm 45–90 U/L, child normal 60–170 U/L)	
Exudate	> Client's serum LD	
pH	7.4	
Specific gravity		
Transudate	< 1.016	< 1.016
Exudate	> 1.016	> 1.016
Total protein		
Transudate	< 2.5 g/dL	<25 g/L
Exudate	> 3 g/dL	> 30 g/L
Volume	< 25 mL	
White blood cells		
Transudate	< 100/mm^3	< 100 × 010^9/L
Exudate	> 1000/mm^3	> 1000 × 10^9/L

Abnormal Pleural Fluid Accumulation, or Pleural Effusion may be Caused by

1. Increased capillary permeability due to inflammation; this typically is associated with increased pleural fluid protein (over 3 g%).
2. Decreased plasma colloid osmotic pressure due to hypoproteinemia, this typically is associated with pleural fluid protein, about 1 g%.
3. Increased hydrostatic pressure due to increased systemic and/or pulmonary venous pressure, as in congestive heart failure. Pleural fluid protein concentration is variable in these cases.
4. Decreased lymphatic drainage due to tumor, inflammation, or fibrosis involving mediastinal lymph nodes (also systemic venous hypertension). This typically is associated with increased pleural fluid protein (over 3 g%).

Indications for Thoracentesis

1. Effusion of unknown etiology.
2. Effusion of known etiology causing symptoms.
3. Intrapleural instillation of drugs for treatment of infection or malignancy.
4. Hemothorax or empyema (to prevent organization).

Complications of Thoracentesis may include:

1. Hemopneumothorax due to lung laceration.
2. Mediastinal shift or pulmonary edema (if large amounts are aspirated at one time).

Do not Remove More than 1 liter of Fluid at One Time

Collect pleural fluid in three sterile anticoagulated EDTA tubes labeled sequentially: First tube for culture and Gram's stain, the rest for cell counts, differential counts, total protein, glucose, cytology, etc. If malignancy or tuberculosis is suspected, several hundred mL of anticoagulated fluid should be given for examination.

Gross Examination

Hemorrhagic fluids can be distinguished from traumatic tap by noting the color of aspirate in the successive tubes filled with fluid. In traumatic tap the later tubes become clearer.

Hemorrhagic Pleural Fluid Can be Found in

- Intrapleural malignancy (60% cases)
- Pancreatitis
- Pulmonary infarction
- Pleural infection
- Closed chest trauma
- Tuberculosis
- Postmyocardial infarction syndrome
- Congestive heart failure occasionally
- Hepatic cirrhosis.

Hemothorax

Hemothorax can be distinguished from hemorrhagic effusion. Similar PCV of fluid and blood implies hemothorax. Cloudy, turbid fluid is usually due to large numbers of leukocytes associated with septic/nonseptic inflammation.

Milky Fluid

Pseudochylous effusion may occur in:

- Tuberculosis
- Rheumatoid arthritis, etc.

True Chylothorax

True chylothorax is rare, occurs due to leakage of thoracic duct contents, is creamy fluid with consistency of milk, which clears and decreases in volume with alkalinization and ether extraction.

Pleural fluid should be *observed for clotting* in plain tube or after adding $CaCl_2$ to EDTA fluid tube. Presence of fibrinogen suggests damage to capillary walls caused by inflammation or neoplasm.

Microscopic Examination

This should be done as has been told in CSF examination (TLC and DLC). A WBC count over 1000/cu mm or over 50% of neutrophils, suggests inflammation (septic or non-septic). A high percentage of lymphocytes (> 50%) suggests tuberculosis, lymphoma or carcinoma.

Sometimes lymphocytic effusion may be seen in:

- Cardiopulmonary disease
- Cirrhosis
- SLE
- Infectious mononucleosis
- Subacute bacterial pulmonary infection.

RA Cells

RA cells may sometimes be seen in rheumatoid pleural effusions. *LE cells* may be seen in *SLE.*

Eosinophilic pleural effusions may be seen in:

- Convalescent pneumonia
- Pneumothorax
- Pulmonary infarction
- Hypersensitivity diseases
  - Asthma
  - Loeffler's syndrome
  - Periarteritis
  - Parasitic diseases.

Immature blood cells may be seen in:

- Chronic myeloid leukemia
- Myeloid metaplasia (extramedullary hemopoiesis).

Chemical Examination

Pleural effusions are classified as transudates or exudates with the former having protein content less than 3 g% and the latter more than 3 g%.

Normal pleural fluid glucose is about equal to whole blood glucose. Blood glucose changes are reflected in pleural fluid after a lag period of 1 to 3 hours. A pleural fluid glucose concentration 30–40 mg% less than whole blood suggests bacterial infection (including tuberculosis), nonseptic inflammation (especially rheumatoid pleuritis), or malignancy. In contrast to rheumatoid disease, the effusions due to SLE typically have normal glucose concentrations.

Microbiologic Examination

All effusions should be examined for bacteria by Gram's stain and culture. The possibility of tuberculosis should always be considered with idiopathic pleural effusions.

PERICARDIAL FLUID (PF)

The pericardial sac under normal circumstances contains 20–50 mL of clear, straw-colored fluid. A rapid abnormal accumulation of 200 mL may produce cardiac tamponade, while gradual accumulation of 1000 mL or more may be relatively asymptomatic (Table 12.6).

TABLE 12.6: Presence of pericardial fluid

Pericardial fluid	*Observation*	*SI units parameter*
Apperance	Clear to pale yellow	
Glucose		
Transudate	Approximate whole blood levels (Whole blood adult norm 60–80 mg/dL, Whole blood child normal 51–85 mg/dL)	
Exudate	Lower than whole blood levels	
Lactate dehydrogenase		
Transudate	≤ Client's serum LD (serum adult normal 45–90 U/L, serum child normal 60–170 U/L)	

Indications for Pericardial Fluid Aspiration

1. Acute or chronic cardiac tamponade.
2. To confirm diagnosis and establish cause for pericardial effusion of unknown etiology.

Complications of the (Blind) Pericardial Fluid Aspiration

1. Cardiac arrhythmias, especially ventricular fibrillation.
2. Infection of pleural spaces by purulent pericardial fluid.
3. Laceration of an atrium or coronary artery.
4. Pneumothorax.
5. Inadvertent injection of air into the cardiac chamber.

Pericardial fluid aspiration: This should be in 3 sterile tubes at least—EDTA tube for gross and microscopic examination, plain or heparinized tube for microbiologic examination, and heparinized tube for chemical examination. Aspiration should be done under CT scan guidance.

Gross Examination

Gross appearance of PF may be clear, cloudy, blood-tinged, grossly bloody, milky (chylous or pseudochylous) or similar to gold paint.

Increased amounts of normal-appearing pericardial fluid may be found in:

- Congestive heart failure
- Early stages of inflammation
- Some patients with idiopathic (viral) pericarditis.

Cloudy appearance may be associated with:

- Septic/nonseptic inflammation (bacterial, rheumatoid or rheumatic)
- Chronic effusions of any etiology
- Myxedema
- Idiopathic
- Postmyocardial infarction syndrome.

Blood-tinged pericardial fluid is seen in:

- Traumatic tap, but it clears on aspirating more fluid.

Grossly bloody fluid may be caused by:

- Idiopathic hemorrhagic pericarditis (? viral)
- Postmyocardial infarction syndrome
- Postpericardiectomy syndrome
- Tuberculosis
- Rheumatoid arthritis
- Systemic lupus erythematosus
- Metastatic carcinoma
- Bacterial pericarditis
- Leaking aortic syndrome → (hemopericardium → acute cardiac tamponade (hemopericardium has a PCV similar to that of peripheral blood).

Milky pericardial fluid (unusual) may be due to:

- True chylopericardium
- Chronic pericarditis from any cause, e.g.
  - Bacterial
  - Fungal
  - Tuberculous
  - Rheumatoid pericarditis
  - Rheumatic
  - Myxedema.

Microscopic Examination

Total and differential counts done as for CSF. Increased leukocytes with preponderance of neutrophils are characteristic of bacterial pericarditis but may also be seen in viral pericarditis or chronic postmyocardial infarction syndrome. A high percentage of lymphocytes suggest tuberculous pericarditis.

Microbiologic Examination

Cultures for bacteria, fungi, and tuberculosis should be performed in all effusions of unknown etiology.

Chemical Examination

Pericardial fluids should be classified as transudates or exudates.

Transudates are typically seen in:

- Congestive heart failure
- Hypoproteinemic states
- Myxedema
- Viral pericarditis
- Early septic/nonseptic inflammation.

PERITONEAL FLUID

Normally, the peritoneal cavity contains less than 100 mL of clear, straw-colored fluid (Table 12.7.

TABLE 12.7: Presence of peritoneal fluid

Peritoneal fluid	*Observation*	*SI units parameter*
Appearance	Clear or pale yellow	
Albumin	Negative	
Alkaline phosphatase		
Adult female	76–250 U/L	
Adult male	90–239 U/L	
Ammonia	< 50 g/L	
Cholesterol		
Transudate	< 46 mg/dL	< 1.19 mmol/L
Exudate	> 46 mg/dL	> 1.19 mmol/L
Glucose	60–100 mg/dL	3.3–6.1 mmol/L
Transudate	Lower than whole blood levels (Whole blood adult normal 60–89 mg/dL, child norm 51–85 mg/dL)	
Lactic acid	10–20 mg/dL	1.1–2.3 mmol/L
Lactate dehydrogenase		
Transudate	≤ Client's serum LD (serum adult normal 45–90 U/L, child normal 60–170 U/L)	
Exudate	> Client's serum LD	
pH	7.4	7.4

Contd...

Contd...

Peritoneal fluid	*Observation*	*SI units parameter*
Specific gravity		
Transudate	< 1.016	< 1.016
Exudate	> 1.016	> 1.016
Total protein		
Transudate	< 2.5 g/dL	< 25 g/L
Exudate	> 3 g/dL	> 30 g/L
Volume	< 100 mL	
White blood cells		
Transudate	< 100/mm^3	< 100 × 10^9/L
Exudate	> 1000/mm^3	> 1000 × 10^9/L

Indications for Abdominal Paracentesis

To be done under ultrasound guidance.

1. Ascites of unknown etiology.
2. Symptomatic ascites, e.g. dyspnea.
3. Possible ruptured viscus or intra-abdominal hemorrhage due to trauma.
4. Acute abdominal pain of unknown etiology.
5. Postoperative hypotension and pain of unknown etiology.
6. Instillation of cytotoxic drugs in ascites due to malignancy.

(The chief complication of abdominal paracentesis is intestinal perforation, perforation of other viscera is rare. If aspiration reveals gross blood or intestinal contents—laparotomy must be done).

Gross Examination

Color of Peritoneal Fluid

Pale yellow to amber in:

- Congestive heart failure
- Hepatic vein obstruction
- Cirrhosis
- Nephrotic syndrome.

Similar appearance in:

- Ruptured urinary bladder.

Turbid fluid suggests peritonitis due to:

- Appendicitis
- Pancreatitis
- Strangulated/infarcted intestine
- Torn/ruptured bowel due to trauma/primary bacterial infection.

Blood-tinged or grossly bloody fluid may be seen in:

- Ruptured spleen
- Ruptured liver
- Torn mesenteric vessels (trauma)
- Aortic aneurysm rupture
- Splenic artery, leaking aneurysm
- Hepatic vessel rupture
- Hemorrhagic pancreatitis
- Peritoneal laceration following muscular effort
- Traumatic tap—clears as more fluid is aspirated.

Greenish in:

- Perforated duodenal ulcer
- Perforated intestine
- Cholecystitis
- Perforated gallbladder
- Acute appendicitis (Biliary peritonitis is usually rapidly fatal).

Milky fluid is due to chylous ascites, various causes are:

- lymphoma
- Carcinoma
- Tuberculosis
- Parasitic infestations
- Adhesions
- Hepatic cirrhosis
- Nephrotic syndrome.

(If surgical treatment is not indicated, elimination of dietary long-chain fatty acids will decrease accumulation of chylous fluid in abdomen, pericardium or pleural cavity).

Microscopic Examination

In peritoneal fluid TLC > 500/cu mm or RBC count > 100,000/cu mm are considered abnormal. Increased TLC, chiefly neutrophils, typically occur with acute peritonitis from any cause and may be the only evidence of intestinal rupture due to blunt trauma. A high incidence of lymphocytes should suggest the possibility of tuberculous peritonitis, but may also be found with chylous ascites.

Cytology examination with Papanicolaou stained films should be done. Sometimes, differentiation between reactive shed mesothelial cells and true neoplastic cells may be difficult.

Microbiologic Examination

Gram's stain and AFB stain should be done as usual. Cultures should also be done to know the actual pathogenic organism and its sensitivity.

Chemical Examination

Ascitic transudate is seen in:

- Congestive heart failure
- Constrictive pericarditis
- Hepatic vein obstruction
- Cirrhosis
- Nephrotic syndrome.

An acid pH of the peritoneal fluid should suggest perforated peptic ulcer.

With pancreatitis, the fluid amylase level is raised.

Elevated ammonia levels in peritoneal fluid above 3 g/mL are not found in pancreatitis and suggest intestinal necrosis, perforation, or urinary extravasation.

Jejunal and ileal fluids have very high alkaline phosphatase (100–10,000 times serum level).

Ascitic fluid LDH is raised in:

- Malignancy of peritoneum
- Hemorrhagic peritoneal fluid of any etiology.

Differential diagnosis of peritoneal transudate vs. aspirated urine

Simultaneous measurements of creatinine and urea nitrogen on blood and peritoneal fluid are helpful. High levels of peritoneal fluid urea and creatinine with normal serum levels suggest inadvertent aspiration from the urinary bladder. High levels of peritoneal fluid urea and creatinine with elevated urea but normal creatinine in peripheral blood suggest rupture of the urinary bladder, since urea diffuses more rapidly than creatinine across the peritoneal surface.

AMNIOCENTESIS AND AMNIOTIC FLUID ANALYSIS, DIAGNOSTIC

Normal Value

Routine Analysis (Table 12.8)

Color: Colorless, straw-colored, or clear to milky.

TABLE 12.8: Presence of amniocentesis and amniotic fluid

Parameter	*Observation*	*SI unit*
Acetylcholinesterase	Negative	
Alpha$_1$-Fetoprotein		
12 weeks' gestation	≤42 µg/mL	
14 weeks' gestation	≤35 µg/mL	
16 weeks' gestation	≤29 µg/mL	
18 weeks' gestation	≤20 µg/mL	
20 weeks' gestation	≤18 µg/mL	
22 weeks' gestation	≤14 µg/mL	
30 weeks' gestation	≤3 µg/mL	
35 weeks' gestation	≤2 µg/mL	
40 weeks' gestation	≤1 µg/mL	
Normal values may also be reported in multiples of the median (MOM) or 0.5–3.0 MOM.		
Bilirubin		
Trimester 1, 2	≤ 0.074 mg/dL	≤ 1.2 µmol/L
40 weeks' gestation	≤ 0.024 mg/dL	<0.4 µmol/L
Calcium	4 mEq/L	4 mmol/L
Carbon dioxide	16 mEq/L,	16 mmol/L
Chloride	102 mEq/L	102 mmol/L
Creatinine		
≤ 27 weeks' gestation	0.8–1.1 mg/dL	72–99 µmol/L
30–34 weeks' gestation	1.1–1.8 mg/dL	99–162 µmol/L
35–40 weeks' gestation	1.8–4.0 mg/dL	162–360 µmol/L
Oestriol		
Trimester 1, 2	≤ 9 µg/dL	<309 µmol/L
Term	≤59 ng/dL	<2023 µmol/L
Glucose	30 mg/dL	2 mmol/L
Lecithin		
≤ 35 weeks' gestation	6–9 mg/dL	
≥ 35 weeks' gestation	15–20 mg/dL	
Lecithin/sphingomyelin (L/S)	Ratio	
Immaturity	≤ 1.5	
Borderline maturity	1.5–1.5	
Maturity	2.0–4.0	
Postmaturity	≥4.1	
Meconium	Negative	
pH		
Trimester 1, 2	7.12–7.38	7.12–7.38
Term	6.91–7.43	6.91–7.43
Potassium	4.9 mEq/L	4.9 mmol/L
Sodium	133 mEq/L	133 mmol/L
Sphingomyelin	4–6 mg/dL	
Total protein	2.5 g/dL	25 g/L
Urea		
Trimester 1, 2	12–24 mg/dL	
Term	19–42 mg/dL	
Uric acid		
Trimester 1, 2	2.76–4.68 mg/dL	0.17–2.8 mmol/L
Term	7.67–12.13 mg/dL	0.46–0.72 mmol/L

Abnormalities that may be Found Upon Routine Analysis

Abnormal color	*Possible Cause*
Yellow	Due to fetal bilirubin, erythroblastosis fetalis
Green	Due to meconium, breech presentation, fetal death, defecation, distress, hypoxia, intrauterine growth retardation, post-maturity, vagal stimulation
Red	Due to presence of blood, intrauterine hemorrhage
Port wine	Acute fetal distress, abruptio placentae
Brown	Oxidized hemoglobin, maternal tissue trauma, fetal death, fetal maceration

Abnormal Bilirubin		*SI Units*
Fetal involvement	0.10–0.28 mg/dL	= 1+ 1.6–4.5 µmol/L
Later fetal involvement	0.29–0.36 mg/dL	= 2+ 4.7–5.8 µmol/L
Fetal distress	0.47–0.95 mg/dL	= 3+7.6–15.4 µmol/L
Fetal death	>0.95 mg/dL	= 4+ > 15.4 µmol/L

Abnormal Creatinine

35–40 weeks' gestation: Large muscle mass, possible diabetes	> 2 mg/dL	> 180 µmol/L
Low birthweight	<2 mg/dL	< 180 µmol/L

Increased alpha$_1$-fetoprotein: Anencephaly, cystic fibrosis, duodenal atresia, esophageal atresia, fetal bladder neck obstruction with hydronephrosis, fetal death, meningomyelocele, multiple pregnancy, nephrosis (congenital), neural tube defects, spina bifida, omphalocele, and Turner's syndrome.

Increased bilirubin: Anencephaly, erythroblastosis fetalis, hemolytic disease of the newborn, hydrops fetalis, intestinal obstruction, and Rh sensitization.

Positive acetylcholinesterase: Neural tube abnormalities that allow cerebrospinal fluid (which contains acetylcholinesterase) to leak into the amniotic sac.

Positive meconium: Fetal distress.

Decreased alpha$_1$-fetoprotein: Not applicable.

Decreased bilirubin: Not clinically significant.

Decreased creatinine: Fetal lung immaturity.

Chromosome analysis: Interpretation required.

Description

Detection of fetal jeopardy or genetic disease and determination of fetal maturity. *Amniocentesis* is a 20–30 minute procedure in which an aspiration of amniotic fluid is taken transabdominally and usually performed after week 12 of gestation. In routine analysis, amniotic fluid is examined for levels of calcium, chloride, carbon dioxide, creatinine, estriol, glucose, pH, potassium, sodium, protein, urea, uric acid, culture, and or genetic defects, chromosomal studies, detection of fetal jeopardy or distress (via color, bilirubin) and to measure lung maturity (via L/S ratio) and age (via creatinine) of the fetus. *Alpha$_1$-alpha-fetoprotein* is a globulin protein secreted by the yolk sac and by fetal liver cells during hepatic cell multiplication. Highest amounts are found during pregnancy and in hepatic cancer. Measurement is usually performed from week 16 to 20 to help identify fetal neural abnormalities, gastroesophageal atresia, and nephrosis. *Chromosome analysis* of amniotic fluid cells is performed by examining karyotyped cells for genetic abnormalities such as Down syndrome, Tay-Sachs disease, and other inborn errors of metabolism. Amniotic fluid is examined for color and bilirubin level for purposes of detecting fetal jeopardy or distress due to hemolysis of fetal red blood cells. Erythroblastosis fetalis occurs when maternal antibodies attack fetal RBCs, causing fetal anemia. This occurs when the mother's blood contains the Rh factor that reacts with fetal erythrocyte antigens. The test is usually performed at gestation week 24 or later and can help determine the need for intrauterine fetal blood transfusion.

Risks

Bleeding, intrauterine death, premature labor, spontaneous abortion.

Contraindications

Abruptio placentae, incompetent cervix, placenta previa, and a history of premature labor.

Preparation

1. Obtain an aminocentesis tray, surgical scrub solution, a light-protected container, and povidone-iodine solution. Also, obtain RhoGAM for Rh-negative mothers.
2. Obtain maternal vital signs. Auscultate baseline fetal heart tones.
3. Note the estimated date of conception and week of gestation on the laboratory requisition.
4. Procedure should be performed in a darkened room if the specimen will be tested for bilirubin.
5. See patient and family teaching.

Procedure

1. The position of the fetus and a pocket of amniotic fluid are determined using ultrasound and palpation, with the mother in a supine position.
2. The mother's abdominal area is cleansed with surgical scrub solution and povidone-iodine and allowed to dry.
3. The aspiration site is draped, demarcating a sterile field.
4. The mother is instructed to place her hands behind her head, and the aspiration site is anesthetized with 1 mL of 1 or 2% lidocaine intradermally and subcutaneously.
5. A 20 to 22-gauge, 5-inch-long spinal needle with a stylette is inserted through the abdominal wall into the intrauterine cavity, and the stylette is withdrawn.
6. About 10–15 mL of amniotic fluid is aspirated through the spinal needle into a syringe, and the needle is withdrawn. Use a 20 mL amniotic fluid sample for direct genetic analysis for the four most common mutations responsible for Tay-Sachs disease.

Postprocedure Care

1. Apply a dry sterile dressing to the aspiration site.
2. Inject 5 mL of amniotic fluid into a light-protected (foil-covered or amber) test tube to test for bilirubin. Inject 10 mL of amniotic fluid into a sterile, siliconized glass container or a polystyrene container for culture and genetic and other studies (AFP). Specimens to be transported to another site for testing should be packed in a cool, insulated container to maintain a temperature of 2–5°C. Freezing temperatures should be avoided.
3. Obtain the mother's vital signs. Auscultate fetal heart tones for changes from the baseline.
4. The mother should rest on her right side for 15–20 minutes after the procedure.
5. RhoGAM may be prescribed for Rh-negative mothers.
6. Transport the amniotic fluid specimen to the laboratory immediately and refrigerate.

Patient and Family Teaching

1. Empty your bladder immediately prior to the procedure if gestation is 21 weeks or more. You must have a full bladder during the procedure if gestations is 20 weeks or less.
2. It is important to lie motionless throughout the procedure. You may experience a strong contraction with the needle insertion.
3. Chromosome analysis results may take up to 4 weeks.
4. Inform the patient with abnormal genetic findings of choices regarding pregnancy and pregnancy termination. Also, refer the patient for genetic counseling prior to future attempts to become pregnant.
5. After the procedure, notify the physician for cramping, abdominal pain, unusual vaginal drainage/fluid loss, fever, chills, dizziness, or more or less than the usual amount of fetal activity.

Factors that Affect Results

1. Reject frozen or clotted specimens.
2. Inadvertent aspiration of maternal urine can be ruled out by testing the specimen for blood urea nitrogen (BUN) and creatinine. Urine BUN is >100 mg/dL, whereas amniotic fluid is well under 100 mg/dl. Urine creatinine is usually 0.80 mg/dL, whereas amniotic fluid creatinine is usually $\leq$4 mg/dL.
3. Nonsiliconized glass containers for routine analysis may result in cell adherence on the sides of the container.
4. Amniotic fluid testing must be performed within 3 days of collection.
5. Amniocentesis should be performed between weeks 24 and 28 when checking for hemolytic disease of the newborn and Rh sensitization.
6. Falsely low bilirubin levels may result from failure to protect the specimen from light.
7. Specimens contaminated with blood should be tested for fetal hemoglobin to determine whether the blood is of maternal or fetal origin. Fetal blood contamination results in falsely high bilirubin levels. Fetal or maternal blood will interfere with measurements of fetal lung maturity and amniotic fluid constituents that are also constituents of plasma, such as protein, potassium, and glucose.
8. Creatinine levels are affected by maternal creatinine clearance and maternal creatinine levels. A concurrent maternal serum creatinine should be drawn. Maternal serum to amniotic fluid creatinine should be about 2:1.
9. Elevated AFP results may be caused by contamination of the specimen with fetal blood.
10. Small and closed neural tube defects may not cause elevated AFP levels.
11. Accurate L/S ratio measurement is not possible if the specimen is contaminated with blood (fetal or maternal) or meconium.

Other Data

1. Direct karyotyping of placental villi samples obtained by needle aspiration has been found to yield faster results than amniotic fluid chromosome analysis.

2. Chromosomal aberration has been found in 4.6% of fetuses in women over 38 years old, the most common being trisomy 21 (62%), Klinefelter's syndrome (11%), and Edward's syndrome {(trisomy 18) (11%)}.
3. For diamniotic twin pregnancies, each amniotic sac should be sampled.
4. A 1995 study suggested that early amniocentesis is feasible from 11 weeks of gestation and "can be performed for the usual indications" as an alternative to chorionic villus sampling. In the future, results will be available in less than 1 week using cytogenetic techniques.
5. Prenatal cystic fibrosis profile may be performed by polymerase chain reaction.

CHAPTER 13

Semen Analysis

INTRODUCTION

Semen examination is an integral part of the evaluation of infertility. As a result of its relative simplicity, semen examination is often requested before the more complicated and expensive examination of the female. Repeat examination should be done if once it is found to be abnormal.

Semen consists of spermatozoa suspended in seminal plasma. Spermatozoa comprise about 5% of semen volume (derived from testis). Approximately, 60% of the semen volume is derived from the seminal vesicles. This viscid, neutral, or slightly alkaline fluid is often yellow or even deeply pigmented because of its high flavin content. Prostate contributes 20% of the volume of semen. This milky fluid is slightly acidic, with a pH of about 6.5 largely because of its high content of citric acid. The prostatic secretion is also rich in proteolytic enzymes and acid phosphatase. These proteolytic enzymes are believed to be responsible for the coagulation and liquefaction of semen. Less than 10–15% of semen volume is contributed by epididymidis, vasa deferentia, bulbourethral and urethral glands.

SEMEN ANALYSIS

A semen analysis measures the amount of semen a man produces and determines the number and quality of sperm in the semen sample.

A semen analysis is usually one of the first tests done to help determine whether a man has a problem fathering a child (infertility). A problem with the semen or sperm affects more than one-third of the couples who are unable to have children (infertile).

Tests that may be done during a semen analysis include:

- *Volume:* This is a measure of how much semen is present in one ejaculation.
- *Liquefaction time:* Semen is a thick gel at the time of ejaculation and normally becomes liquid within 30 minutes after ejaculation. Liquefaction time is a measure of the time it takes for the semen to liquefy.
- *Sperm count:* This is a count of the number of sperm present per milliliter (mL) of semen in one ejaculation.
- *Sperm morphology:* This is a measure of the percentage of sperm that have a normal shape.
- *Sperm motility:* This is a measure of the percentage of sperm that can move forward normally. The number of sperm that show normal forward movement in a certain amount of semen can also be measured (motile density).
- *pH:* This is a measure of the acidity (low pH) or alkalinity (high pH) of the semen.
- *White blood cell count:* White blood cells are not normally present in semen.
- *Fructose level:* This is a measure of the amount of a sugar called fructose in the semen. The fructose provides energy for the sperm.

Why it is Done?

A semen analysis is done to determine whether:

- A man has a reproductive problem that is causing infertility
- A vasectomy has been successful
- The reversal of a vasectomy has been successful.

How to Prepare?

The patient may be asked to avoid any sexual activity that results in ejaculation for 2 to 5 days before a semen analysis.

This helps ensure that his sperm count will be at its highest, and it improves that reliability of the test. If possible, the patient should not avoid sexual activity for more than 1 to 2 weeks before this test, because a long period of sexual inactivity can result in less active sperm.

Ask the patient to avoid drinking alcohol for a few days before the test.

Ask the patient about any medications or herbal supplements he may be taking.

How it is Done?

Ask the patient to produce a semen sample, usually by ejaculating into a clean sample cup. He can do this in a private room or in a bathroom at your office or clinic. If patient lives close to your health professional's office or clinic, he may be able to collect the semen sample at home and then transport it to the office or clinic for testing.

- The most common way to collect semen is by masturbation, directing the semen into a clean sample cup
- The patient can collect a semen sample during sex by withdrawing his penis from his partner just before ejaculating (coitus interruptus). He then ejaculates into a clean sample cup. This method can be used after a vasectomy to test for the presence of sperm, but other methods will likely be recommended if the patient is being tested for infertility.
- The patient can also collect a semen sample during sex by using a condom. If he uses a regular condom, he will need to wash it thoroughly before using it to remove any powder or lubricant on it that might kill sperm. He may also be given a special condom that does not contain any substance that kills sperm (spermicide). After he has ejaculated, carefully remove the condom from his penis. Tie a knot in the open end of the condom and place it in a container that can be sealed in case the condom leaks or breaks.

If the patient, collect the semen sample at home, the sample must be received at the laboratory or clinic within 1 hour. Keep the sample out of direct sunlight and do not allow it to get cold or hot. If it is a cold day, carry the semen sample container against his body to keep it as close to body temperature as possible. Do not refrigerate the semen sample.

Since semen samples may vary from day to day, 2 or 3 different samples may be evaluated within a 3-month period for accurate testing.

A semen analysis to test the effectiveness of a vasectomy is usually done 6 weeks after the vasectomy.

How it Feels?

Producing a semen sample does not cause any discomfort. However, the patient may feel embarrassed about the method used to collect it. If masturbation is against his religious beliefs, discuss alternate methods of collection with the patient.

Risks

There are no risks associated with collecting a semen sample.

Results

A semen analysis measures the amount of semen a man produces and determines the number and quality of sperm in the semen sample. Results of a semen analysis are usually available within a day. Normal values may vary from laboratory-to-laboratory.

Certain conditions may be associated with a low or absent sperm count. These conditions include orchitis, varicocele, Klinefelter syndrome, radiation treatment to the testicles, or diseases that can cause shrinking (atrophy) of the testicles (such as mumps). If a low sperm count or a high percentage of sperm abnormalities is found, further testing may be done. Other tests may include measuring hormones, such as testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), or prolactin. A small sample (biopsy) of the testicles may be needed for further evaluation if the sperm count or motility is extremely low.

What Affects the Test?

Factors that can interfere with semen test or the accuracy of the results include:

- Medicines, such as cimetidine (Tagamet), male and female hormones (testosterone, estrogen), sulfasalazine, nitrofurantoin, and some chemotherapy medicines
- Caffeine, alcohol, cocaine, marijuana, and smoking tobacco
- Herbal medicines, such as St. John's wort and high doses of Echinacea
- A semen sample that gets cold, the sperm motility value will be inaccurately low
- Exposure to radiation, some chemicals (such as certain pesticides or spermicides), and prolonged heat exposure
- An incomplete semen sample: This is more common if a sample is collected by methods other than masturbation
- Not ejaculating for several days: This may affect the semen volume.

Semen Analysis		
Semen volume	Normal:	1.0–6.5 milliliters (mL) per ejaculation
	Abnormal:	An abnormally low or high semen volume is present, which may sometimes cause fertility problems
Liquefaction time	Normal:	Less than 60 minutes, ideally < 30 minutes
	Abnormal:	An abnormally long liquefaction time is present, which may indicate an infection
Sperm count	Normal:	20–150 million sperm per milliliter (mL) 0 sperm per milliliter if the man has had a vasectomy
	Abnormal:	A very low sperm count is present, which may indicate infertility
		However, a low sperm count does not always mean that a man cannot father a child. Men with sperm counts below 1 million have fathered children
Sperm shape	Normal:	At least 70% of the sperm have normal shape and structure
(morphology)		
	Abnormal:	Sperm can be abnormal in several ways, such as having two heads or two tails, a short tail, a tiny head (pinhead), or a round (rather than oval) head. Abnormal sperm may be unable to move normally or to penetrate an egg. Some abnormal sperm are usually found in every normal semen sample. However, a high percentage of abnormal sperm may make it more difficult for a man to father a child
Sperm movement	Normal:	At least 60% of the sperm show normal forward movement
(motility)		At least 8 million sperm per milliliter (mL) show normal forward movement
	Abnormal:	Sperm must be able to move forward (or "swim") through cervical mucus to reach an egg. A high percentage of sperm that cannot swim properly may impair a man's ability to father a child
Semen pH	Normal:	Semen pH of 7.1–8.0
	Abnormal:	An abnormally high or low semen pH can kill sperm or affect their ability to move or to penetrate an egg
White blood cells	Normal:	No white blood cells or bacteria are detected
	Abnormal:	Bacteria or a large number of white blood cells are present, which may indicate an infection
Fructose level	Normal:	300 milligrams (mg) of fructose per 100 milliliters (mL) of ejaculation
	Abnormal:	The absence of fructose in the semen may indicate that the man was born without seminal vesicles or has blockage of the seminal vesicles

What to Think About?

- A semen sample collected at home must be received at the laboratory or clinic within 1 hour. Keep the sample out of direct sunlight and do not allow it to get cold or hot. If it is a cold day, carry the semen sample container against your body to keep it as close to body temperature as possible. Do not refrigerate the semen sample
- Consistently detecting sperm in the semen of a man who has had a vasectomy indicates that his surgery was not successful, and another form of birth control should be used to prevent pregnancy. A low number of sperm may be present in a semen sample taken initially after a vasectomy. However, sperm should not be present in subsequent samples
- A man whose mother took the medicine diethyl stilbestrol (DES) during her pregnancy with him has a greater-than-normal risk of being unable to father a child (infertile)
- Additional tests may include measuring hormone levels, such as testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), or prolactin. For more information, see the medical tests testosterone, luteinizing hormone, follicle-stimulating hormone, and prolactin
- Other fertility testing, including sperm penetration, the presence of antisperm antibodies, or analysis after sexual intercourse (postcoital), may be recommended for infertility problems. For more information, see the medical test infertility testing.

Collection

A 3-day period of abstinence is recommended before collecting the semen sample. Prolonged abstinence from

intercourse should be avoided. The most satisfactory specimen is that collected in the laboratory by masturbation. If specimen will be delivered in a condom, the condom should first be cleaned and washed thoroughly, dried and then used. During transportation of the specimen, it should not be exposed to extremes of temperature and in no case, the delay after collection till submission to the laboratory be more than 2 hours.

Gross Examination

Physical Characteristics

Freshly ejaculated semen is a highly viscid, opaque, white or gray-white coagulum, which may have a distinct musty or acrid odor. After 10 to 20 minutes, the coagulum will spontaneously liquefy to form a translucent, turbid, viscous fluid, which is mildly alkaline, with a pH of about 7.7. The pH may be slightly acidic in congenital aplasia of the vasa deferentia and seminal vesicles. Increased or decreased turbidity is not of much significance, except when increased turbidity is because of leukocytes associated with an inflammatory process in some parts of the reproductive tract.

All the parameters mentioned above should be checked for in every specimen received. Also important is the volume of the ejaculate.

Viscosity: Can be assessed by pouring semen, if it falls drop by drop, its viscosity is normal. Increased viscosity is important if it compromises the sperm motility.

Liquefaction: Liquefaction of the specimen should be complete within 30 minutes. It is important to distinguish persistent viscosity from delayed liquefaction.

Volume: The normal semen volume averages 3.5 mL, with a usual range of 1.5 to 5.0 mL. Paradoxically increased semen volume is more often (causes reduced sperm count) associated with infertility. Less volume may result in poor penetration of the cervical mucus. Semen volume does not vary significantly with the period of abstinence.

Microscopic Examination

Sperm Counts

Diluting fluid consists of:

- Sodium bicarbonate 5 g
- Formalin neutral 1 mL
- Distilled water 100 mL.

Safety Precautions

Safety precautions should be observed when handling seminal fluid. The following guidelines should be followed:

- If non-disposable items are used, soak contaminated items (e.g. hemacytometers and coverslips) in 70% alcohol
- All disposable items should be placed in a biohazard bag for autoclaving
- Gloves must be worn and hands thoroughly washed when the examination is completed
- Seminal fluids that are to be discarded should be placed in biohazard bags for autoclaving.

Sperm Counting Methods

Sperm can be counted either manually or by automated methods. Although automated counting has some advantages for assessment of motility parameters, manual counting is still performed by most laboratories.

There are several manual counting methods available for semen. These include:

- Neubauer hemacytometer
- Makler chamber
- CellVu (Millennium Sciences, Inc)
- MicroCell (Conception Technologies).

The Makler, CellVu, and MicroCell methods have the advantage of requiring no dilution of the semen. Since semen is viscous, accurate dilution can be problematic. These methods also allow counting of motile and non-motile sperm at the same time and thus avoid the need for separate assessment via wet mount. Each laboratory should determine the best reproducible method for their own situation, equipment, and expertise.

Calculating sperm count on a hemacytometer.

The formula for calculating the sperm count when 5 small squares within the large center square are counted is:

Number of sperm counted in 25 squares on each of 2 sides × dilution factor/volume × 1000 = sperm/mL.

Example: 100 sperm are counted in the five small squares of one side of the hemacytometer, 110 sperm are counted in 5 small squares of the other. The dilution is 1:20.

Number of sperm in 25 squares on 2 sides = 210 × 5 = 1050

Sperm/mL = 1050 × 20 (dilution factor) divided by 0.2 mm^3 × 1000 = 105 million sperm/ml.

Diluting a specimen for counting on a hemacytometer.

Following liquefaction (20–30 minutes), mix the sample manually by swirling the container several times. Thorough mixing is essential for accurate counting. Calibrated automatic pipettes are used to prepare a dilution. Because of the viscosity of semen, the semen should be added to the diluent using a positive pressure pipettor.

The dilution often used for routine sperm counts is 1:20 but the actual dilution factor will vary depending on the

total sperm count. For high concentration specimens, a greater dilution will be necessary. For low concentrations, an undiluted or minimally diluted specimen may be required. The appropriate dilution is determined by estimating the concentration needed to do a count of at least 100 cells per side of the loaded hemacytometer.

The diluent that may be used for sperm counts on a hemacytometer can be as follows: 5 g of sodium bicarbonate in 100 mL of distilled water, plus 1 mL of formalin (neutral).

Other Counting Chambers

Some professionals believe that sperm counts done by hemacytometer are not accurate because of the need to dilute the viscous semen prior to counting. There are several other counting methods available to assess sperm concentration.

The advantages of the following methods are:

- The specimen does not have to be diluted
- Motile and non-motile sperm can both be counted avoiding the need for wet mount evaluation of motile cells.

Note that counting moving sperm can be difficult and takes significant practice to avoid error.

For each of these methods accurate counts are best obtained when at least 100 sperm per replicate are counted.

- **Makler** (Zygotek Systems, Inc.): An undiluted sample is placed on the chamber and covered with the coverglass. Ten squares on the grid contain 0.000001 mL
- **CellVu** (Millennium Sciences, Inc.): Two sides of a special slide are loaded with a drop of undiluted semen. Coverslips with special grids are placed on top of the sperm according to manufacturer's directions. Sperm on both sides are counted
- **MicroCell** (Conception technologies) has two chambers on a single, disposable slide. A special eyepiece with a grid is needed for counting.

Loading and Counting Using a Hemacytometer

Fill both sides of the hemacytometer. Focus on the large center square with the 20X objective. The counting area consists of five small squares in the large center square. The squares usually counted are the four corner squares and the center square, all of which are marked R. A minimum of 100 sperm should be counted in the five small "R" squares. If the number of sperm is low then 10 squares or all squares may be counted to obtain the 100 per side. Count both sides of the hemacytometer and take the average of the two counts to calculate the actual count per mL.

Neubauer Hemacytometer

The picture on the right shows the counting chamber of the Neubauer hemacytometer. This counting method is used to count many types of cells. To use this chamber for counting sperm the specimen will usually need to be diluted. Proper loading of the hemacytometer is also important for accurate sperm counts to be obtained.

When Count are Towards Lower Limit Use the Method Given as Under

Counting can be done with WBC pipette. Following liquefaction draw semen till '0.5' mark, dilute with the diluting fluid till 11 mark. Mix properly. Charge the chamber, let stand for 2 minutes (sperms settle down). The spermatozoa in 4 sq mm (four large squares) are counted. Multiply this number by 50,000 to get the number of sperms per milliliter of semen. When seminal viscosity is markedly increased, a mucolytic agent prior to pipette dilution can be added in equal volume (1:1 dilution) and then the final count be multiplied by 2. Normal sperm count range is 60 to 150 million/mL with an average of 100 million/mL. Counts of less than 20 million/mL are considered distinctly abnormal.

Motility

Active motility is a must for normal spermatozoa as they have to migrate from cervix to the fallopian tubes where fertilization of the ovum occurs. A drop of liquefied semen should be kept on a prewarmed slide and coverslipped. The coverslip should be ringed by petrolatum. Count at least 200 spermatozoa, the whole depth of the fluid should be screened and the nonmotile sperms settled at the bottom be included also to assess motility. The percentage of sperms showing actual progressive motion should be recorded. Normal semen contains more than 70% motile sperms. Semen should be considered abnormal if fewer than 60% of spermatozoa show progressive motion in specimens examined within 3 hours of collection.

Sperm Morphology (Fig. 13.1)

This is assessed by performing differential count of morphologically normal and abnormal spermatozoa types on stained smears (Fig. 13.1). Smears are made on slides as for blood smears. Place the smear immediately in a fixative 95% ethanol (v/v) or 50% (v/v) ethanol ether before drying has occurred. The most suitable stain is Papanicolaou. Air dried smears can be stained by Mayer's hematoxylin [air dried smears in 10% (v/v) formalin for 1 minute; water rinse; Mayer's hematoxylin 2 minutes; water rinse; air dry], but this is not a very satisfactory method. Other staining

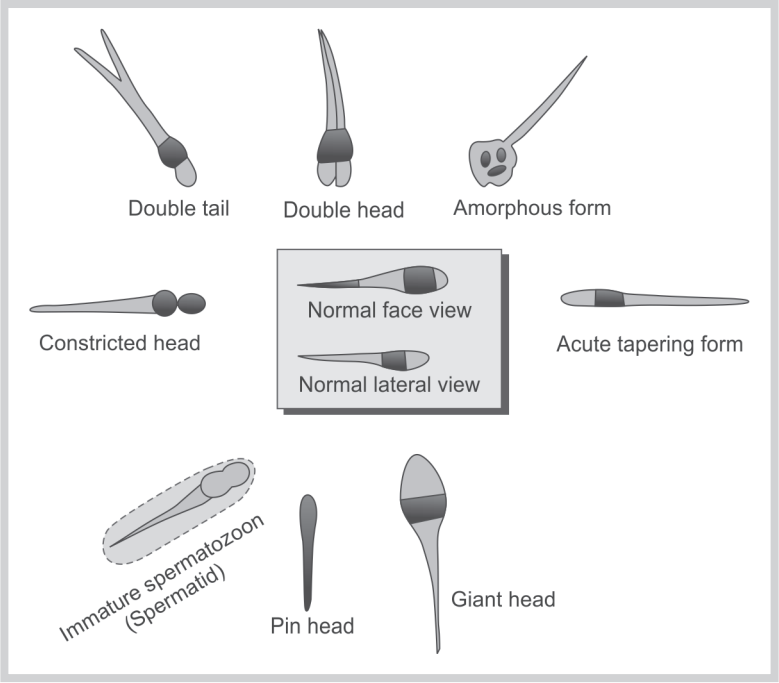


FIG. 13.1: Morphological forms of spermatozoa

techniques include Giemsa, basic fuchsin and crystal violet. Basic fuchsin and crystal violet need heat fixing.

At least 200 spermatozoa should be examined under oil immersion and the percentage of abnormal forms noted. Normal semen has fewer than 30% abnormal forms. In addition to sperm morphology, the presence of RBCs, WBCs and epithelial cells should be noted. Differentiate immature germinal cells from macrophages or leukocytes. Numerous granules and globules are normally present in the semen.

Chemical Examination

Fructose: This is the main sugar of semen and reduced levels of seminal fructose correspond well with diminished androgen deficiency. There is an inverse relationship between fructose level and sperm count. A low fructose concentration is the result of a low testosterone level or seminal vesical insufficiency. Resorcinol method is quite simple and inexpensive.

This principle of the test being that fructose heated with resorcinol in an acidic medium produces a red precipitate. The reaction involves the conversion of fructose to hydroxymethyl furfural that condenses with resercinol to form the real precipitate.

Reagent

Resorcinol 50 mg
Concentrate hydrochloric acid 33 mL.
Dissolve and make to 100 mL with distilled water.

Method

- Take 5 mL of resorcinol reagent in a test tube (15 mL)
- Add 0.5 mL of seminal fluid
- Bring the solution to the boil (take care—the test tube opening should not be facing you)
- Examine the solution and report as mentioned below.

Reporting

Negative: No change in color seen.
Positive: Red colored precipitate forms within 30 seconds. Check the positive reaction with 0.5% aqueous fructose solution. 2% glucose can evoke similar test result, but such quantities of glucose are not seen in semen.

Other Tests

Postcoital (Sims-Huhner) test: Optimum time ovulatory phase at midcycle. The woman reports within 8 hours after coitus. Mucus is aspirated from the endocervical canal and sent to the laboratory.

Measure the volume of the mucus. Evaluate Spinn-Barkeit (refers to tenacity of the mucus). Grasp a portion of mucus with forceps and note the distance, which it can be drawn before breaking. A good Spinn-Barkeit, which should prevail at midcycle, is at least 10 cm. A drop of mucus is then placed on a microscope slide, covered with a coverslip and examined for the presence of sperms. An estimate of the number of sperms per high power field with percentage of motile forms should be reported. At the same time, look for WBCs, RBCs and *Trichomonas*.

Antibodies to Spermatozoa

These can be produced in the male himself or in female. Role of spermatozoal antibodies in infertility is now an established fact in experimental studies, but information pertaining to human spermatozoa is equivocal. Most clinical correlative studies thus far have utilized sperm agglutination tests. The method Franklin and Dukes employed makes use of serum and semen. Results were read (for sperm agglutination) macroscopically after a 4 hours incubation at 37°C. Some medical diagnostic companies provide kits also for assessing antibodies to sperms.

CHAPTER 14

Sputum Examination

SPUTUM

Tracheobronchial secretions are often collectively referred to as sputum. Sputum is constituted by plasma, water, electrolytes and mucin. As it comes out, it is contaminated by nasal and salivary secretions, and normal bacterial flora of the oral cavity. Under appropriate immunologic or inflammatory stimulus, mast cells, eosinophils and plasma cells may contribute to the secretions. Sputum is viscoelastic, i.e. some of the properties of a liquid. Chemical composition reveals sputum is 95% water and only 5% solids. The solid content increases with inflammation. It also shows exfoliation of lining cells.

Specimen Collection

1. Before collecting or expectorating sputum, the mouth should be prerinzed and this removes contaminants from oral cavity especially.
2. For most examinations, a first morning specimen is best as it represents the pulmonary secretions accumulated overnight.
3. To obtain a good specimen, patient's cooperation and understanding is essential. Usually, no problem arises with adults. Children are problematic sometimes. The undermentioned methods can be used for them:
   a. A nasopharyngeal swab may be taken which is quite representative of the bronchial pathogens.
   b. A cough plate is held before the child's mouth and the child is urged to cough.
   c. Cough swab method gives the most representative, noncontaminated sputum sample. The child's mouth is held open by using a tongue depressor. Epiglottis is visualized and is touched with a swab to induce cough. Material expelled from trachea is (coughed) deposited on the swab, which can then be plated on appropriate culture media.
   d. In patients who are uncooperative or cannot produce adequate sputum, induction should be tried. Commonly used inductants are 10% sodium chloride, acetylcysteine and sterile or distilled water aerosols. In persons with a history of bronchospasmodic disorders, bronchodilators should be given after inductants are used. Acetylcysteine breaks the disulfide bonds which maintain the gel structure of mucus. Acetylcysteine can be given in an aerosol form with a bronchodilator.

The specimen should be collected in a sterile disposable, impermeable container with a screw cap.

Sputum Examination

Transfer the specimen in a sterile petridish placed against a dark background. Wooden applicator sticks can be used to spread it thinly and can be seen with the naked eye or by using a hand lens.

Macroscopic Examination

Volume: A 24-hour volume of sputum is measured in patients with chronic bronchitis, lung abscesses or bronchial asthma. A rising volume or decreasing volume indicates worsening and improvement respectively.

Consistency and Appearance

Sputum may be described as serous (liquid), mucoid, purulent, bloody or combinations of these, e.g. seropurulent, mucopurulent.

A normal sputum is clear and watery and any opalescence is because of cellular material suspended

in it. In pulmonary edema, sputum is serous, frothy and blood tinged. Most opaque particles are masses of pus and epithelium. Other materials seen in the sputum can be Curschmann's spirals, Dittrich's plugs, casseous material, bronchial casts, or food substances.

Color: Normal sputum is clear and colorless. A *Yellow color* indicates pus and epithelial cells as seen in a pneumonic process.

Greenish tint implies *Pseudomonas* as the etiologic agent.

Rust colored sputum is due to decomposed hemoglobin and is seen in pneumococcal pneumonia or pulmonary gangrene, whereas a *bright red* sputum is found in recent hemorrhage which can follow acute cardiac infarction, pulmonary infarction, neoplasm invasion and rupture of a vessel.

Odor: Normal sputum is odorless. Suppurative pulmonary disorders such as lung abscesses, cavitary tuberculosis or gangrene produce most putrid odors. A ruptured subphrenic or liver abscess may impart a fecal odor.

Other Findings

1. *Cheesy masses:* Fragments of necrotic pulmonary tissue seen in pulmonary gangrene or tuberculosis.
2. *Bronchial casts:* These are branching tree-like casts of bronchi and their size depends upon the size of bronchi from which they have been expectorated. These can be seen in untreated lobar pneumonias, fibrinous bronchitis. To recognize these casts, they have to be floated on water against a black background.
3. *Broncholiths* (lung stones): These are formed due to calcification of necrotic/infected tissue within a larger bronchus or cavity. The central core of these may be a foreign body or a fungus growth. Though rare, but when seen, chronic tuberculosis should be kept in mind.
4. *Dittrich's plugs:* They are seen in putrid bronchitis and bronchiectasis. When expectorated, they are usually solitary of a variable size. When crushed, they are found to be made of cellular debris, fatty acid crystals, fat globules, and bacteria. These plugs are seen most commonly in chronic bronchitis, bronchiectasis and bronchial asthma.
5. *Foreign bodies:* These are usually objects inhaled by a child. Usually, substances inhaled are peanuts and buttons. Radiologically, they are difficult to see.
6. *Parasites:* Various parasites that can be seen in sputum are *Ascaris lumbricoides, Echinococcus granulosus, Toxocara canis* and *Paragonimus westermani.*

Microscopic Examination

Having done the macroscopic examination, transfer the suspicious looking particles to a clear slide and examine unstained if necessary (one may come across Curschmann's spirals, elastic fibers, fungus and myelin globules). The remaining portion of the sputum is cultured. Smears made on clear slides should be air dried, fixed over a flame and then stained with Gram's stain/Ziehl-Neelsen stain. Wright's stain can be done for blood cells and buffered crystal violet for epithelial cells. Pap's stain is best for studying cytology of sputum (Fig. 14.1).

If cells characteristic of the bronchopulmonary tree are not seen—consider the specimen as inadequate and discard it even for culture. The presence of squamous cells signifies the specimen as being more representative of the oral cavity than the bronchopulmonary tree.

The basal cells are about the size of a lymphocyte with scanty cytoplasm. Columnar bronchial epithelial cells may or may not be ciliated, the nonciliated ones are of the goblet type. The presence of alveolar macrophage is the best indication that the material being examined has arisen from the lower respiratory tract, these cells often show anthracotic pigment which is not of any significance.

Blood cells are best seen by the usual peripheral smear stains. Neutrophils predominating imply an acute pyogenic infection, lymphocytes are predominant in tuberculosis and eosinophils are usually seen in bronchial asthma. Erythrocytes in large numbers indicate exudation or hemorrhage.

Sputum Culture

Each specimen received should be plated on blood agar, chocolate agar, MacConkey's agar and thioglycollate broth.

COMMON RESPIRATORY DISORDERS

Mycobacteria

A culture should always be performed in a previously undiagnosed case of respiratory tuberculosis. In a fulminant form, the sputum is mucopurulent and shows RBCs, caseous and necrotic materials. Elastic fibers in the necrotic tissue indicate pulmonary tissue destruction (e.g. blood vessels; alveoli and bronchi from which they can be derived) and can be seen in abscesses, bronchiectasis, or malignancy. Most often, they are seen in advanced cases of tuberculosis. Within the caseous material, acid-fast bacilli (AFB) can usually be demonstrated. Sputum induction gives a higher recovery rate of tubercle bacilli. The slides can be stained with auramine-rhodamine (AR) stain and/or Ziehl-Neelsen (ZN) stain. AR stain shows nonviable

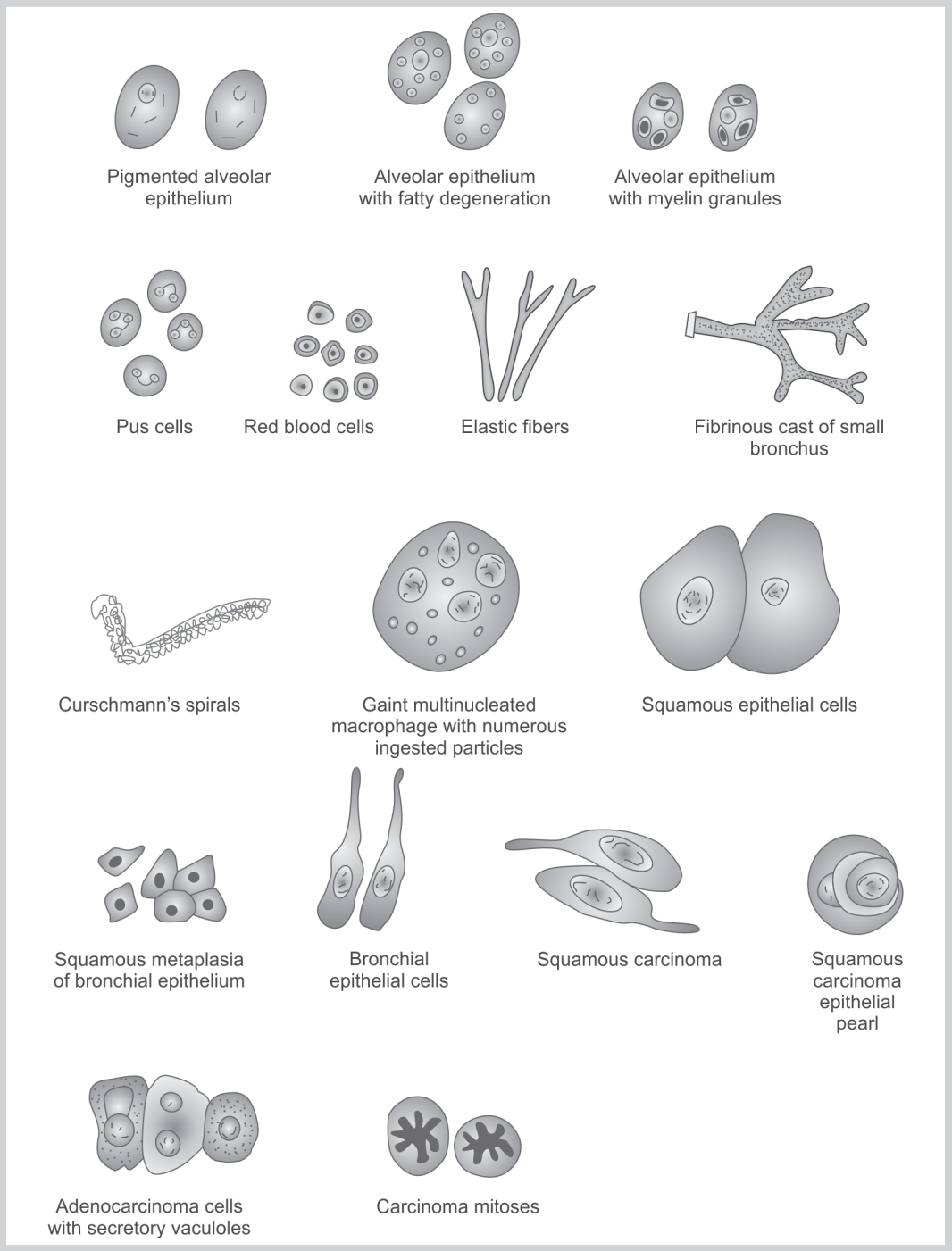


FIG. 14.1: Microscopic structures that may be seen in sputum

bacilli also (ZN does not); so for prognostic evaluation, all AR positive specimens should be stained with ZN also. AR staining is superior to ZN because:

a. AFB have more affinity for AR dye
b. The entire smear can be screened as the low power (X 10) objective is used, and

c. The black background in fluorescent microscopy makes the bacilli stand out sharper to allow more rapid and accurate slide screening.

Mycotic (Fungal) Disease

Respiratory fungal disease often mimics either inflammatory or neoplastic disease in clinical symptoms and X-ray findings. A first morning specimen is preferred as it represents the overnight secretions of the tracheobronchial tree. Place the specimen in a sterile container and view against a dark background. Fungi are usually seen as tiny flecks or particles, which appear yellow or gray in color and more dense than the surrounding sputum.

Make a direct mount with 10% sodium hydroxide and examine under low and high power. If no fungi are seen, the specimen can be concentrated by using 4% NaOH or the enzyme pancreatin. Confirm microscopic finding by cultures.

Pathological Fungi

Actinomyces israelii

Not a true fungus, is a gram-positive organism that grows slowly with branching filaments. It is a commensal but becomes invasive. Macroscopically it appears as yellow (sulfur) granules less than 1 mm in diameter. Microscopically, they are gram-positive mycelial filaments surrounded by a sheath of eosinophilic matter, which imparts a club-shaped appearance to the ends of these filaments.

Nocardia asteroides

These are like *A. israelii* but lack the clubbed ends. The filaments are gram-positive, bacilliform in shape and in some stains are partially acid fast. It may, however, be a saprophyte in the upper respiratory tract. Its repeated presence is diagnostic of pulmonary nocardiosis.

Cryptococcus neoformans

Direct examination with India ink is advocated. The organism appears as a single budding blastospore, 2 to 20 μ in diameter and is surrounded by a capsule from 3 to 5 μ in diameter.

Histoplasma capsulatum

Sputum staining with Wright's/Giemsa's stain reveals macrophages with characteristic intracellular small yeast cells in the cytoplasm.

Coccidioides immitis

Examine sputum by wet direct mounts. The organism appears as a spherule, 5–200 μ in diameter and is filled with endospores. In the chronic cavity, hyphae may be seen.

Blastomyces dermatidis

Infection beginning from lungs may spread hematogenously. In direct wet mounts, the organisms appear as 8–15 μ diameter spherules without a capsule. Budding may be seen with a characteristic sputum. No mycelium occurs in sputum.

Candida albicans

It is a throat commensal but overgrows with excessive use of antibiotics and immunosuppressants and becomes pathogenic (keep in mind that they can grow very well on sputum in vitro also). The report should indicate the number of organisms seen per field. On direct mount, they appear as 4 μ diameter, thin-walled organisms singly, in pairs, or in small clusters. Budding forms and pseudomycelia may be seen. The organisms stain intensely positive with Gram's stain.

Aspergillus fumigatus

These are like *C. albicans,* the organism appears often as a sputum contaminant.

Phycomycetes

Mucormycosis rarely causes pulmonory lesions and occur more commonly in diabetics. Direct wet mount may show huge (15 μ diameter) aseptate hyphae. Isolation on culture is a must.

Bronchial Asthma

The sputum is usually white and mucoid and contains no blood or pus unless an underlying infection is present.

Various findings seen are:

1. *Eosinophilia:* Sputum has eosinophilic staining properties (attributed to increased accumulation of serum proteins), not seen in chronic bronchitis.
2. *Bronchial epithelial cells:* These often occur singly and show hydropic change with poorly defined original morphology. During acute phases, these cells gather in larger clusters, display a vacuolated cytoplasm with ciliated border—known as Creola bodies. In addition, one may see hypersecretory goblet cells singly/clustered.

3. *Charcot-Leyden crystals:* Seen almost only in the sputum of bronchial asthma cases. The crystals are colorless, pointed hexagons and variable in size (may look needle shaped). These are derived from disintegration of eosinophils, hence they stain strongly with eosin.
4. *Blood cells:* Mostly eosinophils are seen, infection brings with it a neutrophilic response. Monocytes and histiocytes appear in significant numbers during the recovery phase.
5. *Creola bodies:* Almost exclusively seen in sputum of bronchial asthma cases. Their appearance is a poor prognostic sign.
6. *Curschmann's spirals:* These are characteristic of bronchial asthma sputum but may be seen in other respiratory disorders. Macroscopically, they can sometimes be recognized by the naked eye and appear as yellow-white, mucoid, wavy threads frequently coiled into little balls. Their length may exceed 1.5 cm. Microscopically, they show a central thread around which mucus is wrapped, supported by a fibril network.

Bronchiectasis

The production of mucopurulent sputum is one of the cardinal signs of bronchiectasis and the amount expectorated varies with the posture. Morning cough is typical. Characteristically, the sputum is putrid, gray green in color (50–250 mL/day), at times blood tinged. On sitting, the sputum separates into three layers: (i) upper frothy layer, which later subsides, (ii) a middle turbid mucous layer and (iii) a bottom layer of pus cells and various organisms. The microscopic examination of bottom layer discloses bronchial epithelial cells, fatty crystals, bacteria and occasionally Dittrich's plugs. When crushed, they emit a foul odor.

Chronic Bronchitis

This may be catarrhal or cellular. Macroscopically the sputum is tenacious, white and mucoid in appearance. Intercurrent infections make it purulent yellow-green in color. The average volume expectorated is about 60 mL/day. A decreasing volume implies improvement. In early chronic bronchitis, large numbers of histiocytes and monocytes indicate a stable phase, during exacerbation these cells disappear. When entering remission, these cells reappear. Leukocytes and epithelial cells are increased during active disease and diminished in number with recovery. Presence of necrotic tissue/elastic fibers indicates abscess formation or bronchiectasis. Examination of the Gram's stain usually reveals the presence of mixed organisms. Active phase is accompanied by raised sputum LDH levels. When bacterial resistance to antibiotic therapy is developing, increased LDH activity may be observed before clinical deterioration. Therefore, appropriate changes in antibiotics may be made sooner rather than waiting for culture or clinical signs. In addition, DNA levels also rise during infections. Levels fall as improvement is noted.

Lung Abscess

Only when it ruptures into a bronchus—it leads to sputum production. The etiologic agent usually isolated are *Klebsiella, Haemophilus, Staphylococcus aureus, Streptococcus hemolyticus.* Following rupture, a large amount of bloody, creamy, foul smelling pus is suddenly and violently expectorated. More often than not, mixed organisms are present. A search for tubercle bacilli or malignant cells must also be made.

Pneumonia

Early diagnosis can be established by a Gram's stain of the sputum. Sputum should be homogenized for a more even distribution of pathogenic organisms on Gram's stain. Of the gram-positive pneumonias, the main pathogen is *Diplococcus pneumoniae,* rarely are staphylococci and streptococci involved.

In pneumococcal pneumonia, the sputum characteristics change with the stage of the disease. Early lobar pneumonia sputum is scanty and transparent with occasional blood flecks. In red hepatization stage the sputum becomes rusty red in color, tenacious and mucopurulent. Microscopically, many intra- and extracellular organisms, epithelial cells, leukocytes and red cells are seen. During resolution stage, the sputum becomes more abundant, less tenacious and assumes the appearance as seen in chronic bronchitis. Reappearance of rusty character should indicate further progression or involvement of the opposite lung. Daily sputum Gram's stains should be performed on these patients for two reasons: (i) to follow the effect of treatment, and (ii) to rule out secondary infection.

In staphylococcal pneumonia, a yellow purulent, voluminous sputum is present. On Gram's stain, large numbers of staphylococci and neutrophils are seen.

Gram-negative pneumonias are often caused by *Klebsiella, Haemophilus, Pseudomonas* and *Escherichia coli*. With the exception of foul green sputum seen in

Pseudomonas infections, no classic macroscopic findings are present in these sputums. As a group, sputums in the various gram-negative pneumonias are purulent and foul smelling. Putrid sputums may be associated with anaerobic organisms and should be cultured accordingly.

In Gram's stain, *Haemophilus* is often missed as safranin does not stain it well but methylene blue stain permits easier recognition of *H. influenzae*.

Pneumoconiosis

1. In *anthrasilicosis* angular black granules will be both intra- and extracellular but are not pathognomonic as they can be seen in urban dwellers and smokers.
2. In *asbestosis,* dumbbell-shaped asbestos needles in clusters are diagnostic. Numerous multinucleated giant cells and histiocytes may also be seen.
3. In *silicosis* the particles are detected by polarization microscopy. The crystals appear sharp, elongated, and fragmented. Numerous neutrophils, macrophages, and multinucleated giant cells may be observed.
4. In *byssinosis* also, polarized light can be used to demonstrate the crystals. They appear as rectangular, prism-shaped crystals that shine brightly with polarized light.

Pulmonary Embolism

Pulmonary embolism causing infarction reveals bright red blood in a very tenacious, mucoid background. As the infarction resolves, the sputum becomes progressively darker in color. Microscopic examination shows erythrocytes, macrophages with denatured hemoglobin in the cytoplasm. Bacterial superinfection occurs at this stage.

Heart Disease

In certain types of heart disease, the sputum has characteristic findings.

In acute edema, the sputum is abundant, frothy and pink (up to 1 liter may be brought out in a day). Microscopically, it shows numerous RBCs and large hyaline masses (protein in nature). In mitral heart disease, the sputum is tenacious and blood is present, either in streaks or in dark masses mixed with mucus.

In chronic congestive heart failure, the sputum is frothy and rust colored. Microscopy reveals the presence of RBCs and heart failure cells. In fresh unstained sputum, these cells appear as round colorless bodies filled with various sized granules of yellow to brown pigment. This pigment (hemosiderin) can be demonstrated by staining with 10% potassium ferrocyanide for a few minutes and then with 0.1 N HCl. Hemosiderin pigment stains a blue color.

Viral Infections

Viruses are responsible for 70% to 90% of all respiratory infections. Preparation of sputum specimens for viral examination is similar to sputum cytology for malignant cells. Instead of examining for malignant changes in cells, the presence of inclusion bodies is looked for.

The inclusion bodies of herpes simplex and adenovirus are intranuclear. Herpes simplex is easier to identify and the changes are seen in the young columnar or squamous exfoliated cells. These mononuclear cells along with giant cells develop intranuclear eosinophilic inclusion bodies surrounded by a halo.

Eosinophilic intracytoplasmic inclusions are seen in parainfluenza and measles virus infections, while basophilic intracytoplasmic inclusions are present in respiratory syncytial and cytomegalic viral infections.

Pulmonary Alveolar Proteinosis

Lung biopsy is confirmatory but microscopic examination of the sputum shows an increase of hypertrophic, hyperplastic alveolar cells with a granular protein deposit in the background.

Cytologic Examination in Malignancy

Cytologic sputum examination forms an extremely important diagnostic test and gives a 50% yield in positive cases. The most ideal specimen is the single, early morning, 'deep cough' sputum and should be collected on 3 or 5 consecutive mornings. The samples (unfixed) should be submitted to the laboratory fresh. Examine the fresh specimen and select the tissue flecks and bloody areas for smearing onto a clean slide. The accepted criterion for a satisfactory sputum sample is the presence of alveolar macrophages. Four slides are prepared for examination and stained with the Papanicolaou stain. If multiple sputum collections are impractical, the most reliable sample then is the postbronchoscopy sputum specimen.

Central bronchogenic carcinoma gives the highest percentage positive results in sputum examination, although peripheral carcinomas and metastatic carcinomas may sometimes yield positive results.

CHAPTER 15

Pregnancy Tests

Pregnancy test is a misnomer as most of the methods employed measure human chorionic gonadotropin (hCG) and not the presence of fetus. hCG is a glycoprotein produced by trophoblastic cells beginning about 10 days after conception. hCG is a dimer—the subunits are nonspecific (shared with LH, FSH and TSH), the β subunits are unique to hCG.

Five weeks after last menstrual period (*LMP), hCG begins to rise rapidly in urine and attains peak levels at 10 weeks of gestation.*

For laboratory confirmation of early pregnancy, hCG is the most logical measurement (for evaluation of fetal distress during the third trimester, estriol is more useful).

BIOASSAYS

Historical Aspects

Aschheium and Zondek Test (1928)

Over a 2-day period give multiple injections of urine to 5 immature female mice about 1 day old, weighing 5 to 7 g each. Sacrifice all animals 4 days after the first injection. Examine their ovaries for corpus luteum formation. This test is reliable but too long and time consuming for general clinical use.

Friedman Test (1931)

A mature female rabbit is injected intravenously with urine; at 48 hours the ovaries are examined for corpora lutea and hemorrhagic follicles (The rabbits used should be isolated for 30 days before use to avoid false positive results).

Bellerby Test (1934)

Female South African clawed toads, *Xenopus laevis*, deposit eggs within 24 hours following injection with hCG. Since, large doses of hCG are required, concentration by alcohol precipitation or kaolin adsorption is necessary for adequate sensitivity.

Rat Ovarian Hyperemia Test of Frank and Berman

Two immature female rats weighing 45 to 60 g each are given two injections of urine/serum and sacrificed 24 hours later using carbon monoxide. Positive result is indicated by ovarian hyperemia due to capillary dilatation associated with thecal cell hyperplasia.

Tests Using Male Toad/Male Frog

Galli-Mainini used male toad *Bufo arenarum,* while Wiltberger and Miller used male frog *Rana pipiens*: 4 to 6 hours after injection with hCG, these animals release sperm which can be detected microscopically. For adequate sensitivity concentration is required.

Normal Values of hCG (Serum/Plasma)

hCG		*SI units*
Males	< 5.0 mIU/mL	< 5.0 IU/L
Females		
Nonpregnant	< 5.0 mIU/mL	< 5.0 IU/L
< 1 week gestation	5–50 mIU/mL	5–50 IU/L
2 weeks gestation	50–500 mIU/mL	50–500 IU/L
3 weeks gestation	100–10,000 mIU/mL	100–10,000 IU/L
4 weeks gestation	1000–30,000 mIU/mL	1000–30,000 IU/L
5 weeks gestation	3500–115,000 mIU/mL	3500–115,000 IU/L
6 to 8 weeks gestation	12,000–27,000 mIU/mL	12,000–27,000 IU/L
12 weeks gestation	15,000–220,000 mIU/mL	15,000–220,000 IU/L

Comments

With all bioassays, drugs excreted in the urine may cause decreased sensitivity and false negative tests or even death of the animal. Quinidine, barbiturates, laxatives, antihistamines, sulfonamides, salicylates, antibiotics, ergot and morphine derivatives may cause interference. So, the patient should discontinue all medication 3 to 4 days prior to the test. High concentrations of urinary electrolytes (especially K^+), bacteria and unspecified endogenous substances, all of these may cause false negative reactions.

Toxic substances may be removed partially or completely from urine by: (i) dialysis in cellophane tubing for 30 to 60 minutes under running water, (ii) acidification with 0.1 N hydrochloric acid to a pH of about 6.0, (iii) extraction with ether, or (iv) absorption with kaolin. False positive reactions may be caused by high titers of LH and/or FSH due to menopause or primary ovarian failure, as well as number of drugs, especially phenothiazines, prochlorperazine, and promazine.

Concentration of hCG is often expressed in animal units. There are rat units, mouse units, male and female toads, frog units, etc. These units are difficult to compare. More accurate is the international unit (IU) of hCG, related to specific gonadotropic activity of 0.1 mg of a dried standard kept at the National Institute for Medical Research, London. This is the amount of activity sufficient to cause cornification of the vaginal epithelium of immature rats.

IMMUNOLOGIC METHODS

1. **Hemagglutination inhibition test.**
2. **Latex particles agglutination inhibition test.**
3. **Radioimmunoassays for the β-subunit of hCG** (this is by far the most sensitive and specific method available).
4. **ELISA/Immunochromatography.**

Most routine pregnancy tests have a sensitivity of about 0.7–1.0 IU/mL. ELISA tests have sensitivity of up to 20 mIU/mL.

Immunologic Tests for Pregnancy

If purified hCG is injected into an animal, a specific hCG-neutralizing antibody will be formed. Several tests for the presence of hCG in urine depend on the specificity of an antigen-antibody reaction.

Before proceeding with the test, perform the heat coagulation test for proteinuria, if positive—a false positive pregnancy test can be anticipated. The test is done in one of the two main ways, which differ in the carrier used for the external source of hCG. In one method, the patient's urine—1 drop—is mixed with 1 drop of an antiserum. The mixture is then incapable of reacting with the hCG carried on a suspension of latex particles which is added next and no agglutination now occurs.

If the test urine does not contain hCG, the added antiserum will not be neutralized, when the hCG-coated latex principle suspension is added, the still active antiserum will react with the hCG on the particles causing them to agglutinate. A positive result is thus shown by an even suspension of the latex particles, a negative result, by clumping. This, then, is a latex agglutination inhibition test (False positive results may occur in proteinuria). In some methods, the latex particles are coated with hCG antibody, hence, the reversal of readings and interpretations should be considered, i.e. agglutination implying a positive result.

In the other type of method, performed in a tube, the external hCG, of the system is carried on red cells, which are said to be sensitized to hCG. The test urine is mixed with anti-hCG serum, and then with a suspension of sensitized red cells. If the urine contains hCG, the anti-hCG serum will be neutralized and the sensitized red cells will be unaffected, they fall to the bottom of the tube, forming a well-defined ring. This is a positive reaction. If the urine does not contain hCG, the still active antiserum will agglutinate the hCG-sensitized red cells, which will form an evenly dispersed layer on the bottom of the test tube. Thus, the test can be regarded as a hemagglutination inhibition test. This is not affected by proteinuria.

Both procedures may be performed with dilutions of urine in order to assess the 24 hours output of hCG in the follow-up for placental-trophoblastic tumors. The hemagglutination test is easier to read and the slide test requires less time and less equipment. The use of known positive and negative controls is strongly recommended for both methods. Latest methods using ELISA (enzyme-linked immunosorbent assay) are being marketed, these are more sensitive tests.

SLIDE TEST FOR PREGNANCY

(*Foretel® from* Tulip Group of Companies)

Latex Agglutination Inhibition Method

Summary

Human chorionic gonadotropin (hCG), a hormone produced by viable placental tissue during pregnancy, is excreted in urine approximately 20 days after the last

menstrual period. The levels of hCG rise rapidly reaching peak levels after 60 to 80 days and then the hCG levels fall suddenly and eventually plateau out.

The hCG molecule consists of two combined dissimilar subunits namely, alpha and beta. The alpha subunit is practically identical to the alpha subunit of luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid stimulating hormone (TSH) and the pituitary glycoprotein hormone. The beta subunit of hCG, by virtue of its unique amino acid sequence and content, confers biological and immunological specificity to the entire hCG molecule.

The appearance of hCG in urine soon after conception and its rapid rise in concentration make it an ideal marker for detection and confirmation of pregnancy. However, elevated hCG levels are frequently associated with trophoblastic and non-trophoblastic neoplasms; these conditions should be considered before a diagnosis of pregnancy can be made.

FORETEL slide test for pregnancy employs monoclonal antibodies specific to the beta subunit of hCG.

Reagents

1. Anti-beta human chorionic gonadotropic antibody (mouse monoclonal); The antibodies are adjusted to provide a sensitivity of about 0.3 IU/mL of hCG.
2. Suspension of polystyrene latex particles to which hCG has been chemically coupled. Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

a. Store the reagents at 2–8°C. Do not freeze.
b. The shelf life of the reagents is as per the expiry date mentioned on reagent vial labels.

Principle

FORETEL slide test for pregnancy utilizes the principle of latex agglutination inhibition. The urine specimen to be tested is first mixed with the antibody reagent containing antibodies directed against the beta subunit of hCG. Then hCG coupled latex reagent is added and the mixture is allowed to react. When the urine specimen is from a non-pregnant woman and does not contain hCG, the anti-beta hCG monoclonal antibodies will be free to react with latex coupled hCG causing agglutination. When the urine specimen is from a pregnant woman and contains atleast 0.3 lU/mL of hCG, the anti-beta hCG monoclonal antibodies will be neutralized and will not react with latex coupled hCG antigen. Hence, no agglutination will be observed.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagents contain sodium azide, 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. The reagents can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of reagents should be verified by testing with known positive and negative urine controls.
4. Use reagents of the same lot numbers. Do not interchange reagents to different lot numbers.
5. Do not interchange vial droppers.
6. Shake the latex antigen vial well before use to disperse the latex particles uniformly and improve test readability.
7. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry. Do not use detergents, soaps or organic solvents to clean the slide.

Sample Collection and Preparation

Qualitative Method

Though random urine specimens can be used, first morning urine specimen is preferable. Specimens should be collected in clean glass or plastic containers free of detergents. Specimens should be tested immediately preferably within 12 hours of collection. Should a delay in testing occur, add thimerosal (0.001%) or sodium azide (0.01%) to the specimen and store at 2–8°C up to 72 hours. Do not use grossly contaminated specimens and if the specimen is cloudy or bloody, centrifuge at 1000 rpm (125 g) for one minute and use clear supernatant for testing.

Semi-quantitative Method

Specimens collected over a 24-hours period should be pooled in a clean detergent free container and refrigerated at 2–8°C. Thimerosal (0.001%) or sodium azide (0.01%) are recommended as urine preservatives.

Material Provided with the Kit

Reagent Pack

Anti-beta human chorionic gonadotropic antibody (mouse monoclonal), hCG latex antigen.

Accessories Pack

Glass slide with three reaction circles, pipettes for dispensing urine specimen, mixing sticks, rubber teats.

Additional Material Required

Positive and negative urine controls, isotonic saline, a high intensity direct light source, stopwatch.

Test Procedure

Bring all reagents and samples to room temperature before use.

Qualitative Method

1. Place one drop of urine (specimen or control) on the glass slide using a disposable pipette provided with the kit. Deliver the drop vertically.
2. Add one drop of anti-beta hCG antibody to the drop of urine on the slide. Deliver the drop vertically.
3. Using a mixing stick, mix the antibody and urine uniformly over the entire circle for 30 seconds.
4. Add one drop of latex reagent to the mixture. Mix uniformly over the entire circle. Do not let the dropper tip touch the liquid on the slide.
5. Immediately start a stopwatch. Rock the slide gently back and forth, observing for agglutination macroscopically at 3 minutes.

Semi-quantitative Method

1. Measure and record the total volume of patient urine collected over a 24-hour period.
2. Using isotonic saline, prepare progressive dilutions from an aliquot of collected urine specimen. (1:2,1:4,1:8 and so on.)
3. Perform the qualitative test procedure using each dilution as specimen.

Interpretation of Results

Qualitative Method

Agglutination is a negative test result indicating the absence of detectable levels of hCG.

No agglutination is a positive test result indicating the presence of detectable levels of hCG.

Semi-quantitative Method

No agglutination in the highest urine dilution corresponds to the amount of hCG/mL. To calculate the concentration of hCG in the specimen, use the following formula, hCG = S × D

where, S = sensitivity of the test, i.e. 0.3 IU/mL

D = highest dilution of urine showing no agglutination.

For determining 24 hour hCG concentration, use the following formula, hCG 24 hours = S × D × V where, V = volume of 24 hours urine specimen.

Remarks

1. Patient specimens, in pathological conditions such as a hydatidiform mole or choriocarcinoma or testicular tumor, may contain hCG and produce a positive test result not necessarily indicating a pregnancy.
2. Values of hCG greater than 250 IU/mL, 110 days after LMP, suggest the presence of a pathological condition such as a hydatidiform mole or choriocarcinoma.
3. Use only urine as test specimen. Do not use serum.
4. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.

SLIDE TEST FOR PREGNANCY

(*Foresight from* Tulip Group of Companies)

Direct Latex Agglutination Method

Summary

Human chorionic gonadotropin (hCG), a hormone produced by viable placental tissue during pregnancy, is excreted in urine approximately 20 days after the last menstrual period. The levels of hCG rise rapidly reaching peak levels after 60 to 80 days and then the hCG levels fall suddenly and eventually plateau out.

The hCG molecule consists of two combined dissimilar subunits namely, alpha and beta. The alpha subunit is practically identical to the alpha subunit of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). The beta subunit of hCG, by virtue of its unique amino acid sequence and content, confers biological and immunological specificity to the entire hCG molecule.

The appearance of hCG in urine soon after conception and its rapid rise in concentration makes it an ideal marker for detection and confirmation of pregnancy. However, elevated hCG levels are frequently associated with trophoblastic and non-trophoblastic neoplasms, these conditions should be considered before a diagnosis of pregnancy can be made. Foresight slide test for pregnancy employs monoclonal antibodies specific to the beta subunit of hCG.

Reagent

1. Foresight latex reagent: A uniform suspension of polystyrene latex particles coated with beta hCG specific mouse monoclonal antibodies.
2. Positive control, reactive with the Foresight latex reagent.
3. Negative control, non-reactive with the Foresight latex reagent.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

Store the reagent at 2–8°C. Do not freeze.

The shelf-life of reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Foresight slide test for pregnancy utilizes the principle of direct latex agglutination. The urine specimen to be tested is mixed with the latex reagent coated with beta hCG specific mouse monoclonal antibodies and mixture is allowed to react. When the urine specimen is from a pregnant woman and contains at least 0.2 lU/mL of hCG, it reacts with latex reagent coated with anti-beta hCG causing agglutination.

When the urine specimen is from a non-pregnant woman and does not contain hCG it does not react with the latex reagent coated with beta hCG specific monoclonal antibodies and hence no agglutination is observed.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Reagent contains 0.1% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagent should be verified by testing with known positive and negative urine controls.
4. Do not interchange vial droppers/caps.
5. Shake the latex reagent vial well before use to disperse the latex particles uniformly and improve test readability.
6. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry. Do not use detergents, soaps, or organic solvents to clean the slide.
7. Accessories provided with the kit only must be used for optimum results.

Specimen Collection and Preparation

Qualitative Method

Though random urine specimens can be used, first morning urine specimen is preferable. Specimens should be collected in clean glass or plastic containers free of detergents. Specimens should be tested immediately preferably within 12 hours of collection. Should a delay in testing occur, add thimerosal (0.001%) or sodium azide (0.01%) to the specimen and store at 2–8°C up to 72 hours. Do not use grossly contaminated specimens and if the specimen is cloudy or bloody, centrifuge at 1000 rpm (125 g) for one minute and use clear supernatant for testing.

Semi-quantitative Method

Specimens collected over a 24-hour period should be pooled in a clean detergent free container and refrigerated at 2–8°C. Thimerosal (0.001%) or sodium azide (0.01%) are recommended as urine preservatives.

Material Provided with the Kit

Latex reagent coated with beta hCG-specific mouse monoclonal antibodies, Positive control, negative control, glass slide with three reaction circles, Pipettes for dispensing urine specimen, mixing sticks, rubber teats.

Additional Material Required

Isotonic saline, A high intensity direct light source, stopwatch.

Test Procedure

Bring reagent and urine specimen to room temperature before testing.

Qualitative Method

1. Place one drop of urine (specimen or control) on the glass slide using the disposable pipette provided with the kit. Deliver the drop by holding the dropper vertically.
2. Even for dispensing controls, the use of sample dispensing pipettes is recommended.
3. Add one drop of latex reagent to the specimen. Mix uniformly over the entire circle. Do not let the dropper tip touch the liquid on the slide.
4. Immediately start the stopwatch. Rock the slide gently back and forth, observing for agglutination macroscopically at 2 minutes.

Semi-quantitative Method

Measure and record the total volume of patient's urine collected over a 24-hour period. Using isotonic saline, prepare progressive dilutions from an aliquot of collected urine specimen (1:2, 1:4, 1:8 and so on). Perform the qualitative test procedure using each dilution as specimen.

Interpretation of Test Results

Qualitative Method

Agglutination is a positive test result and indicates presence of detectable levels of hCG in the specimen indicating pregnancy. No agglutination is a negative test result and indicates absence of detectable levels of hCG.

Semi-quantitative Method

To calculate the concentration of hCG in the specimen, use the following formula:

hCG = S × D

where, S = Sensitivity of the test, i.e. 0.2 IU/mL

D = Highest dilution of urine under test showing agglutination.

Remarks

1. Patient specimen, in pathological conditions such as hydatidiform mole or choriocarcinoma, may contain hCG and produce a positive test result not necessarily indicating a pregnancy.
2. During the first trimester of a normal pregnancy, hCG levels as high as 160–180 lU/mL may be obtained.
3. Values of hCG greater than 250 lU/mL, 110 days after LMP, may suggest the presence of pathological condition such as hydatidiform mole or choriocarcinoma.
4. Foresight is designed to detect hCG levels attained during the course of a normal pregnancy. As in case of similar pregnancy tests, prozoning may occur with hCG levels above 260 lU/mL.
5. Use only urine as test specimen. Do not use serum.
6. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
7. If the result is negative and pregnancy is still suspected, the test should be repeated with a fresh specimen collected after a week.

ELISA PREGNANCY TEST

(*Vectra from* Tulip Group of Companies)

Introduction

Vectra pregnancy test is a rapid, visual, qualitative, enzyme immunoassay for the determination of human chorionic gonadotropin (hCG), a marker for pregnancy in urine/serum.

Principle

Vectra pregnancy test utilizes the principle of sandwich enzyme immunoassay, with a unique mono-antibody combination specific against hCG present in urine/serum.

The patient urine/serum specimen is allowed to react with the monoclonal antibody directed against hCG, coated on the microtiter wells and the monoclonal antibody-enzyme conjugate complex. If hCG is present in the test specimen, antibody-hCG-antibody-enzyme complex will be formed on the surface of the microtiter well. Washing the well under running tap water will clear off the unbound complex and the unreacted conjugate. Incubating the well with substrate reagent results in development of blue color. The intensity of the blue color is proportional to the concentration of hCG present in the urine/serum specimen. Visual comparison of the intensity of the blue color with test specimen well as against the positive control well indicates the concentration of hCG greater than or equal to 25 mlU/mL of hCG in the test specimen.

DIPSTICK ICT PREGNANCY TEST

(*Clue from* Orchid Biomedical Systems)

Clue one step pregnancy test is a rapid, self-performing, qualitative, two-site sandwich immunoassay for the determination of human chorionic gonadotropin (hCG), a marker for pregnancy, in urine specimens.

Summary

Human chorionic gonadotropin (hCG), a glycoprotein hormone secreted by viable placental tissue during pregnancy, is excreted in urine approximately 20 days after the last menstrual period. The levels of hCG rise rapidly reaching peak levels after 60 to 80 days.

The appearance of hCG in urine soon after conception and its rapid rise in concentration makes it an ideal marker for the early detection and confirmation of pregnancy. However, elevated hCG levels are frequently associated with trophoblastic and non-trophoblastic neoplasms and hence these conditions should be considered before a diagnosis of pregnancy can be made.

Clue one step pregnancy test detects the presence of hCG in urine specimens, qualitatively, at concentrations as low as 10 mlU/mL in less than 5 minutes.

Principle

Clue one step pregnancy test utilizes the principle of immunochromatography, a unique two site immunoassay on a membrane. As the test sample flows through the membrane assembly of the dipstick, the colored anti-hCG-colloidal gold conjugate complexes with the hCG in the sample. This complex moves further on the membrane to the test region where it is immobilized by the anti-hCG coated on the membrane leading to formation of a pink-colored-band which confirms a positive test result. Absence

of this colored-band in the test region indicates a negative test result. The unreacted conjugate and unbound complex if any move further on the membrane and are subsequently immobilized by the anti-mouse antibodies coated on the membrane at the control region, forming a pink band. This control band serves to validate the test results.

Reagents and Materials Supplied

Each individual pouch contains :

1. Dipstick: Membrane assembly predispensed with anti-hCG antiserum-colloidal gold conjugate and anti-hCG antiserum and anti-mouse antiserum at the respective regions.
2. Desiccant pouch.

Storage and Stability

The sealed pouches in the test kit may be stored between 4–30°C till the duration of the shelf life as indicated on the pouch.

Note

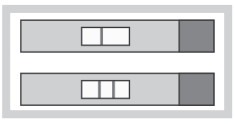
1. For in vitro diagnostic use only. Not for medicinal use.
2. Do not use beyond expiry date.

Specimen Collection and Preparation

Though random urine specimens can be used, first morning urine specimen is preferable as it contains the highest concentration of hCG. Specimens should be collected in clean glass or plastic containers. If testing is not immediate, the urine specimens may be stored at 2–8°C for up to 72 hours. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Test Procedure and Interpretation of Results

1. Collect urine specimen in a clean test tube. Ensure that only sufficient quantity of the specimen is collected to allow submerging the red area of the dipstick (About 1 cm high).
2. Bring the sealed pouch to room temperature, open the pouch and remove the dipstick. Once opened, the dipstick must be used immediately.
3. Dip the red area of the dipstick in the urine specimen submerging only the red area.
4. Observe for the release of the colloidal gold complex on the membrane. This would be seen as a pink moving front on the membrane and could take 10 to 15 seconds to appear depending upon the sample.
5. Remove the dipstick and place horizontally on a flat surface. Alternatively the dipstick may be left to stand in the specimen for the entire duration of the test ensuring only the red area is left submerged in the specimen.
6. At the end of 5 minutes read the results as follows:



Negative: Only one pink-colored-band appears on the dipstick.
Positive: Two distinct pink-colored bands appear on the dipstick.

7. The test should be considered invalid if neither the test band nor the control band appears. Repeat the test with a new dipstick ensuring sufficient dip time as mentioned in point no. 4.

Limitation of the Test

1. A number of conditions other than pregnancy including trophoblastic and non-trophoblastic neoplasms such as hydatidiform mole, chorio-carcinoma, etc. cause elevated levels of hCG. Such clinical conditions must be ruled out before diagnosis of pregnancy can be made.
2. Highly dilute urine specimens and specimens from very early pregnancy may not contain representative levels of hCG. If pregnancy is still suspected, repeat the test with first morning urine after 48–72 hours.
3. As with all diagnostic tests, the results must be correlated with clinical findings.

DEVICE ICT PREGNANCY TEST

(*Clue® from* Orchid Biomedical Systems)

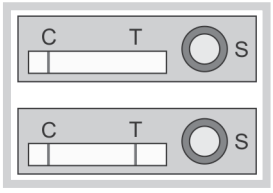
Specimen Collection and Preparation

Though random urine specimens can be used, first morning urine specimen is preferable as it contains the highest concentration of hCG. Specimens should be collected in clean glass or plastic containers. If testing is not immediate, the urine specimens may be stored at 2–8°C for up to 72 hours. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Test Procedure and Interpretation of Results

1. Bring the sealed pouch to room temperature, open the pouch and remove the device. Once opened, the device must be used immediately.
2. Dispense two drops of urine specimen into the sample well'S' using the dropper provided. Refrigerated specimens must be brought to room temperature prior to testing.

3. At the end of 5 minutes read the results as follows:



Negative: Only one colored-band appears on the control region 'C'.
Positive: In addition to the control bands, a distinct colored-band also appears on the test region T.

4. The test should be considered invalid if neither the test band nor the control band appear. *Repeat the test with a new device.*
5. Although, depending on the concentration of hCG in the specimen, positive results may start appearing as early as 30 seconds, negative results must be confirmed only at the end of 5 minutes.

Limitation of Tests

1. A number of conditions other than pregnancy including trophoblastic and non-trophoblastic neoplasms such as hydatidiform mole, choriocarcinoma, etc. cause elevated levels of hCG. Such clinical conditions must be ruled out before a diagnosis of pregnancy can be made.
2. Highly dilute urine specimens and specimens from very early pregnancy may not contain representative levels of hCG. If pregnancy is still suspected, repeat the test with first morning urine after 48–72 hours.
3. As with all diagnostic tests, the results must be correlated with clinical findings.

ICT Techniques for Urine/Serum Sample

For routine pregnancy testing, urine is the most preferred specimen, requiring no specialized skills for collection, processing and storage. However, during problem pregnancies and bad obstetrics cases, levels of hCG in urine are very low. Due to factors such as fluid intake, time of collection of specimen, urine may not contain representable amounts of hCG, thereby affecting the sensitivity of membrane based one step assays.

Advantages of Serum Testing

- During normal pregnancies, hCG is present as an intact molecule in maternal serum.
- Serum hCG levels can be detected within 24 hours post-implantation.
- Serum specimens facilitate early pregnancy detection.
- hCG is uniformly distributed in serum and is not affected by fluid intake and can be collected at any time of the day.
- hCG is a very stable molecule when stored in serum.

DIPSTICK ICT, URINE/SERUM PREGNANCY TEST

(*Gravi check from* Orchid Biomedical Systems)

Specimen Collection and Preparation

Urine as Sample

Though random urine specimens can be used, first morning urine specimen is preferable as it contains the highest concentration of hCG. Specimens should be collected in clean glass or plastic containers. If testing is not immediate, the urine specimens may be stored at 2–8°C for up to 72 hours. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Serum as Sample

No special preparation of the patient is necessary prior to specimen collection by approved techniques. Though fresh serum is preferable, serum specimens may be stored at 2–8°C for up to 24 hours, in case of delay in testing. Do not use hemolyzed or contaminated specimens. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Test Procedure and Interpretation of Results

1. Collect urine/serum specimen in a clean test tube. Ensure that only sufficient quantity of the specimen is collected to allow submerging the orange area of the dipstick (About 1 cm high).
2. Bring the sealed pouch to room temperature, open the pouch and remove the dipstick. Once opened, the dipstick must be used immediately.
3. Dip the orange area of the dipstick in the urine/serum specimen submerging only the orange area.
4. For urine samples: Dip the dipstick in the urine sample for 10–15 seconds and place horizontally on a flat surface. Alternatively, the dipstick may be left to stand in the specimen for the entire duration of the test ensuring only the orange area is left submerged in the specimen. At the end of 5 minutes, read the results as described below.
   For serum samples: Leave the dipstick in the specimen for entire duration of the test ensuring only the orange

area is submerged in the specimen. Read the results at the end of 15 minutes as follows:

Negative: Only one colored-band appears on the dipstick.
Positive: Two distinct colored bands appear on the dipstick.

5. The test should be considered invalid if neither the test band nor the control band appears. Repeat the test with a new dipstick ensuring sufficient dip time.

Limitations of the Test

1. A number of conditions other than pregnancy including trophoblastic and non-trophoblastic neoplasms such as hydatidiform mole, choriocarcinoma, etc. cause elevated levels of hCG. Such clinical conditions must be ruled out before a diagnosis of pregnancy can be made.
2. Highly dilute urine specimens and specimens from very early pregnancy may not contain representative levels of hCG. If pregnancy is still suspected, repeat the test with first morning urine after 48–72 hours after the initial test.
3. As with any assay employing animal antibodies, presence of cross-reacting heterophilic antibodies may yield discrepant results.
4. As with all diagnostic tests, the results must be correlated with clinical findings.

DEVICE ICT URINE/SERUM PREGNANCY TEST

(*Gravi check from* Orchid Biomedical Systems)

Specimen Collection and Preparation

Urine as Sample

Though random urine specimens can be used, first morning urine specimen is preferable as it contains the highest concentration of hCG. Specimens should be collected in clean glass or plastic containers. If testing is not immediate, the urine specimens may be stored at 2–8°C for up to 72 hours. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Serum as Sample

No special preparation of the patient is necessary prior to specimen collection by approved techniques. Though fresh serum is preferable, serum specimens may be stored at 2–8°C for up to 24 hours, in case of delay in testing. Do not use hemolyzed or contaminated specimens. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Altered Laboratory Results in Normal Pregnancy

Test	*Alteration*	*Demonstrated or possible reason*
Hematology		
Hb	Falls but not lower than 11 g%	Plasma volume expands more than RBC mass
WBC	Mild neutrophilia; no lymphocytosis	Similar response seen in stress, strenuous exercise
Platelets	Slight decrease or no change	Plasma volume expansion
Reticulocytes	Rise to 2–5%	Need to increase RBC mass
Blood volume	Rises by 40–50%	Increase in plasma and red cells
Serum iron	Falls modestly, even if stores are adequate	Loss to fetal blood supply; increased plasma volume; supplementation is desirable
Iron binding capacity	Increases	Estrogen-induced increase in protein synthesis
Folate levels	Serum concentration falls; RBC concentration should be constant	Fetal use; estrogen associated folate block Supplementation desirable
ESR	Increases markedly	Elevated fibrinogen levels

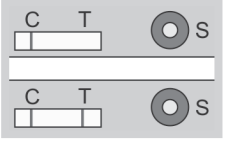
Contd...

Contd...

Test	*Alteration*	*Demonstrated or possible reason*
Coagulation		
Fibrinogen	Rises about 50%	? Acute phase reaction
PT and PTT	Normal or slightly shortened	Increased factor levels affect tests relatively little
Plasminogen	Increases	?
Antithrombin III	Decreases moderately	Comparable effect seen with therapy
Fibrin degradation products	Increased slightly	? Effect of increased plasminogen and decreased antithrombin III ? Effect of fibrin deposition in placenta
Chemistry		
Serum albumin	Decreases by as much as 1 g%	Increased degradation, hemodilution
Immunoglobulins	Ig G, Ig A drop	? Degradation ? Altered immunologic responsiveness
Serum alkaline phosphatase	↑ by 200–300%	Placental alkaline phosphatase in serum
Serum cholesterol	↑ 30–40%	? Effect of placental hormones
Serum free fatty acids	↑ 50–60%	? Effects of placental hormones, altered insulin reactivity
Serum creatinine	↑	Hemodilution; ↑ Glomerular filtration rate (GFR)
Plasma bicarbonate	↑ 10–15%	Compensation for chronic hyperventilation
Urine glucose	Mild glycosuria with normal blood sugar levels	Lowered renal threshold
Renal function	GFR increases, Concentrating capacity ↑	Mobilization of retained fluid
Endocrine		
Thyroid Total and		
Free T_4	↑ and normal respectively	
Total and free T_3	↑ and normal respectively	
ACTH	Markedly ↑ in early months	?
Serum total cortisol	↑	Estrogen—associated rise in binding protein
Serum testosterone	↑	? ↑ ovarian synthesis
Parathormone	↑	?
Aldosterone	Progressive ↑	Altered plasma volume, renal sodium load
Renin—angiotensin	↑ in latter half	With ↑ Na or ↑ blood pressure, level decreases

Test Procedure and Interpretation of Results

1. *Bring the sealed pouch to room temperature,* open the pouch and remove the device. Once *opened, the device must be used immediately.*
2. Dispense two drops of urine/serum specimen into the sample well 'S' using the dropper provided. Refrigerated specimens must be brought to room temperature prior to testing.
   Read the results at the end of 5 minutes for urine samples and at the end of 15 minutes for serum samples as follows:



Negative: Only one colored-band appears on the control region 'C.'
Positive: In addition to the control band, a distinct colored-band also appears on the test region.

3. The test should be considered invalid if no band appears. Repeat the test with a new device.
4. Although, depending on the concentration of hCG in the specimen, positive results may start appearing as early as 30 to 60 seconds, negative results must be confirmed only at the end of the stipulated time.

TROUBLESHOOTING

Latex Methods

Indirect/Latex Agglutination Inhibition Method

Foretel

Problem: False Positive Results		
Possible causes		*Solutions*
1.	Latex reagent not working	The reagents should be stored at 2–8°C. Do not freeze the reagents Check the working of the latex reagent by mixing with the anti-β hCG reagent No agglutination indicates deterioration of the latex reagent
2.	Anti-β hCG reagent vial is contaminated with known positive sample. This may be due to the dropper tip having touched the specimen	Check the anti-β hCG reagent with the latex reagent. If no agglutination is observed, it indicates that the anti-β hCG reagent is not working Vial droppers must not be interchanged and reagents of the same lot numbers should be used
3.	Presence of detergent on the slide or in the sample	Wash the slide thoroughly with distilled water, wipe dry and retest Collect urine specimen in a detergent free container
4.	Very high hCG levels are found in pathological conditions like choriocarcinoma and hydatidiform mole	Do a semiquantitative test and check for high abnormal hCG levels Check for patient's history
Problem: Delayed Agglutination		
Possible causes		*Solutions*
1.	Borderline case with hCG levels just about 0.3 IU/mL	Use the first morning sample. Retest fresh sample after a week
2.	Improper mixing of urine and anti-β hCG reagent	Mix urine and anti-β hCG reagent as per instructions given in the test protocol
3.	Reagents not brought to room temperature before testing	All reagents must be brought to room temperature before testing. This is necessary for the antigen-antibody to react optimally
4.	Delayed testing	Sample should be tested within 12 hours of collection
5.	Contaminated urine specimen is used for testing	Contaminated urine specimens should not be used for testing
6.	Too early diagnosis of hCG levels below detection limit	Retest after a week using 1st morning sample
Problem: False Negative Results		
Possible causes		*Solutions*
1.	Case of ectopic pregnancy	Follow-up with the patient's history
2.	Threatened abortion progressing into inevitable abortion	Repeat test and correlate results with clinical findings
3.	The urine sample is contaminated, cloudy and bloody or is used after a long period of time	Specimen should be collected in clean glass of plastic containers free of detergents or any such material Specimen should be tested preferably within 12 hours of collection They can be stored at 2–8°C for up to 72 hours after adding preservatives thimerosal (0.001%) or sodium azide (0.01%). If the specimen is cloudy or bloody, centrifuge it at 1000 rpm for one minute and use only the clear supernatant for testing

Latex Agglutination/Direct Method

Foresight®

Problem: False Positive Results		
Possible causes		*Solutions*
1.	Urine sample may have heavy bacterial growth	Check the container used for specimen collection. Specimen should be collected in a clean and dry container Do not use grossly contaminated specimens. If the specimen is hazy or bloody, centrifuge at 1000 rpm for one minute and use clear supernatant for testing
2.	Wrong sample tested	Label and test correct sample. Record results accordingly
3.	Latex reagent contaminated with positive control/ positive sample	Ensure that the dropper tip does not touch the sample/control on the slide while dispensing Take one or two drops of the latex reagent on a slide, and check for granulation, aggregation or agglutination. There should be no granulation, aggregation or agglutination
4.	Presence of detergent on slide	Wash slide thoroughly with water, dry and retest
5.	Test results are correct but reporting may be wrong	Check and verify final report correctly
6.	Test interpretations are wrong	Results should be interpreted as per test protocol Agglutination = Positive No agglutination = Negative
7.	Drying of the reagent on slide	Do not read results beyond 2 minutes Do not perform the test directly under a fan
8.	Male urine used as negative control to check the working of the kit	Do not use male urine as negative control as it will give false positive results Since the hormones, protein and salt composition of male urineis different and foresight being a direct test system is calibrated to yield accurate results on male urine. Use the negative control provided with the kit or use known negative female urine
Problem: False Negative Results		
Possible causes		*Solutions*
1.	Wrong sample used	Collect, label the samples appropriately and test accordingly
2.	Sample stored for a long period of time 0.01% should be used as preservatives. The samples can then be stored	Specimens should be tested preferably within 12 hours of collection Should a delay in testing occur, thimerosal 0.001% or sodium azide. at 2–8°C for up to 72 hours
3.	Reagent may have deteriorated due to thermal damage	Avoid exposure of the reagents to high temperatures. Store at 2–8°C. Do not freeze
4.	Prozoning due to hCG levels above 250 IU/mL (In pathological conditions such as choriocarcinoma and hydatidiform mole)	In such cases, the sample should be diluted and used. Check the patient's history
5.	Serum is being used as specimen	Use only urine and not serum as specimen.
6.	Sample drop size insufficient	Ensure that the sample is dispensed using dispensing pipette provided. Ensure that there are no air bubbles while dispensing the sample.
7.	Reagents not brought to room temperature before testing	Bring all reagents to room temperature before testing.
8.	Presence of detergent on the slide	Wash thoroughly with water and retest

RAPID FORMATS

Device/Dipstick

Clue

Problem: False Positive Results		
Possible causes		*Solutions*
1.	Presence of trophoblastic and non-trophoblastic neoplasms such as choriocarcinoma and hydatidiform mole	Check the clinical history of the patient
2.	The flow properties of the nitrocellulose membrane are partially affected leading to the nonspecific movement of partially aggregated gold-sol particles	Check the device/dipstic pouch for pinholes and observe the dessicant for any color change. The results of the test should be correlated with clinical findings
Problem: Delayed Positive Results		
Possible causes		*Solutions*
1.	Urine samples were tested immediately after removing from the refrigerator	Bring the urine samples to room temperature before testing
Problem: False Negative Results		
Possible causes		*Solutions*
1.	Inadequate quantity of sample used for testing	Dispense exactly 2 drops of the sample using the dropper provided with the kit
2.	The kit is exposed to very high temperatures leading to the deterioration of the antibodies coated on the device/dipstick	Store the kit at 4–30°C when not in use
3.	Turbid or contaminated urine samples used for testing	Do not use contaminated urine samples. In case there is a delay in testing urine samples can be stored at 2–8°C up to 72 hours. Turbid samples should be centrifuged before testing
4.	Highly diluted samples or samples of very early pregnancy used for testing	Highly diluted samples or samples of very early pregnancy may not contain representative levels of hCG. In such cases, if pregnancy is suspected, the test should be repeated with first morning urine after 48–72 hours after the initial test
Problem: Invalid Results		
Possible causes		*Solutions*
1.	Pinholes or defect in the pouch. The nitrocellulose membrane has lost its flow properties due to the absorbance of moisture	Check the pouch for pinholes and also check the color of the dessicant (silica gel) accompanying the pouch A change in color from deep blue to white/pink indicates absorbance of moisture. In such cases, discard the test device and rerun the test using a fresh device
2.	The device/dipstick is removed from the refrigerator and tested immediately before attaining room temperature. This leads to the hydration of the sites on the nitrocellulose membrane thereby adversely affecting its flow properties	The test device/dipstick should be brought to room temperature before being tested

Gravicheck®

Problem: False Positive Results		
Possible causes		*Solutions*
1.	A number of other conditions including trophoblastic and non-trophoblastic neoplasms such as hydatidiform mole, choriocarcinoma cause elevated levels of hCG	Check the clinical history of the patient, before diagnosing for pregnancy
2.	The flow properties of the nitrocellulose membrane are partially affected leading to the nonspecific movement of partially aggregated gold-sol particles	Check the device/dipstick pouch for pinholes besides also note color changes if any, in the dessicant pouch. The results of the test must be correlated with clinical findings
Problem: Delayed Positive Results		
Possible causes		*Solutions*
1.	Urine/serum sample used for testing is used immediately after removal from the refrigerator	Bring the urine/serum samples to be tested to room temperature before commencing the test procedure
Problem: False Negative Results		
Possible causes		*Solutions*
1.	Urine/serum stored for a long time is used for testing	Fresh early morning urine sample is preferable for testing, as it contains the highest concentration of hCG, however, if testing is not immediate, the urine specimen may be stored at 2–8°C for up to 72 hours Similarly, if fresh serum if used as sample is preferable, however, serum samples may be stored at 2–8°C for up to 24 hours, in case of delay in testing
2.	Inadequate quantity of sample dispensed in the sample well of the test device	Dispense exactly 2 drops of urine/serum sample in the sample well.
3.	In case of dipstick, improper submerging into the urine/serum specimen	Dip the orange area of the dipstick in the urine/serum specimen submerging only the orange area.
4.	Error in interpreting results	For urine samples, read results at the end of 5 minutes and for serum samples at the end of 15 minutes.
5.	The kit containing the test device is exposed to very high temperatures leading to the deterioration of antibodies coated on the device/dipstick	The kit should be stored at 4–30°C when not in use
6.	Turbid or contaminated samples are used for testing	Avoid using contaminated urine/serum samples. Turbid samples should be centrifuged, allowed to settle and only the clear supernatant should be used for testing
7.	Highly diluted samples or samples of very early pregnancy used for testing	Highly diluted samples or samples of very early pregnancy may not contain representative levels of hCG. In such cases, if pregnancy is suspected, the test should be repeated with first morning urine after 48–72 hours after the initial test
Problem: Invalid Results		
Possible causes		*Solutions*
1.	The pouch may be having pinholes or is in a defected condition due to which the nitrocellulose membrane has a tendency of loosing its flow properties	Check the condition of the pouch and observe for pinholes in the pouch if any before performing the test
2.	Change in color of the desiccant accompanying the pouch	A change in color of the desiccant from deep blue to white/pink indicates absorbance of moisture. In such cases, discard the test device and rerun the test using a fresh device

CHAPTER 16

Examination of Gastrointestinal Contents

NORMAL SALIVA—CONSTITUENTS

Constituents

Volume secreted/24 h	1000–1500 mL
pH	6.3 to 6.85
pH in mouth is usually	7.5–8.0
Specific gravity	1.002 to 1.008
Total solids	0.5 g%
Sodium	17.4 (8.7–24) mEq/L
Potassium	14.1 (13–16) mEq/L

GASTRIC JUICE

Constituents

Digestive enzymes/factors

Pepsin and hydrochloric acid (for protein digestion).

Renin for curdling milk, and gastric lipase—a weak lipolytic ferment.

Normal Gastric Constituents in Infants and Children

The stomach of neonates secretes small amounts of pepsin, renin, and free acid. Almost 4% of otherwise normal children have achlorhydria, this percentage gradually rises with age (30% have achlorhydria at age 60 years and above). During the first year of life, the volume of the residuum is 2–5 mL (pH = 2.6–3.0). Both these rise to adult levels at 15–20 years of age.

Abnormal Gastric Constituents

These may include the following:

1. Blood: An important abnormal finding.
2. Food remnants many hours after eating.
3. Large amounts of mucus or bile.
4. Sarcinae, pyogenic bacteria, lactobacilli, yeast cells.
5. Tissue fragments, large amounts of epithelium.
6. Parasites and ova.
7. Organic acids, e.g. lactic acid, seen in absence of hydrochloric acid.
8. Tubercle bacilli, in pulmonary tuberculosis, by swallowing of sputum containing *Mycobacterium tuberculosis.*

Routine Gastric Juice Examination

Gross Examination

1. *Amount:* Normal fasting content is 50–100 mL.
2. *Color:*
   a. Blood is red or the color of coffee ground if acid hematin is formed.
   b. Fresh bile is yellow; old bile is green.
   c. In stasis, food colors may persist.
3. *Odor:*
   a. Normal is sour or slightly rancid.
   b. Fecal in intestinal obstruction.
   c. Ammoniacal in uremia.
4. *Character:* Let stand, note the three layers:
   a. Top—Mucus
   b. Middle—Opalescent fluid
   c. Bottom—Bread-like residue.
5. *Reaction:* Acidic—normal pH.
6. *Rate of secretion:*
   a. Mean values for basal rate of secretion of acid

Age (years)	*mEq/L*
20–49	2.5
50–59	2.0
> 60	1.5

b. Mean values for 12 hours nocturnal secretion in a normal person
   Volume—580 mL
   Free acid—29 mEq/L or 16.85 mEq/12 hours.

Chemical Examination

1. Blood

May be due to one of the causes of hematemesis, or may be due to trauma of passing a tube. Do guaiac or benzidine tests.

2. Qualitative Test for Free HCl (Topfer's Test)

To 5 drops of gastric juice in evaporating dish, add 1 or 2 drops of 0.5% alcoholic solution of dimethylaminoazobenzene (Topfer's reagent). Cherry-red color occurs with HCl.

3. Titration for Acid

Method

Transfer 5 mL of gastric juice to an evaporating dish add 20 mL water. Add 3 drops of Topfer's reagent and 3 drops of phenolphthalein, and titrate with N/10 NaOH until the last trace of red color disappears. This is the amount of NaOH needed to neutralize the free HCI—this value multiplied by 20 equals the mEq/L of free HCl. Carry on titrating until red color of phenolphthalein reappears. The total number of mL of NaOH used (in both titrations) multiplied by 20 equals the mEq/L of total acidity.

Comments

In the fasting state, gastric contents ordinarily contain 0–15% HCl. For gastric juice, the maximum concentration of HCl is about 0.160 or 160 mEq of HCl/L. The free rather than total acid primarily determines the pH, which usually varies in fasting contents from 2.0-1.0, and this pH range corresponds to an HCl normality (normality = mEq/L/1000) of about 0.05–0.10. If the normality drops to 0.01 or so, the pH may be about 3.0, and it takes very little amount of food or any other diluent to bring the pH to 7.0.

Lactic Acid (Kelling's Test)

Seen usually in achlorhydria only. Add 2 drops of 10% ferric chloride to one test tube full of water, mix and divide into 2 test tubes. Add 1 mL of gastric juice to one tube and compare. Lactic acid gives canary yellow color. Much lactic acid (over 0.1%) suggests gastric carcinoma.

Microscopic Examination

Place one drop of sediment on a slide and coverslip it. Look for undigested food particles, blood, mucus, bacteria, tissue fragments, parasites, sarcinae, yeasts. Lactobacilli are large nonmotile rods, which stain brown with Gram's

Normal gastric constituents in adults

Constituents	Normal residuum	Appetite juice (postseeing/smelling/ tasting of food)
Water (%)	99.02	99.45
Total solids (%)	0.98	0.55
Organic solids (%)	0.53	0.41
Inorganic solids (%)	0.45	0.14
Specific gravity	1.006–1.009	1.007
pH	0.9–1.5	0.9–1.5
Total acidity (mEq/L)	10–50	20–100
Free HCI (mEq/L)	0–30	25–50
Chlorides (g%)	0.5–0.6	
Total nitrogen (avg: 66 mg%)	51–75	
NPN	20–30	
Urea nitrogen	1.3–4	
Total sulfur	7	
Total phosphorus	5	5
Amino acid N	3–9	3–9
Ammonia N	2–3	2–3

stain and form lactic acid, they occur in stasis in the absence of HCl.

Exfoliative cytologic preparations of fresh gastric washings should be used in the search for gastric neoplasms.

Gastric Test Meals

Procedures

If the test is to be performed in the morning, give nothing orally after supper the previous night.

Tubeless Gastric Analysis

This test employs azure-A resin as the indicator (Diagnex Blue Test).

Azure-A carbacrylic resin dissociates in the presence of acid to yield free azure-A, which is then excreted in the urine. In the absence of free acid in the stomach, no azure-A will be released and hence none will appear in the urine.

The test meal consists of caffeine with sodium benzoate to stimulate gastric secretion and azure-A resin granules as the indicator substance. Urine is analyzed for azure-A by a simple colorimetric method. On getting up in the morning, urine is micturated and discarded. Nil orally till the completion of the test. The gastric stimulant, either 500 mg caffeine sodium benzoate or 50 mg Histalog is taken with a glass of water or else histamine or Histalog can be administered subcutaneously. One hour later, patient urinates and discards the sample. Immediately thereafter, 2 g of azuresin are ingested with half glass of water. Two hours later, the patient urinates and saves the entire sample. The sample is diluted to 300 mL with water and a 10 mL aliquot is placed in each of 3 test tubes. Two of the tubes serve as color controls and to each of these approximately 300 mg of L-ascorbic acid is added. This reduces the azure-A to a colorless form. The tubes are then placed in a comparator block containing azure-A standards of 0.3 mg/300 mL and 0.6 mg/300 mL. If the color of the test urine is more intense than that of 0.6 mg standard, the test is completed and the patient is presumed to secrete hydrochloric acid. If the color of the test urine is less than that of 0.6 mg standard, a drop of solution containing 195 mg $CuSO_4.5H_2O$ in 100 mL of 18% HCl (Diagnex blue reagent) is added to each of the 3 urine tubes. All three tubes are placed in a boiling water bath for 10 minutes. After cooling at room temperature for 2 hours, the color development is again compared to the standard solutions. The results are reported as less than 0.3 to 0.6 mg, or greater than 0.6 mg.

Interpretation

This is strictly a qualitative test. An excretion of greater than 0.6 mg azure-A in 2 hours is considered to be indicative of HCl secretion, while values less than 0.3 mg are considered presumptive evidence of anacidity. Values between 0.3 and 0.6 mg represent borderline secretion.

Basal Gastric Secretion

This represents the response of the stomach to endogenous stimuli, which are continually present in the interdigestive or fasting state.

The minimum requirements include the following:

1. The patient must be in the fasting state and free from the sight or odor of food.
2. All medications influencing gastric secretion must be withheld for 24 hours.
3. The patient must be removed from environmental situations evoking untoward psychological reactions, such as fear, anger, or depression.

The 1 hour morning aspiration has replaced the cumbersome and inherently less precise 12 hours nocturnal aspiration.

Method

1. Following a 12 hours overnight fast, the patient is intubated. Water may be taken until 8 hours prior to intubation.
2. The residual volume of gastric secretion is measured and qualitatively examined.
3. Continuous aspiration is begun, preferably manually with a syringe. Segregate the aspirate into 15 minute samples. Usually, the first 1 or 2 samples are discarded to allow the patient to adjust to the intubation procedure. Subsequent to this adjustment period, four 15 minutes samples are taken.
4. If the basal secretion study is to be followed by the augmented histamine test, a suitable dose of antihistamine be given parenterally 30 minutes before completing the collection of basal secretion.
5. For each 15 minutes sample, the volume, pH, and titrable acidity are measured and the acid output calculated. The sum of the acid outputs in the 4 samples, expressed in milliequivalents, represents the 1 hour basal acid output.

Interpretation

The mean basal acid output reported for normal males ranges from 1.3 to 4.0 mEq/h. Lower values occur in females and with ageing. Somewhat lower values are reported in most large series for gastric carcinoma and benign gastric

ulcer and distinctively higher values for duodenal ulcer or jejunal ulcer following partial gastrectomy with gastrojejunostomy. Extremely high acid output is present in patients with the Zollinger-Ellison syndrome.

Augmented Histamine Test (AHT)

A dose of 0.04 mg per kg body weight is the optimum dosage that can be given, and any further increase in dosage does not increase the gastric acid output. All parietal cells capable of acid secretion are stimulated by histamine (functioning parietal cell mass). The AHT or the analogous Histalog test are now established as definitive tests for the diagnosis of anacidity.

The side effects of histamine are overcome by previous administration of antihistamine. A history of bronchial asthma or urticaria, the presence of severe cardiac, pulmonary or renal disease and paroxysmal hypertension or other possible signs and symptoms of pheochromocytoma are contraindications to the performance of this test.

Method

1. Following a 12 hours fast, basal secretion is collected for 1 hour as previously described.
2. Thirty minutes before completion of the basal secretion collection, a suitable dose of antihistamine is given IM, e.g. 10 mg chlorpheniramine maleate or 50 mg diphenhydramine hydrochloride.
3. After the conclusion of the basal secretion study, histamine acid phosphate is administered subcutaneously in a dose of 0.04 mg per kg body weight.
4. Gastric contents are then collected in 15 minute samples for 1 hour.
5. The volume, pH and titrable acidity are measured for each sample and the acid output is calculated. From these, the 1 hour or maximal acid output in mEq is computed.

Interpretation

The maximum rate of acid secretion is characteristically attained within 15 minutes after histamine injection and is maintained for approximately 30 minutes. By 60 minutes after histamine injection, acid secretion usually falls to the basal level. The maximum output, representing the sum of the acid.

The upper limit of normal is 30 mEq HCl secreted in the 30 minutes period between 15 and 45 minutes after the histamine injection. Values higher than the stated upper normal limit are usually found in duodenal ulcer and Zollinger-Ellison syndrome. Anacidity in the augmented histamine test is most commonly found in adults with pernicious anemia or gastric carcinoma, it has also been reported in other conditions, e.g. hypochromic anemia, rheumatoid arthritis, steatorrhea, aplastic anemia, myxedema, nutritional megaloblastic anemia and the asymptomatic relatives of patients with pernicious anemia.

The basal and AHT are used as determining factors for gastrectomy or vagotomy. It has been suggested that an increased functioning parietal cell mass evidenced by an elevated maximal acid output indicates the need for gastric resection. Whereas, raised basal secretion with normal or only slightly elevated maximal secretion is taken as an indication for vagotomy.

Histamine Infusion Test

The use of a slow IV infusion of histamine allows measurement of acid output in a sustained steady state.

Advantages

1. It obviates the need for doing both basal and augmented histamine tests.
2. The greater acid output achieved in the sustained steady state facilitates the detection of low levels of acid output.
3. This is a highly reproducible test.
4. The slow histamine infusion has lesser side effects.

Method

1. The patient is intubated following a 12 hours overnight fast.
2. A basal hour collection is obtained.
3. Thirty minutes before completion of the basal hour, a suitable dose of antihistamine is given intramuscularly.
4. After completion of the basal hour, an IV infusion of histamine in physiologic saline is begun and the dose rate is adjusted to deliver 0.04 mg of histamine phosphate per kg body weight per hour.
5. The infusion is continued until four 15 minute steady state samples have been collected. The initiation of the steady state is evident from the plateau reached in volume output and usually requires about 30 to 45 minutes to obtain after the start of the infusion.
6. Each sample of the basal hour and steady state is analyzed for volume, pH and titrable acidity.

Interpretation

The normal values of acid output in mEq/hour for males is 16 to 32 and for females 18 to 25. The values are markedly higher in duodenal ulcer patients.

Histalog Test

Histalog (3 β-aminoethyl pyrazole dihydrochloride, Betazole), an analog of histamine can be used instead of histamine.

Advantage: Lesser side effects and obviation of the need to give antihistamine.

The augmented Histalog dosage is 1.7 mg/kg given IM. The test is similar as AHT except that: (i) no antihistamine is needed, and (ii) eight instead of four 15 minute post-Histalog samples are collected.

The peak acid secretion in Histalog test is reached in the second to fifth 15-minute period. The peak secretory rate may last for 45 to 90 minutes.

Insulin Hypoglycemia Test

Acid secretion is stimulated by hypoglycemia caused by insulin administration. The major stimulus is transmitted via vagus nerve and can be removed by vagotomy.

Hypoglycemic response—for about 30 minutes after insulin injection there is a slight depression of gastric secretion.

The predominant effect during the remainder of the first 2 hours consists of marked enhancement of gastric secretion.

The final effect is manifested after 2 hours and is also stimulatory to gastric secretion (the second phase is via the vagus and the third is humoral via the adrenocortical hormones—hence the second but not the third stage can be abolished by vagotomy).

Method

1. Following a 12 hours overnight fast the patient is intubated. Two hours basal secretion is obtained in 15 minutes samples.
2. Blood samples for glucose estimation are obtained upon completion of the basal secretion study and at 30, 60 and 90 minutes after insulin injection.
3. Insulin is given IV either at a fixed dosage of 15 or 20 units or at a calculated dosage of 0.20 units per kg of body weight (keep a 50 mL syringe filled with 50%-w/v-glucose solution readily available to counteract any serious hypoglycemic effects).
4. Gastric secretion is collected in 15 minute samples for 2 hours after insulin.
5. For each basal and postinsulin gastric sample, the volume and titrable acidity are determined, and the acid output is computed.

Interpretation

This test is valid only if the blood glucose falls below 50 mg% at some point of the test, which will usually be 30 minutes after insulin administration. Validity of the test also depends upon the capability of the stomach to secrete hydrochloric acid. Hence, if no acid is present in either the basal or postinsulin periods, it is necessary to perform an augmented histamine test in an attempt to evoke acid secretion. If the stomach is truely anacidic, no conclusion can be drawn regarding the completeness of vagotomy, but the question of simple peptic ulceration is then effectively excluded.

The patient can be considered completely vagotomized if the acid output in the greater of the two postinsulin hours is less than the greater of the two basal hours. Incomplete vagotomy is likely if the acid output in the 2 hour postinsulin period exceeds that of the 2 hour basal period by more than 0.5 mEq. Incomplete vagotomy is also suggested by an acid output of greater than 2 mEq in either basal hour. In incomplete vagotomy if acid output is elevated in the first postinsulin hour—the prognosis is bad in the sense that recurrence may occur; whereas, elevation in the second hour is less likely to be followed by a recurrence.

Gastrin Secretory Test

One mg of gastrin (prepared from gastric antrum of Swine) per kg of body weight can be given subcutaneously or else a single 50 g IV injection can be given. Most subjects will show a maximum output beginning about 20 minutes after gastrin injection and will maintain this level of acid output for 20 to 40 minutes. The response is quite rapid with IV administration, with peak levels occurring in 5 to 10 minutes.

Pentagastrin (a synthetic pentapeptide with gastrin nucleus) can be used instead of gastrin, the results are reproducible and without the side effects of histamine.

Miscellaneous Investigations

Mycobacterial culture: Individuals having pulmonary tuberculosis but cannot produce sputum or in children who cannot effectively expectorate, this method of aspirating and culturing the gastric contents is quite useful. It is essential that the gastric contents be collected in the early morning prior to eating or drinking and preferably immediately upon awakening before increased gastric motor activity has largely emptied its contents. The sample withdrawn should be immediately submitted for culturing.

Exfoliative cytology: For diagnosing gastric carcinomas—gastric cytology, gastroscopy and roentgenography—can be used, but the most discriminating information is provided by exfoliative cytology (chymotrypsin can be used to facilitate the exfoliation of cells by liquefying the mucus coating). Diagnosis rate is almost 90%.

EXAMINATION OF DUODENAL CONTENTS

Duodenal Drainage

Indications

1. For diagnosis of liver or biliary tract disease. Drainage may be done to help diagnose exacerbations of chronic infections early so that they can be controlled.
2. For other diagnostic purposes relating to parasites, pancreatic enzyme, etc.
3. For therapeutic drainage in cholangitis or biliary obstruction.

Method for Diagnostic Drainage

1. Give nothing orally after midnight.
2. In the morning intubate (Rehfuss or Levintube) to a length of 50 cm (29 inches). Withdraw gastric specimen.
3. With the patient erect or lying on his right side before the fluoroscope, feed and massage tube into middle third of the duodenum. Now aspirate duodenal contents for 5–30 minutes and label "A", this evacuation specimen is of little value for bile study.
4. Slowly inject 50 mL of warm 33% magnesium sulfate through the tube to relax sphincter of Oddi. Clamp tube for 5 minutes then drain for 30 minutes and label "B". Gallbladder bile is first dark, then lighter. If no "B" bile is obtained, inject another 50 mL of magnesium sulfate. If still unsuccessful, inject 30 mL of olive oil.
5. During the final period of 30 minutes, try to collect yellow hepatic bile. Label it "C".

Examination for Diagnosis

1. Note density, color, and flocculi in all three specimens. Test for bile, blood, reaction, and ferments as necessary.
2. *Microscopy*: This is important in detecting early cholelithiasis (gallsand). Note pus cells, bacteria, cellular elements and crystals.
3. Giardia or other parasites may be present.
4. Culture for bacteria, especially typhoid bacilli.

Interpretation

1. Absence of dark "B" bile indicates loss of gallbladder function. No bile may appear in common duct obstruction.
2. In cholelithiasis, many cholesterol and calcium bilirubinate crystals appear in "B" and "C" bile. The cholesterol crystals may be perfect or atypical or may be mixed with cellular detritus. The calcium bilirubinate comes as yellow or reddish particles in the size of a pinhead.
3. In biliary tract inflammation, there is much yellow cellular and bacterial materials in "B" and "C" bile.
4. Blood may be grossly visible in advanced carcinoma.

COMPOSITION OF BILE

Gross and Chemical Characteristics

a. Volume per 24 hours: 700–1000 mL
b. Specific gravity: Hepatic duct—1.01, gallbladder —1.026 to 1.032.
c. *Total Solids*

	Hepatic duct (g%)	*Gallbladder (g%)*
• Bile salts	1.8	8.7
• Fatty acids and lipids	0.24	1.8
• Cholesterol	0.16	0.87

d. pH : Hepatic duct, 7.5 (6.2–8.5); gallbladder, 6.0 (5.6–8.0)
e. Sodium : 134–156 mEq/L
f. Potassium : 3.9–6.3 mEq/L
g. Chloride : 83–110 mEq/L
h. Bicarbonate : 38 mEq/L.

PANCREATIC FUNCTION TESTS

Composition of Pancreatic Juice

Obtain specimen by duodenal drainage, it is mixed with bile. The flow of pancreatic juice is stimulated by an injection of secretin. Secretin is a hormone normally produced by upper intestinal mucosa in response to the presence of acid. The flow of pancreatic juice begins 5 minutes after a meal, is at its height in 2–3 hours, lasts 6–8 hours in all.

Gross and Chemical Characteristics of Pancreatic Juice

a. Volume per 24 hours: 500–800 mL.
b. Specific gravity: 1.007.
c. Total solids: 1.5–2.5 g%.
d. Alkalinity: pH is 7.0–8.2; 10 mL of pancreatic juice = 10–13 mL of 0.1 N NaOH and is more effective than bile or succus entericus in neutralizing acidic gastric juice.
e. Bicarbonate: 70–100 mEq/L.
f. Sodium: 100–150 mEq/L.
g. Potassium: 2–8 mEq/L.
h. Chlorides: 50–95 mEq/L.

Digestive Enzymes

Proteolytic Enzymes

1. Trypsin is a pancreatic protease. There are 100-200 units/L. It is much more active than pepsin. The inactive trypsinogen secreted is activated by enterokinase or by trypsin itself. Trypsin hydrolyzes proteins at peptide bonds.
2. *Chymotrypsin:* Two forms, A and B are secreted and are activated by trypsin. Its action is like that of trypsin.
3. *Collagenase:* It digests collagen and is the one that initiates tissue destruction in necrotizing pancreatitis.
4. *Elastase:* Digests elastin, which is the most resistant of all body proteins to lytic agents.

Peptidases

1. *Carboxypeptidase:* The active enzyme removes amino acids one by one from the carboxyl ends of the peptide chains.
2. *Aminopeptidase:* The active enzyme removes amino acids one by one from the ends of the peptide chains bearing the free amino groups.

Nucleases

Ribonuclease and deoxyribonuclease are secreted in probably more than one or perhaps several forms, they hydrolyze the respective nucleic acids.

Amylolytic Enzymes

Amylase: Alpha amylase attacks the alpha-1-4-glycosidic bonds of starches breaking them down to the disaccharide maltose.

Lipolytic Enzymes

1. *Lipase:* It partially hydrolyzes neutral fats, splitting off one fatty acid at a time, thus forming diglycerides and monoglycerides along with liberated free fatty acids. Its optimum pH range is 7 to 9. This enzyme is activated by biliary contents. It shows optimal activation when the substrate is in emulsified form rather than in true solution form. The emulsifying action of bile salts and bile acids is very helpful for optimal enzymic action.
2. *Lecithinase* (*phospholipase*): Phospholipases A and B act in succession. Both of these remove fatty acids, the end products formed from lecithin and cephalin are glyceryl phosphoryl choline, glyceryl phosphoryl ethanolamine and glyceryl phosphoryl serine.

Acute Pancreatitis

Acute pancreatic necrosis (acute hemorrhagic pancreatitis) has over 50% mortality rate. It is known to be related to gallstones, alcoholism, trauma, infection (mumps), renal transplantation, various metabolic disorders, e.g. hyperlipidemia, uremia and hyperparathyroidism.

Serum amylase estimation has been widely used in the diagnosis of acute pancreatitis. Serum amylase activity rises within hours following an episode. Values over 5 times the upper limit of normal are suggestive of the diagnosis. Values may return to normal within 5 days following a mild edematous attack. Persisting elevated values longer than this suggest continuing necrosis or possible pseudocyst formation. The urine amylase activity rises promptly, often within several hours of the rise in serum activity. Values over 1,000 units per hour (in urine) or higher are seen, almost exclusively in patients with acute pancreatitis.

Amylase activity in blood (or in peritoneal fluid in certain conditions) may be raised to 1,000 Somogyi units in various (nonpancreatic) disorders as: (i) intestinal obstruction, strangulation, or perforation, (ii) following upper abdominal surgery, (iii) ruptured ectopic pregnancy, (iv) mumps, (v) renal insufficiency, and (vi) following morphine administration. Values over 5,000 units suggest a diagnosis of acute pancreatic necrosis.

Chronic Pancreatitis (Cirrhosis of Pancreas)

There is variable degree of fibrosis and atrophy in the pancreatic parenchyma. Diagnosis as in the case of acute pancreatitis, depends in part on determination of amylase activity in serum and urine.

Carcinoma of Pancreas

Serum amylase may be elevated but is of little diagnostic importance.

Lipase

This is an esterase acting on ester linkages in triglycerides. Bile salts and calcium enhance its activity. Lipase occurs predominantly in the pancreas, but small amounts are produced in the gastric and small bowel mucosa.

Principle (Lipase estimation in serum)

The classic method of serum lipase determination is that of Cherry and Crandall using olive as substrate, overnight incubation (24 hours), and titration of liberated fatty acids with sodium hydroxide, using phenolphthalein as indicator. Normal range of values is up to 1.5 units in serum.

The method given above takes a long time and if the report is to be given on emergency basis—a rapid (20 minute incubation) specific turbidimetric method is available. The disadvantage of this method is spuriously high results

obtained in the presence of jaundice. By this method, values above 10 units are doubtful, above 19 units definitely abnormal.

Interpretation

Acute pancreatitis: Serum lipase activity rises slower than that of amylase, sometimes as late as 24 to 48 hours after onset, often peaking on the fourth day. It may remain elevated longer than the serum amylase. Eventhough, it is less sensitive than the serum amylase, it provides confirmatory evidence for the diagnosis when positive. Elevation in patients with mumps strongly suggests significant pancreatic as well as salivary gland involvement by the disease.

Chronic pancreatitis: Serum lipase estimation is of relatively little value in the diagnosis of chronic pancreatitis.

Pancreatic carcinoma: Serum lipase is elevated more often in patients with pancreatic carcinoma than is serum amylase, although not with sufficient frequency to make it of much value diagnostically.

Secretin Test

A double lumen tube, providing for separate aspiration of gastric and duodenal contents, is passed into the duodenum, using fluoroscopic guidance and maintaining constant aspiration of gastric contents. Duodenal contents are aspirated until clear. The patient is then given IV one unit of secretin per kg of body weight, and pancreatic secretion entering the duodenum is collected for 80 minutes. The aspirate is examined for volume, bicarbonate content, and amylase activity.

The test is not employed for the diagnosis of acute pancreatic necrosis (it would be hazardous). Patients with chronic pancreatitis are unable to secrete juice of high bicarbonate content (less than 90 mEq/L). As in the case of chronic pancreatitis, this test may assist in diagnosis of pancreatic carcinoma tumors of head of pancreas tend to depress the overall volume flow (lower limit of normal—2 mL per kg body weight per 80 minutes). In carcinoma body of pancreas half the patients may show normal volume, carcinoma of tail does not affect the volume.

Patients with ductal obstructive lesion may exhibit elevation of serum amylase during and following the test, normally there is no elevation of serum amylase activity. The pattern of increased volume with decreased bicarbonate and normal amylase has been associated with hemochromatosis. Rarely, an increase in the amylase with normal bicarbonate concentration and volume flow has been noted in patients with nutritional and metabolic pancreatic fibrosis as well as in pancreatitis associated with inflammatory disease of the intestines. In some patients of pancreatic ductal obstruction, levels may rise.

Tumors of the head of the pancreas associated with jaundice must be differentiated from nonsurgical cholestatic liver disease, from carcinoma, obstructing stone, or other obstructing pathologic lesions of the common bile duct, and from ampullary carcinoma.

Duodenal aspirate containing cholesterol crystals or calcium bilirubinate pigment and pus, especially when associated with a normal secretin test, suggest gallstone etiology. However, a duodenal aspirate containing calcium bilirubinate pigment is not specific for cholelithiasis. Unremittent jaundice, alcoholic duodenal fluid and stools, consistently negative urine urobilinogen tests, and less than 5 mg fecal urobilinogen per 24 hours, associated with a normal secretin test, suggest carcinoma of the common bile duct or gallbladder. Intermittent jaundice and presence of blood in the aspirate suggest carcinoma of the duodenal papilla, especially when associated with an abnormal secretin test. Cytologic examination of aspirate may be helpful in the diagnosis of carcinoma, as are the results of enzyme and volume outputs.

Other Laboratory Tests in Acute Pancreatitis

- Leukocytosis in patients with acute pancreatitis (up to 30,000/mm^3).
- Hemoconcentration, so raised hemoglobin.
- Serum levels of lecithinase A, trypsin and deoxyribonuclease activity are also elevated.
- A falling serum calcium points to the more serious form of pancreatitis as does turbidity of serum.
- In alcohol-related pancreatitis serum bilirubin may rise.
- Transient hyperglycemia may also occur.

Miscellaneous Tests for Chronic Pancreatitis

Various tests for malabsorption can be done

- Serum carotenoid level
- Glucose tolerance test
- Three-day fecal fat determination
- Gross and microscopic examination of stool
  - ^{131}I triolein test
  - D-xylose test.

SWEAT ELECTROLYTES PILOCARPINE IONTOPHORESIS

Pilocarpine is iontophoresed into the skin to stimulate locally increased sweat gland secretion. The resulting

sweat is absorbed by filter paper, diluted with distilled water and analyzed for sodium and chloride contents. The method is painless and reliable. Total body sweating is hazardous in cystic fibrosis patients.

Diagnostic Application of Sweat Testing

Fibrocystic disease of pancreas (Mucoviscidosis)

This is a familial, Mendelian recessive disease characterized by abnormal secretion by the various exocrine glands of the body, including pancreas, salivary glands, peritracheal, peribronchial and peribronchiolar glands, lacrimal glands, sweat glands, mucosal glands of small intestine and even the bile ducts.

Laboratory diagnosis depends largely upon demonstration of increased sodium and chloride in the sweat, found in almost 99% of patients. Screening tests for sweat chloride have also been used and depend upon hand imprints on silver nitrate containing agar or paper. The sweat chloride precipitates with silver, and the intensity of the print is roughly proportional to the sweat chloride precipitation. However, chemical estimation of sweat chloride is more accurate.

In adult males values of sweat chloride up to 70 mEq/L and in females up to 65 mEq/L are normal.

Normal values in children

Chloride	*Sodium*
Below 50 mEq/L: Normal	Below 70 mEq/L: Normal
50-60 mEq/L: Equivocal	70-90 mEq/L: Equivocal
Over 60 mEq/L: Abnormal	Over 90 mEq/L: Abnormal

CHAPTER 17

Diabetes Mellitus: Laboratory Diagnosis

DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disorder with vascular components that is characterized by disturbances in carbohydrate, lipid and protein metabolism. So, hyperglycemia and glycosuria reflect the major metabolic lesion in carbohydrate metabolism, with secondary metabolic disturbances in proteins (gluconeogenesis) and lipids (ketosis and hypercholesterolemia).

With hyperglycemia, renal glycosuria occurs with an osmotic diuresis (polyuria) ultimately leads to dehydration and associated polydipsia (increased thirst). Glycogenolysis and gluconeogenesis (protein depletion) are augmented to generate glucose that contributes to or sustains hyperglycemia. Muscle glycogen cannot contribute glucose directly to the blood because of the absence of glucose-6-phosphatase. A failure of glucose to penetrate adipose tissue cells mobilizes fat and produces a rise in the free fatty acid and triglycerides in the liver. A diabetic fatty liver may result from the absence of lipoprotein synthesis when protein synthesis is compromised by accelerated gluconeogenesis (negative nitrogen balance). When glucose oxidation is impaired, fatty acids form the major source of energy and generate an excess of acetyl coenzyme A that cannot be oxidized to water and carbon dioxide or be disposed of in other metabolic routes. The condensation of two carbon fragments of acetyl coenzyme A results in formation of ketone bodies, ketonemia and ketonuria. The three ketone bodies are acetone, β hydroxybutyric acid and acetoacetic acid. Ketoacidosis is the hallmark of potentially fatal complications of diabetes mellitus.

Classification and Causes of Diabetes

Primary

- Maturity-onset (adult) type
- Growth-onset (juvenile) type
- Hyperpituitarism
  - Pituitary basophilism
  - Acromegaly.
- Hyperadrenalism
  - Cortical: Cushing's syndrome, aldosteronism
  - Medullary: Pheochromocytoma.
- Hyperthyroidism
- Iatrogenic
  - Corticosteroids and ACTH
  - Growth hormone
  - Thyroid extract and triiodothyronine.

Destruction of Pancreatic Islets

- Surgical removal of pancreas
- Hemochromatosis
- Fibrocystic disease of pancreas
- Neoplasm.

Miscellaneous

- Diuretics and derivatives (thiazide therapy)
- Stress reactions, surgery and pregnancy
- Starvation and low carbohydrate intake.

The course of the disease can be divided into four stages:

1. Prediabetes
2. Suspected diabetes
3. Chemical or latent diabetes
4. Overt diabetes.

The period from birth until the first evidence of the disease characterizes prediabetes. In suspected diabetes, the patient displays an abnormal glucose tolerance test (GTT) or even diabetic symptoms after stressful influences (e.g. obesity, pregnancy, trauma and infections), but usually is normal in all respects. In chemical or latent diabetes, there are no signs or symptoms of disease but an abnormal GTT or fasting hyperglycemia are evident when the patient is not under stress. With overt diabetes, symptoms of polyuria, polydipsia and weight loss (and possibly ketoacidosis) are often associated with fasting hyperglycemia and glycosuria. For diagnosis of diabetes in individuals with marked glucose intolerance, the provocative tests should not be performed (as in an insulin requiring diabetic). In patients who have neither glycosuria nor fasting hyperglycemia—in these individuals provocative tests may be needed to demonstrate impaired glucose tolerance. Glycosuria associated with ketonuria is almost always pathognomonic of diabetes mellitus.

Screening Tests

These include urine and blood glucose estimations:

Urine Glucose (Methods Mentioned Elsewhere)

While evaluating glycosuria, it should be remembered that venous "true glucose" must exceed 180 mg% of blood before any glucose will spill over into urine (renal threshold). In diabetic nephropathy, the renal threshold may be elevated considerably (very little filtration apparatus left, i.e. glomeruli) even in the presence of hyperglycemia. Also, the renal threshold increases with age, and in some elderly patients no glycosuria will be present with serum levels of 200 mg% of glucose.

Fasting Blood Sugar

For this, plasma is the blood fraction of choice. Fasting plasma glucose values in excess of 120 mg% (true glucose) are considered indicative of diabetes mellitus; values between 110-120 mg% are equivocal and should be confirmed with a GTT. The 2 hours postprandial (PP) test should be done instead of fasting glucose levels. Emotional hyperglycemia from secretion of epinephrine as well as cerebral lesions (skull fractures, tumors, vascular accident, and encephalitis) and carbon monoxide poisoning, often provoke hyperglycemia and glycosuria, must be considered in the evaluation of blood glucose measurements.

Two-hours Postprandial Blood Glucose

After an overnight fast (12 hours), the patient is given a breakfast of 100 g of carbohydrate or a 100 g glucose load. Previous to the test, the patient should have been on an adequate carbohydrate diet (300 g daily) and all medications that influence glucose tolerance should have been discontinued 3 days prior to the test. Two hours later [2 hours PP or PC, post cibum] a single blood sample is withdrawn for analysis. A value within normal limits makes the diagnosis of diabetes mellitus unlikely, plasma glucose values in the range of 120-140 mg% are suspicious and in excess of 140 mg% (true glucose), diagnosis is most likely and should be confirmed by GTT.

The limitations of a single 2 hours PP glucose value include the following:

1. Slow absorption, which may delay the peak.
2. Rapid absorption with early hyperglycemia, rapid fall in concentration of blood glucose (due to insulin release), and then a second hyperglycemic peak due to the effects of counter regulatory responses (epinephrine, glucagon, growth hormone).
3. Errors in timing specimen collection.

Diagnosis and Classification of Diabetes Mellitus: New Criteria

New recommendations for the classification and diagnosis of diabetes mellitus include the preferred use of the terms "type 1" and "type 2" instead of "IDDM" and "NIDDM" to designate the two major types of diabetes mellitus: simplification of the diagnostic criteria for diabetes mellitus to two abnormal fasting plasma determinations: and a lower cutoff for fasting plasma glucose [126 mg per dL (7 mmol per L) or higher] to confirm the diagnosis of diabetes mellitus. These changes provide an easier and more reliable means of diagnosing persons at risk of complications from hyperglycemia. Currently, only one half of the people who have diabetes mellitus have been diagnosed. Screening for diabetes mellitus should begin at 45 years of age and should be repeated every three years in persons without risk factors, and should begin earlier and be repeated every 3 years in persons without risk factors, and should begin earlier and be repeated more often in those with risk factors. Risk factors include obesity, first-degree relatives with diabetes mellitus, hypertension, hypertriglyceridemia or previous evidence of impaired glucose homeostasis. Earlier detection of diabetes mellitus may lead to tighter control of blood glucose levels and a reduction in the severity of complications associated with this disease.

Diabetes mellitus is a group of metabolic disorders with one common manifestation: hyperglycemia. Chronic hyperglycemia causes damage to the eyes, kidneys, nerves, heart and blood vessels. The etiology and pathophysiology

leading to the hyperglycemia, however, are markedly different among patients with diabetes mellitus, dictating different prevention strategies, diagnostic screening methods and treatments. The adverse impact of hyperglycemia and the rationale for aggressive treatment have recently been reviewed.

In June 1997, an international expert committee released a report with new recommendations for the classification and diagnosis of diabetes mellitus. These new recommendations were the result of more than two years of collaboration among experts from the American Diabetes Association and the World Health Organization (WHO). The use of classification systems and standardized diagnostic criteria facilitates a common language among patients, physicians, other healthcare professionals and scientists.

Previous Classification

In 1979, the National (American) Diabetes Data Group produced a consensus document standardizing the nomenclature and definitions for diabetes mellitus. This document was endorsed one year later by WHO. The two major types of diabetes mellitus were given names descriptive of their clinical presentation: "insulin-dependent diabetes mellitus" (IDDM) and "noninsulin-dependent diabetes mellitus" (NIDDM).

Diabetes mellitus that is characterized by absolute insulin deficiency and acute onset, usually before 25 years of age, should now be referred to as type 1 (not type I, IDDM or juvenile) diabetes mellitus.

However, as treatment recommendations evolved, correct classification of the type of diabetes mellitus became confusing. For example, it was difficult to correctly classify persons with NIDDM who were being treated with insulin. This confusion led to the incorrect classification of a large number of patients with diabetes mellitus complicating epidemiologic evaluation and clinical management. The discovery of other types of diabetes with specific pathophysiology that did not fit into this classification system further complicated the situation. These difficulties, along with new insights into the mechanisms of diabetes mellitus, provided a major impetus for the development of a new classification system.

The National Diabetes Data Group also established the oral glucose tolerance test (using a glucose load of 75 g) as the preferred diagnostic test for diabetes mellitus. However, this test has poor reproducibility, lacks physiologic relevance and is a weaker indicator of long-term complications compared with other measures of hyperglycemia. Furthermore, many high-risk patients are unwilling to undergo this time-consuming test on a repeat-basis. The new diagnostic criteria also address this issue.

Changes in the Classification System

The new classification system identifies four types of diabetes mellitus: type 1, type 2, other specific types and gestational diabetes. Arabic numerals are specifically used in the new system to minimize the occasional confusion of type "II" as the number "11". Each of the types of diabetes mellitus identified extends across a clinical continuum of hyperglycemia and insulin requirements.

Any patient with two fasting plasma glucose levels of 126 mg per dL (7.0 mmol per L) or greater is considered to have diabetes mellitus.

Type 1 diabetes mellitus (formerly called type I, IDDM or juvenile diabetes) is characterized by beta cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency. The onset is usually acute, developing over a period of a few days to weeks. Over 95% of persons with type 1 diabetes mellitus develop the disease before the age of 25, with an equal incidence in both sexes and an increased prevalence in the white population. A family history of type 1 diabetes mellitus, gluten enteropathy (celiac disease) or other endocrine disease is often found. Most of these patients have the "immune-mediated form" of type 1 diabetes mellitus with islet cell antibodies and often have other autoimmune disorders, such as Hashimoto's thyroiditis, Addison's disease, vitiligo or pernicious anemia. A few patients, usually those of African or Asian origin, have no antibodies but have a similar clinical presentation; consequently, they are included in this classification and their disease is called the "idiopathic form" of type 1 diabetes mellitus.

Type 2 diabetes mellitus (formerly called NIDDM, type II or adult-onset) is characterized by insulin resistance in peripheral tissue and an insulin secretory defect of the beta cell. This is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise. It is more common in women, especially women with a history of gestational diabetes. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance. Defective beta cells become exhausted, further fueling the cycle of glucose intolerance and hyperglycemia. The etiology of type 2 diabetes mellitus is multifactorial and probably genetically based, but it also has strong behavioral components.

The etiologic classifications of diabetes mellitus are listed in Table 17.1.

TABLE 17.1: Etiologic classification of diabetes mellitus

Type 1 diabetes mellitus*
Type 2 diabetes mellitus*
Other specific types:
Genetic defects of beta-cell function
Genetic defects in insulin action
Diseases of the exocrine pancreas
Pancreatitis
Trauma/pancreatectomy
Neoplasia
Cystic fibrosis
Hemochromatosis
Others
Endocrinopathies
Acromegaly
Cushing's syndrome
Glucagonoma
Pheochromocytoma
Hyperthyroidism
Somatostatinoma
Aldosteronoma
Others
Drug-or chemical-induced
Vacor
Pentamidline
Nicotinic acid
Glucocorticoids
Thyroid hormone
Diazoxide
Beta-adrenergic antagonists
Thiazides
Phenytoin
Infections
Congenital rubella
Cytomegalovirus
Others
Uncommon forms of immune-mediated diabetes
Other genetic syndromes sometimes associated with diabetes
Down syndrome
Klinefelter's syndrome
Turner's syndrome
Wolfram syndrome
Friedreich's ataxia
Huntington's chorea
Laurence-Moon Beidel syndrome
Myotonic dystrophy
Porphyria
Prader-Willi syndrome
Others
Gestational diabetes mellitus

Contd...

Contd...

Types of diabetes mellitus of various known etiologies are grouped together to form the classification called "other specific types." This group includes persons with genetic defects of beta-cell function (this type of diabetes was formerly called MODY or maturity-onset diabetes in youth) or with defects of insulin action; persons with diseases of the exocrine pancreas, such as pancreatitis or cystic fibrosis; persons with dysfunction associated with other endocrinopathies (e.g. acromegaly); and persons with pancreatic dysfunction caused by drugs, chemicals or infections.

The definition and diagnosis of gestational diabetes mellitus was not altered in these new recommendations. Gestational diabetes mellitus is an operational classification (rather than a pathophysiologic condition) identifying in women who develop diabetes mellitus during gestation (Women with diabetes mellitus before pregnancy are said to have "pregestational diabetes" and are not included in this group). Women who develop type 1 diabetes mellitus during pregnancy and women with undiagnosed asymptomatic type 2 diabetes mellitus that is discovered during pregnancy, are classified with gestational diabetes mellitus. However, most women, classified with gestational diabetes mellitus, have normal glucose homeostasis during the first half of the pregnancy and develop a relative insulin deficiency during the last half of the pregnancy, leading to hyperglycemia. The hyperglycemia resolves in most women after delivery but places them at increased risk of developing type 2 diabetes mellitus later in life.

New Diagnostic Criteria for Diabetes Mellitus

The new diagnostic criteria for diabetes mellitus have been greatly simplified in Table 17.2.

The oral glucose tolerance test previously recommended by the National (American) Diabetes Data Group has been replaced with the recommendation that the diagnosis of diabetes mellitus be based on two fasting plasma glucose levels of 126 mg per dL (7.0 mmol per L) or higher.

TABLE 17.2: Criteria for the diagnosis of diabetes mellitus and impaired glucose homeostasis

Diabetes Mellitus	
Positive findings from any two of the following tests on different days:	
a.	Symptoms of diabetes mellitus* plus casual † plasma glucose concentration ≥ 200 mg per dL (11.1 mmol per L)
	or
b.	FPG ≥ 126 mg per dL (7.0 mmol per L)
	or
c.	2 h PPG ≥ 200 mg per dL (11.1 mmol per L after a 75 g glucose load)
Impaired Glucose Homeostasis	
a.	Impaired fasting glucose: FPG from 110 < 126 (6.1 to 7.0 mmol per L)
b.	Impaired glucose tolerance: 2 h PPG from 140 to < 200 (7.75 to < 11.1 mmol per L)
c.	Normal FPG < 110 mg per dL (6.1 mmol per L) 2 h PPG < 140 mg per dL (7.75 mmol per L)

†Casual is defined as any time of day without regard to time since last meal

*Symptoms include polyuria, polydipsia or unexplained weight loss. FPG = fasting plasma glucose; 2 hr PPG = two-hour postprandial glucose

Other options for diagnosis include two 2-hour postprandial plasma glucose (2 h PPG) readings of 200 mg per dL (11.1 mmol per L) or higher after a glucose load of 75 g (essentially, the criterion recommended by WHO) or two casual glucose readings of 200 mg per dL (11.1 mmol per L) or higher. Measurement of the fasting plasma glucose level is the preferred diagnostic test, but any combination of two abnormal test results can be used. Fasting plasma glucose was selected as the primary diagnostic test because it predicts adverse outcomes (e.g. retinopathy) as well as the 2 h PPG test but is much more reproducible than the oral glucose tolerance test or the 2 h PPG test and easier to perform in a clinical setting.

The choice of the new cutoff point for fasting plasma glucose levels is based on strong evidence from a number of population linking the risk of various complications to the glycemic status of the patient. As per the study on the risk of diabetic retinopathy based on the glycemic status of 40 to 74 years old participants in the National Health and Nutritional Epidemiologic Survey (NHANES III) the risk of retinopathy greatly increases when the patient's fasting plasma glucose level is higher than 109 to 116 mg per dL (6.05 to 6.45 mmol per L) or when the result of a 2 h PPG test is higher than 150 to 180 mg per dL (8.3 to 10.0 mmol per L) or when the result of a 2 hr PPG test is higher than 150 to 180 mg per dL (8.3 to 10.0 mmol per L). However, the committee decided to maintain the cutoff point for the 2 h PPG test at 200 mg per dL (11.1 mmol per L) because so much literature has already been published using this criterion. They selected a cutoff point for fasting plasma glucose of 126 mg per dL (7.0 mmol per L) or higher. This point corresponded best with the 2 h PPG level of 200 mg per dL (11.1 mmol per L). The risk of other complications also increases dramatically at the same cutoff points.

A normal fasting plasma glucose level is less than 110 mg per dL (6.1 mmol per L) and normal 2 h PPG levels are less than 140 mg per dL (7.75 mmol per L). Blood glucose levels above the normal level but below the criterion established for diabetes mellitus indicate impaired glucose homeostasis. Persons with fasting plasma glucose levels ranging from 110 to 126 mg per dL (6.1 to 7.0 mmol per L) are said to have impaired fasting glucose, while those with a 2 h PPG level between 140 mg per dL (7.75 mmol per L) and 200 mg per dL (11.1 mmol per L) are said to have impaired glucose tolerance. Both impaired fasting glucose and impaired glucose tolerance are associated with an increased risk of developing type 2 diabetes mellitus. Lifestyle changes, such as weight loss and exercise, are warranted in these patients. The committee chose not to address the current controversies surrounding the diagnosis of gestational diabetes mellitus and did not alter the diagnostic criteria in this area. Screening for gestational diabetes mellitus is generally accomplished with administration of a 50 g glucose load one hour before determining a plasma glucose level. A positive screen [defined as a plasma glucose level of 140 mg per dL (7.75 mmol per L) or higher] should prompt a diagnostic test; fasting plasma glucose levels should be measured after a 100 g glucose load at baseline and at 1, 2 and 3 hours after the glucose load. Two of the four values must be abnormal [105 mg per dL (5.8 mmol per L) or higher; 190 mg per dL (10.5 mmol per L) or higher; 165 mg per dL (9.15 mmol per L) or higher; and 145 mg per dL (8.05 mmol per L) or higher) for a patient to be diagnosed with gestational diabetes mellitus. The WHO criteria use a glucose load of 75 g with a test two hours after the glucose load, using the same criterion for the diagnosis of gestational diabetes mellitus.

Glycated Hemoglobin

Measurements of glycated hemoglobin have commonly been used to monitor the glycemic control of persons already diagnosed with diabetes mellitus. Measurements of this hemoglobin, also called glycosylated hemoglobin,

glycohemoglobin, hemoglobin A1c or hemoglobin A1, aid in the evaluation of the stable linkage of glucose to minor hemoglobin components. There is currently no agreement on standardization, so a variety of measurement methods and normal ranges are being used.

Some experts argue that a glycated hemoglobin test could be used for the diagnosis of diabetes mellitus. Glycated hemoglobin levels are as highly correlated to adverse clinical outcomes (e.g. retinopathy) as are fasting plasma glucose or postprandial plasma glucose levels and are as reproducible as fasting plasma glucose levels. The major advantage of measuring glycated hemoglobin is that the specimen can be collected without regard to when the patient last ate.

The expert committee, however, did not include glycated hemoglobin measurement in the recommendations for international standards for the diagnosis of diabetes mellitus. They noted the lack of standardization and normal ranges among the various tests, making it difficult to dictate a standard cutoff point.

The test for measuring glycated hemoglobin is not widely available in developing countries; consequently, it was not favored for use as an international criterion. There is also some overlap in the levels of glycated hemoglobin in patients with diabetes mellitus and those without it.

Although, it was not specifically recommended by the National Diabetes Data Group (US) as a diagnostic test for diabetes mellitus, glycated hemoglobin may, in some case, be used to diagnose diabetes mellitus.

The diagnosis of diabetes mellitus is made in the following fashion. A glycated hemoglobin level of 1% above the reference laboratory's upper range of normal is consistent with diabetes mellitus and has a specificity of 98%. People with normal glycated hemoglobin levels (i.e. within the laboratory's published normal range) either do not have diabetes mellitus or have well-controlled diabetes mellitus (i.e. a false-negative test). However, incorrectly diagnosing these persons as normal would not alter their treatment because exercise and diet are adequately controlling their blood glucose levels.

People who are not diagnosed with diabetes mellitus and who have near-normal glycated hemoglobin levels (less than 1% above the normal range) may be advised of the high probability that they have diabetes mellitus and may be offered the same treatment as a person with mild diabetes mellitus (i.e. dietary and exercise counseling), followed by repeat testing of glycated hemoglobin several months later.

This method of screening and counseling high-risk persons is easier for many patients and clinicians because the blood specimen can be drawn at the time of the patient visit.

> Glycated hemoglobin (also known as glycohemoglobin, glycosylated hemoglobin or HbA_{1c}) is used to monitor treatment in patients with diabetes mellitus; however, it is not recommended for routine diagnosis of this condition because of a lack of standardization of tests and results.

Impact of the New Diagnostic Criteria

Physicians may be concerned that the new diagnostic criteria for diabetes mellitus, including the lower cutoff for fasting plasma glucose levels, may greatly increase the number of people who are diagnosed with diabetes mellitus in their practices.

Concerns about overdiagnosis include the harm created by anxiety, the risks and costs of unnecessary treatment, and possible insurance discrimination, especially if the condition that is being diagnosed is relatively benign or if no effective treatment is available.

On the other hand, underdiagnosing a condition is harmful if early treatment can make a difference in patient outcome, especially if the treatment is relatively benign and inexpensive.

It is true that a rigorous screening program will increase the number of persons who are diagnosed with diabetes mellitus. However, currently only half of the people who have diabetes mellitus according to the old criteria have not been diagnosed and may remain undiagnosed for up to 10 years.

People who are asymptomatic and underdiagnosed continue to develop the complications of diabetes mellitus.

Screening Recommendations

The expert committee provided guidelines governing the selection of patients to be tested for diabetes and the frequency of that testing (Table 17.3). Testing should be considered for all persons who are 45 years of older and should be repeated at 3 years intervals.

Testing should be considered at a younger age and be performed more frequently in persons who are obese (120% of desirable body weight or greater or a body mass index of 27 kg per m^2 or greater); who have a first-degree relative with diabetes mellitus; who have delivered a baby weighing more than 4.032 g (9 lb), or who were diagnosed with gestational diabetes mellitus during pregnancy; are hypertensive; or have a high-density lipoprotein level of 35 mg per dL (0.90 mmol per L) or lower and/or a trigly-ceride level of 250 mg per dL (2.83 mmol per L) or higher. In addition, any patient with

TABLE 17.3: Recommendations for diabetes screening of asymptomatic persons

Timing of the first test and repeat tests	
• Test at age 45: Repeat every three years (patients 45 years of age or older)	
• Test before age 45: Repeat more frequently than every three years if patient has one or more of the following risk factors:	
a.	Obesity ≥ 120% of desirable body weight or BMI ≥ 27 kg per m^2
b.	First-degree relative with diabetes mellitus
c.	Member of high risk-ethnic group (Black, Hispanic, Native American, Asian)
d.	History of gestational diabetes mellitus or delivering a baby weighing more than 4.032 g (9 lb)
e.	Hypertensive (≥ 140/90 mm Hg)
f.	HDL cholesterol level ≥ 35 mg per dL (0.90 mmol per L) and/or triglyceride level ≥ 250 mg per dL (2.83 mmol per L)
g.	History of IGT or IFG on prior testing

BMI = body mass index: HDL = high density lipoprotein;
IGT = impaired glucose tolerance; IFG = impaired fasting glucose

impaired glucohomeostasis should be reevaluated on a more frequent basis.

The expert committee recommended that screening for gestational diabetes mellitus should be reserved for use in women who meet one or more of the following criteria: 25 years of age older, obese (defined as more than 120% above their desirable body weight), a family history of a first-degree relative with diabetes mellitus, and belong to high-risk ethnic population.

Final Comment

The changes recommended by the expert committee for the diagnosis of diabetes mellitus should prove beneficial to patients. Measurement of fasting plasma glucose levels should be more acceptable to the patients than the oral glucose tolerance test and can be readily incorporated with fasting lipid determinations. Identifying asymptomatic persons earlier in the disease process will allow earlier institution of lifestyle changes and medical therapy that may decrease the complications of hyperglycemia. The National Diabetes Data Group (US) emphasizes that these changes in diagnostic criteria have not changed the treatment goals in patients with diabetes mellitus. These goals include maintaining a fasting plasma glucose level of less than 120 mg per dL (6.65 mmol per L) and a glucose hemoglobin measurement of less than 7.0%.

Conventional Diagnostic Tests

Oral Glucose Tolerance Test (OGTT)

This is performed to establish a diagnosis in:

1. Patients with transient or sustained glycosuria who have no clinical symptoms of diabetes (polyuria) and with normal fasting and post-prandial blood glucose levels.
2. Patients with symptoms of diabetes but with no glycosuria and normal fasting level.
3. Persons with a strong family history of diabetes but with no overt diabetes.
4. Patients whose glycosuria is associated with pregnancy, thyrotoxicosis, liver disease, and/or infections.
5. Women who have characteristically large babies (> 9 lbs) or individuals who were large babies.
6. Patients with neuropathies and retinopathies of undetermined origin.

The patient should ingest a daily diet of atleast 300 g of carbohydrate for 3 days prior to the test. Therefore, on an acutely ill-hospitalized patient, this test should not be conducted. As far as possible it should be performed on an ambulatory patient. Preferably, the test should be performed in the morning. Various malignancies, fever, cachexia, liver dysfunction and renal failure may be associated with mild to moderate degrees of abnormal GTT. There is an age-related factor that decreases glucose tolerance and hence makes the interpretation of OGTT in elderly subjects difficult. Timing of glucose administration and blood sampling must be accurate.

Patient Preparation

1. Instruct the patient about the purpose and procedure of the test:
   a. Stress a normal diet with high carbohydrate (150–300 g) for 3 days preceding the test.
   b. Fasting is required for at least 10 hours before the test and not more than 16 hours.
   c. Water is permitted and encouraged.
2. Determine the patient's weight and record it.
3. Collect urine and blood samples and test for glucose, recording exact time of collection. Have the patient empty his or her bladder for each specimen:
   a. No liquids other than water can be taken. Have the patient empty his or her bladder for each urine sample.
   b. No food is to be taken during the test period.
   c. No alcohol to be consumed the previous evening.

d. Encourage the patient to stay in bed or rest quietly during the test period. Weakness or feeling faint may occur during test, and exercise also changes, glucose results.
e. No smoking is allowed during the test.
f. Coffee and unusual physical exercise should be avoided for at least 8 hours before the test.

Carbohydrate meal (or glucose) to be given in 25% (w/v) solution according to the age.

Age	*Dose*
0–18 months	2.5 g/kg
1½–8 years	2.0 g/kg
8–12 years	1.75 g/kg
> 12 years	1.25 g/kg

Preferably, the samples of urine and whole blood be taken at fasting, 30 minutes, 1, 1½, 2, 3, and 4 hours after ingestion of the carbohydrate meal. If nausea and vomiting occur during the test, the interpretation becomes difficult.

Interpretation

Three popular methods for evaluating GTT for diabetes mellitus are:

1. Wilkerson point system

Time	*mg% plasma*	*Points*
Fasting	130 or more	1
1 h	195 or more	½
2 h	140 or more	½
3 h	130 or more	1

Two or more points are judged diagnostic of diabetes mellitus (DM).

2. The Fajans-Conn criteria

Time	*mg% plasma*
Fasting	
1 h	195 or more
1½ h	165 or more
2 h	140 or more

A diagnosis of DM in otherwise healthy and ambulatory individuals under age 50 is made if the above criteria are met.

3. The university group diabetes mellitus program

The fasting 1 h, 2 h and 3 h blood glucose levels are adjusted for plasma glucose as above, and the subject is judged diabetic if the sum of values obtained equals 500 or more.

Abnormally, high values in the first hour with a rapid fall to normal values or a flat curve with no appreciable rise usually reflect primary alterations in intestinal absorption of glucose. The former is characteristic of hyperthyroidism and the latter of hypothyroidism or malabsorptive states. A very flat rise in blood glucose followed by a prolonged and pronounced hypoglycemic phase may be observed in primary (islet cell adenoma or hyperplasia) and secondary hyperinsulinism (hypoadrenocorticism). In the elderly, especially in females, interpretation of OGTT must be made in light of what is an age-dependent carbohydrate intolerance.

Interfering Factors

1. Smoking will increase glucose level.
2. Inadequate diet (such as a weight-reducing diet) before testing can diminish carbohydrate tolerance and suggest a false diabetes.
3. Levels tend to increase normally in older people, the maximum can reach 200 mg/dL.
4. Prolonged administration of oral contraceptives will give significantly higher glucose levels in the second hour or in later blood samples.
5. Bedrest over a lengthy period of time will influence glucose tolerance. For this reason, the test should be performed on ambulatory patients, not on patients whose condition requires bedrest.
6. Infectious diseases and surgery will affect tolerance. Two weeks of recovery should be permitted before the test.
7. Drugs that impair glucose tolerance:
   - Insulin
   - Oral hypoglycemics
   - Salicylates in larger doses
   - Oral contraceptives
   - Thiazide diuretics
   - Corticosteroids
   - Estrogen
   - Ferrous ascorbinate
   - Nicotinic acid
   - Phenothiazines
   - Lithium
   - Metapyrone.

Discontinue these drugs for 3 days prior to test. This test is contraindicated in patients who have had a recent history of surgery, myocardial infarction, a labor and delivery, for these conditions can cause erroneous results. Record and report any reactions that may occur during the test. Weakness, faintness, and sweating may occur between the second and third hours. If this occurs, a blood sample for sugar is drawn and the test is discontinued. Test should be postponed in the event of unexpected illness, such as fever or gastritis or if there has been ingestion of food within 8 hours. If the fasting blood sugar is over 200 mg/dL, the GTT is usually not done. If it is done, the patient should be monitored very carefully for severe reaction or even coma.

IV GTT

In patients with gastrointestinal disorders an intravenous GTT may be done. These patients may be suffering from sprue or malabsorption syndrome or may be post-gastrectomy patients. A sterile glucose solution is given IV (20% w/v) over a 30 minutes period in an amount of 0.5 g/kg of ideal body weight. Similar blood collection intervals, including a fasting specimen, are followed, and a curve is plotted for evaluation (F, 1/2, 1, 1½, 2, 2½ and 3 hours). In a normal individual, the fasting specimen of blood contains a normal amount of glucose, the concentration of any single specimen does not exceed 250 mg%, and by 1 hour 30 minutes to 2 hours, the blood glucose level approximately comes to the fasting level (this test, however, is a less sensitive indicator of mild abnormalities of carbohydrate tolerance than the standard OGTT).

Rapid IV GTT

Here a rapid IV (50% w/v) GTT (0.5 g glucose/kg ideal body weight) to a maximum dose of 25 g may be given over a 3-4 minute period. Blood samples are obtained at intervals of 10 minutes for at least 2 hours. Under these conditions, disappearance of glucose from blood follows an exponential curve and a glucose disappearance constant can be calculated. In normal subjects, glucose disappearance usually exceeds 1.5% of the administered dose per minute, values below 1% are compatible with diabetes mellitus.

Cortisone Glucose Tolerance Test

This may reveal prediabetic patients, especially in relatives of known diabetics. Cortisone promotes intolerance in a latent or mild diabetic. After performance of an initial GTT, a standard dose of cortisone (50 mg) for adults is given parenterally 8½ hours and again 2 hours before a regular GTT. A positive test shows a blood glucose concentration of 140 mg% or higher with 2 hours specimen. Follow-up studies are necessary for such individuals.

Parenteral Administration of Glucagon or Epinephrine

Will cause a slight elevation of blood glucose concentration from glycogenolysis in normal subjects—this is much greater and more sustained in diabetics. It is also a measure of glycogen storage and release, so it may be used to study patients suspected of having a glycogen storage disease.

IV Tolbutamide Test

Oral hypoglycemic agent administration results in secretion of insulin from pancreas. This principle is taken advantage to indicate (active) insulin reserve in patients. Sodium tolbutamide (1 g is given IV in 20 mL of saline over 2 minutes to a (12 hours) fasting subject. A fasting preinjection blood specimen and postinjection samples at 2, 5, 10, 20, 30, 60, 120, and 180 minutes are collected for glucose estimation. Juvenile diabetics (insulinopenic) reveal virtually no response, while adult (maturity-onset) diabetics show a delayed increase in blood glucose concentrations. Patients with an insulin-secreting tumor (islet cell adenoma or hyperplasia) reveal a profound depression of blood glucose values, which persists below 50 mg% at 2 hours, this is associated with maximum insulin values as early as 15 minutes. Appropriate medical precautionary measures must be readily available (sterile glucose injection) and used promptly with any stress tolerance test whenever a patient's condition warrants intervention and cessation of test.

IV Insulin Tolerance Test

Here insulin 0.1 unit/kg of ideal body weight is administered IV in a fasting state, blood specimens are collected at appropriate intervals over a 2 hour period for glucose analysis. Within 30 minutes the blood glucose concentration falls to about 50 or 60% of the fasting level and returns to normal fasting levels between 1 hour 30 minutes, and 2 hours. A failure to observe such a depression in blood glucose concentration may imply insulin resistance. This may be occasionally seen in adult type diabetes, as well as in acromegaly and Cushing's syndrome. In panhypopituitarism and adrenocortical insufficiency (Addison's disease) a more profound and sustained decrease in blood glucose may be observed, hence caution should be exercised in patients suspected of having these disorders.

Glycosylated Hemoglobin (HbA_{1c}); Glycohemoglobin (GHb); Diabetic Control Index

Kits Available Commercially

Increased

Diabetes mellitus, glycosuria, and hyperglycemia.

Decreased

See, below, Factors that affect results.

Description

Glycosylated hemoglobin is blood glucose bound to hemoglobin (Hb) and includes from HbA_1a, HbA_1b, and HbA_1c. HbA_1c is formed as hemoglobin, is gradually glycosylated throughout the 120 days; red blood cell lifespan, and forms the largest portion of the three

glycosylated Hb fractions. The amount of glycosylated hemoglobin found and stored in erythrocytes depends on the amount of glucose available. HbA_1c is a reflection of how well blood glucose levels have been controlled for up to the prior 4 months. Hyperglycemia in diabetics if usually a cause of an increase in HbA_1c.

Factors that Affect Results

a. Reject hemolyzed specimens.
b. Falsely increased values may be due to fetal-maternal transfusion, hemodialysis, hereditary persistence of fetal hemoglobin, neonates and pregnancy.
c. Falsely decreased values may be due to anemia (hemolytic, pernicious, sickle cell); chronic loss of blood ; effects of splenectomy; renal failure (chronic); and thalassemias.

Other Data

a. Glycosylated hemoglobin cannot be used to monitor control of diabetic clients with chronic renal failure, as levels are significantly lower due to shortened erythrocyte survival.

Approximately 8.5% of total hemoglobin: HbA_1

Glycohemoglobin is one of the types of minor hemoglobins found in every individual. Hemoglobin A, undergoes change or glycosylation to hemoglobin A_{1a}, A_{1b}, A_{1c} by a slow, nonenzyme process within the RBCs during their circulating lifespan of 120 days. Simply putting it, glycohemoglobin is blood glucose bound to hemoglobin. The RBC, as it circulates, combines, some of the glucose from the bloodstream with its own content of hemoglobin to form glycohemoglobin in a one-way reaction. The amount of glycosylated hemoglobin found and stored by the RBC depends on the amount of glucose available to it over the RBCs 120 days lifespan. In diabetics with hyperglycemia, the increase in GHb is usually caused by an increase in HbA_{1c}. The glucose concentration will increase when hyperglycemia caused by insulin deficiency develops. This glycosylation is irreversible.

Test Significance

This test is an index of long-term glucose control. GHb monitoring reflects the average blood sugar level for the 2 to 3 months period before the test. The more glucose the RBC is exposed to, the higher the percentage of GHb. The test provides vital information about the success of treatment of diabetes such as the adequacy of dietary or insulin therapy, allows determination of duration of hyperglycemia in new cases of juvenile onset diabetes with acute ketoacidosis, provides a sensitive estimate of glucose imbalance in mild cases of diabetes, and is an evaluation of effectiveness of old and new forms of therapy such as oral hypoglycemic agents, single or multiple insulin injections, and B-cell transplantation. Test results are not affected by time of day, meal intake, exercise, just administered diabetic drugs, emotional stress, patient cooperation or accuracy.

The estimation of GHb is of greater importance for specific groups of patients. These groups include diabetic children, diabetics in whom the renal threshold for glucose is abnormal, unstable insulin-dependent diabetics in whom blood sugars vary markedly from day to day, patients who do not test urine regularly for glucose, and people who, before their scheduled appointments, will change their usual habits, dietary or otherwise, so that their metabolic control appears better than it actually is.

Clinical Relevance

1. Values are increased in poorly controlled and newly diagnosed diabetes. In these instances, HbA_{1c} levels comprise 8 to 12% of the total hemoglobin.
2. With optimal insulin control, the HbA_{1c} levels return toward normal.
3. A diabetic patient who has only recently come under good control may still have a high concentration of glycosylated hemoglobin. This level will only gradually decline as newly formed RBCs with nearly normal GHb replace older RBCs with high concentrations of GHb.

Interfering Factors

1. Spurious results should be expected in every case of hemoglobinopathy distinguishable from hemoglobin A by electrophoresis.
2. Decreased value in pregnancy and sickle cell anemia, increased value in thalassemia.

Confusion in interpretation of results may occur because there are two tests for determining glycosylated hemoglobin. The most specific test measures Hb_{A1}, which includes hemoglobin A_{1a}, A_{1b} and A_{1c}. There are different expected values for each test. Keep in mind that HbA_1, is always 2% to 4% higher than HbA_{1c}.

GLYCOSYLATED HEMOGLOBIN KIT (ION EXCHANGE RESIN METHOD) FOR THE QUANTITATIVE DETERMINATION OF GLYCOHEMOGLOBIN IN BLOOD (FOR IN VITRO DIAGNOSTIC USE ONLY)

(*Courtesy:* Tulip Group of Companies)

Summary

Glycosylated hemoglobin (GHb) is formed continuously by the adduction of glucose by covalent bonding to the

aminoterminal valine of the hemoglobin beta chain progressively and irreversibly over a period of time and is stable till the life of the RBC. This process is slow, non-enzymatic and is dependent on the average blood glucose concentration over a period of time.

A single glucose determination reflects the glucose level at the time. GHb on the other hand reflects the mean glucose level over an extended period of time. Thus GHb reflects the metabolic control of glucose level over a period of time unaffected by diet, insulin, other drugs, or exercise on the day of testing. GHb is now widely recognized as an important test for the diagnosis of diabetes mellitus and is a reliable indicator of the efficacy of therapy.

Principle

Glycosylated hemoglobin (GHb) has been defined operationally as the fast fraction hemoglobins HbA1 (Hb $A1_a$, $A1_b$, $A1_c$) which elute first during column chromatography. The nonglycosylated hemoglobin, which consists of the bulk of hemoglobin, has been designated HbA0.

A hemolyzed preparation of whole blood is mixed continuously for 5 minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the hemolysate preparation and during the binding. During this mixing, HbA0 binds to the ion exchange resin leaving GHb free in the supernatant. After the mixing period, a filter separator is used to remove the resin from the supernatant. The percent glycosylated hemoglobin is determined by measuring absorbances of the glycosylated hemoglobin (GHb) fraction and the total hemoglobin (THb) fraction. The ratio of the absorbances of the Glycosylated hemoglobin and the Total hemoglobin fraction of the Control and the Sample is used to calculate the percent Glycosylated hemoglobin of the sample.

Normal Reference Values

Normal : 4.5–8.0%
Good control : 8.0–9.0%
Fair control : 9.0–10.0%
Poor control : > 10.0%.

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*10 Tests*	*25 Tests*
Ion Exchange Resin (Predispensed Tubes)	10 × 3 mL	25 × 3 mL
Lysing Reagent	5 mL	12.5 mL
Control (10% GHb)	1 × 1 mL	1 × 1 mL
Resin Separators	10 Nos.	25 Nos.

Storage/Stability

Contents stable at 2–8°C till the expiry mentioned on the label. Do not freeze. The Resin separators can be removed on opening the kit and stored at RT.

Reagent Preparation

The ion exchange resin tubes and the lysing reagent are ready to use.

Reconstitute the control with 1 mL of distilled water. Allow to stand for 10 min with occasional mixing. The reconstituted control is stable for at least 7 days when stored at 2-8°C tightly sealed, and at least 4 weeks when stored at -20°C. Do not thaw and refreeze.

Sample Material

Whole blood. Preferably fresh and collected in EDTA. GHb in whole blood is reported to be stable for one week at 2-8°C.

Procedure

Wavelength/Filter : 415 nm (Hg 405 nm)
Temperature : RT
Light path : 1 cm

A. Hemolysate preparation
   1. Dispense 0.5 mL lysing reagent into tubes labeled as control (C) and test (T).
   2. Add 0.1 mL of the reconstituted control and well-mixed blood sample into the appropriately labeled tubes. Mix until complete lysis is evident.
   3. Allow to stand for 5 minutes.

B. Glycosylated hemoglobin (GHb) Separation
   1. Remove cap from the ion-exchange resin tubes and label as control and test.
   2. Add 0.1 mL of the hemolysate from Step A into the appropriately labeled Ion exchange resin tubes.
   3. Insert a resin separator into each tube so that the rubber sleeve is approximately 1 cm above the liquid level of the resin suspension.
   4. Mix the tubes on a rocker, rotator or a vortex mixer continuously for 5 minutes.
   5. Allow the resin to settle, then push the resin separator into the tubes until the resin is firmly packed.
   6. Pour or aspirate each supernatant directly into a cuvette and measure each absorbance against distilled water.

C. Total hemoglobin (THb) fraction
   1. Dispense 5.0 mL of distilled water into tubes labeled as control and test.

2. Add to it 0.02 mL of hemolysate from Step A into the appropriately labeled tube. Mix well.
3. Read each absorbance against distilled water.

Calculations

$$\text{Ratio of Control (RC)} = \frac{\text{Abs. Control GHb}}{\text{Abs. Control THb}}$$

$$\text{Ratio of Test (RT)} = \frac{\text{Abs. Test GHb}}{\text{Abs. Test THb}}$$

$$\text{GHb in \%} = \frac{\text{Ratio of Test (RT)}}{\text{Ratio of Control (RC)}} \times 10 \text{ (Value of Control)}$$

Linearity

The glycosylated hemoglobin procedure shows linearity for GHb levels in the range of 4.0–20.0%.

Notes

Blood samples with hemoglobin greater than 18 g/dL should be diluted 1 + 1 with normal saline before the assay.

Samples from patients with hemoglobinopathies, decreased red cell survival times, gross lipemia may show incorrect results.

Do not use ion exchange resin tubes in case of turbidity or visible discoloration.

Diabetics with metabolic imbalance may have extremely high levels of the labile aldimine form. In such cases the incubation time during hemolysate preparation may be increased to 15 minutes to ensure elimination of this instable fraction.

For mean blood glucose level based upon GHbA1/HbA1c refer to Table 17.4.

Insulin

Normal Values

		SI Units
Adult		
Fasting level	< 17 µU/mL	42–243 pmol/L or 1.00 mg/L
Newborn	3–20 µU/mL	21–139 pmol/L
Infant	< 13 µU/mL	< 89 pmol/L
Prepubertal child	< 13 µU/mL	< 89 pmol/L
Panic levels	> 30 µU/mL	> 290 pmol/L
Last trimester, amniotic fluid	11.3 µU/mL	78 pmol/L

Insulin is a hormone produced in pancreas by the beta cells of the islets of Langerhans, regulates the metabolism of carbohydrates along with liver, adipose, muscle, and other target cells and is responsible for maintaining a constant level of blood glucose. The rate of insulin secretion is determined primarily by the level of blood glucose perfusing the pancreas and is affected by hormonal status, the autonomic nervous system, and nutritional status.

Test Significance

This measurement of the insulin secretory response to glucose may be of value in establishing the diagnosis of insulinoma and in the evaluation of abnormal carbohydrate and lipid metabolism. Insulin levels are also helpful in supporting the diagnosis of diabetes in persons with borderline abnormalities of the GTT. This determination is invaluable in the investigation of fasting hypoglycemic patients and may be useful in the differentiation of islet cell neoplasms. Insulin levels may be ordered along with GTT.

Clinical Relevance

Increased values are associated with:

A. Insulinoma: diagnosis of insulinoma is based on
   1. Association of insulinoma with hypoglycemia
   2. Persistent hypoglycemia along with hyperinsulinemia between 2 and 3 hours after injection of tolbutamide.
   3. Failure of C-peptide suppression when plasma glucose is 40 mg/dL or less. After 100 g of glucose, normal insulin will rise less than 2 µU/ml to 25 to 231 in half hour, 18 to 276 in one hour, 16 to 166 in 2 hours, 4 to 38 in 3 hours. The results may be too variable to be of diagnostic importance.
B. Acromegaly
C. Cushing's syndrome.

Interfering Factors

Falsely increased values are associated with food intake, obesity, and use of oral contraceptives.

(Method: see Endocrinology chapter).

C-Peptide

Normal Values

		SI Units
Qualitative	*Negative*	
Quantitative		
Adult	68–8200 ng/mL	68–8200 µg/L
	or 20 mg/dL	
	or < 8 µg/mL	
Cord blood	10–350 ng/mL	10–350 µg/L

TABLE 17.4: For the conversion of glycosylated hemoglobin A1 (GHbA1) to gylcosylated hemoglobin A1c (HbA1c) and to the mean blood glucose level (MBG)

GHbA1	*HbA1c*	*MBG*	*GHbA1*	*HbA1c*	*MBG*	*GHbA1*	*HbA1c*	*MBG*	*GHbA1*	*HbA1c*	*MBG*
5.0	3.46	—-	9.0	6.81	141	13.0	10.16	252	17.0	13.51	—-
5.1	3.54	—-	9.1	6.89	144	13.1	10.25	255	17.1	13.60	—-
5.2	3.63	—-	9.2	6.98	146	13.2	10.33	258	17.2	13.68	—-
5.3	3.71	—-	9.3	7.06	149	13.3	10.41	261	17.3	13.68	—-
5.4	3.79	—-	9.4	7.15	152	13.4	10.50	264	17.4	13.85	—-
5.5	3.88	—-	9.5	7.23	155	13.5	10.58	266	17.5	13.93	—-
5.6	3.96	—-	9.6	7.31	158	13.6	10.66	269	17.6	14.02	—-
5.7	4.04	—-	9.7	7.40	160	13.7	10.75	272	17.7	14.10	—-
5.8	4.13	—-	9.8	7.48	163	13.8	10.83	275	17.8	14.18	—-
5.9	4.21	—-	9.9	7.56	166	13.9	10.92	278	17.9	14.27	—-
6.0	4.30	57	10.0	7.65	169	14.0	11.00	280	18.0	14.35	—-
6.1	4.38	60	10.1	7.73	171	14.1	11.08	—-	18.1	14.44	—-
6.2	4.46	63	10.2	7.82	174	4.2	11.17	—-	18.2	14.52	—-
6.3	4.55	65	10.3	7.90	177	14.3	11.25	—-	18.3	14.60	—-
6.4	4.63	68	10.4	7.98	180	14.4	11.34	—-	18.4	14.69	—-
6.5	4.71	71	10.5	8.07	183	14.5	11.42	—-	18.5	14.77	—-
6.6	4.80	74	10.6	8.15	185	14.6	11.50	—-	18.6	14.85	—-
6.7	4.88	77	10.7	8.23	188	14.7	11.59	—-	18.7	14.94	—-
6.8	4.97	79	10.8	8.32	191	14.8	11.67	—-	18.8	15.02	—-
6.9	5.05	82	10.9	8.40	194	14.9	11.75	—-	18.9	15.11	—-
7.0	5.13	85	11.0	8.49	197	15.0	11.84	—-	19.0	15.19	—-
7.1	5.22	88	11.1	8.57	199	15.1	11.92	—-	19.1	15.27	—-
7.2	5.30	91	11.2	8.65	202	15.2	12.01	—-	19.2	15.36	—-
7.3	5.39	93	11.3	8.74	205	15.3	12.09	—-	19.3	15.44	—-
7.4	5.47	96	11.4	8.82	208	15.4	12.17	—-	19.4	15.53	—-
7.5	5.55	99	11.5	8.91	211	15.5	12.26	—-	19.5	15.61	—-
7.6	5.64	102	11.6	8.99	213	15.6	12.34	—-	19.6	15.69	—-
7.7	5.72	104	11.7	9.07	216	15.7	12.42	—-	19.7	15.78	—-
7.8	5.80	107	11.8	9.16	219	15.8	12.51	—-	19.8	15.86	—-
7.9	5.89	110	11.9	9.24	222	15.9	12.59	—-	19.9	15.94	—-
8.0	5.97	113	12.0	9.32	224	16.0	12.68	—-	20.0	16.03	—
8.1	6.06	116	12.1	9.41	227	16.1	12.76	—-			
8.2	6.14	118	12.2	9.49	230	16.2	12.84	—-			
8.3	6.22	121	12.3	9.58	233	16.3	12.93	—-			
8.4	6.31	124	12.4	9.66	236	16.4	13.01	—-			
8.5	6.39	127	12.5	9.74	238	16.5	13.09	—-			
8.6	6.47	130	12.6	9.83	241	16.6	13.18	—-			
8.7	6.56	132	12.7	9.91	244	16.7	13.26	—-			
8.8	6.64	135	12.8	9.99	247	16.8	13.35	—-			
8.9	6.73	138	12.9	10.08	250	16.9	13.43	—-			

MBG in mg/dL = 33.3 x HbA1c value—86
These values are linear in the range of 6.5–13% of HbA1c values

C-peptide is formed during the conversion of proinsulin to insulin in the beta cells of the pancreas. It is secreted into the bloodstream in almost equal concentration with insulin. Normally, a strong correlation exists between levels of insulin and C-peptide, except possibly in obese subjects and in the presence of islet cell tumors.

Test Significance

C-peptide level measurement provides a reliable indication of beta and secretory function and insulin secretions. This determination has its most useful application in the evaluation of endogenous secretion of insulin when the presence of circulatory insulin antibodies interferes with the direct assay of insulin. This situation is most likely to occur in diabetics who have been treated with bovine pork insulin. The test is also useful in evaluating hypoglycemic states in identifying surreptitious injection of insulin, and in confirmation of remission of diabetes mellitus. Furthermore, monitoring following pancreatectomy for removal of cancer can provide a means of detecting the presence of residual tissue.

Clinical Relevance

1. *Increased values* are associated with endogenous hyperinsulinism in insulin-dependent diabetic persons when a high level of insulin is also present.
2. *Decreased levels* are associated with persons who have been surreptitiously injecting insulin and who have both hypoglycemia and high insulin levels.
3. *Normal levels* are found in persons who have had a remision of diabetes mellitus.
   (Method: see Endocrinology chapter).

Glucagon

Normal Values

1. 50-200 pg/mL plasma
2. Glucagon response in normal people after a standard test meal of carbohydrates, fat and protein is a gradual increase from 92 plus or minus 12 pg/mL to a peak of 125 plus or minus 13 pg/mL.
3. In a glucose tolerance test, glucagon levels will significantly decline from fasting levels during the hyperglycemic first hour in normal people.

Glucagon is a peptide hormone produced by alpha cells of the islets of Langerhans in the pancreas. In the liver, this hormone promotes glucose production. This action of glucagon is opposed to that of insulin. The normal coordinated release patterns of this hormone provide a sensitive control mechanism for glucose production and storage. For example, low glucose levels result in release, whereas conditions of hyperglycemia reduce circulating glucagon levels to approximately 50% of the amount in the fasting state.

Kidneys play an important role in the metabolism of glucagon.

Abnormally high levels of glucagon recede once insulin therapy begins to control diabetes, and levels slowly revert to normal in persons on maintenance doses of insulin. Also, in contrast to the normal glucagon, secretion in diabetics does not decrease following ingestion of a carbohydrate meal. However, an arginine infusion causes greatly increased glucagon secretion in normal persons.

Test Significance

This measurement has clinical significance in two ways. Glucagon deficiency reflects a general loss of pancreatic tissue. Compelling evidence for glucagon deficiency is the failure of glucagon levels to rise during arginine infusion. Hyperglucagonemia occurs in diabetics, acute pancreatitis, and in situations where catecholamine secretion is greatly augmented as in pheochromocytoma and in the presence of infection.

Clinical Relevance

1. Increased levels are associated with:
   a. Acute pancreatitis.
   b. Diabetes mellitus. Persons with severe diabetic ketoacidosis are reported to have levels five times normal fasting levels despite marked hyperglycemia.
   c. Glucagonoma.
   d. Uremia.
   e. Infections.
   f. Pheochromocytoma.
2. Reduced levels are associated with:
   a. Inflammatory disease with loss of pancreatic tissue.
   b. Neoplastic replacement of pancreas.
   c. Surgical removal of pancreas.

Interfering Factors

Increased levels occur in vigorous exercise and in trauma.

Other Important Tests in Diabetics

Urine — Ketone bodies (present in diabetic ketoacidosis).

Serum — Cholesterol (raised) — Triglycerides (raised) } Can be assessed chemically or by serum electrophoresis

— Ketones (raised in presence of ketonuria).

Hypoglycemia

By definition means blood glucose levels less than 50 mg%.

Causes of Hypoglycemia

Spontaneous (fasting) Hypoglycemia

1. Excessive insulin
   - Insulinoma or insulin-secreting carcinoma
   - Erythroblastosis fetalis.
2. Non-endocrine tumor—retroperitoneal fibroma
3. Glycogen storage disease of the liver
4. Malnutrition or malabsorption
5. Adrenocortical or pituitary failure
6. Liver necrosis
7. Hereditary galactosemia
8. Reye's syndrome and other forms of ketotic hypoglycemia in children.

Induced Hypoglycemia

1. Excessive insulin:
   - Overtreated insulin
   - Leucine (includes some islet cell tumors)
   - Sulfonyl ureas
   - Functional:
     - Prediabetic
     - Postgastrectomy.
     - Hemodialysis with hypertonic glucose
     - Idiopathic.
2. Reduced gluconeogenesis.
   - Ethanol
   - Hypoglycin
   - Hereditary fructose intolerance
   - Failure of glucagon secretion.
3. Persistent increase of peripheral glucose uptake:
   - Failure of catecholamine secretion
   - Propranolol blockade of catecholamine effect.
4. Cause uncertain
   - Pentamidine.

RAPID DIAGNOSTICS

1. Urine sugar: See urinalysis chapter for dipstick tests.
2. Blood sugar: Various instant blood glucose meters are available.

Accu-Chek®

(Courtesy: Roche Diagnostics)

Accu-Chek Active System: Virtually Painfree Testing in 5 Seconds

Things to do. Places to go. Whatever pace you live your life at, new Accu-Chek Active is with you all the way. In just 5 seconds, Accu-Chek Active delivers highly accurate results, whenever and wherever you need them. It's the quickest, best-looking system ever. If you don't want diabetes to slow you down, it's definitely the way to go.

FIG. 17.1: Accu-Chek Softclix

Accu-Chek Softclix (Fig. 17.1)

The exclusive Accu-Chek Softclix Lancing Device with its 11 variable depth settings and lancet allows you to draw the minimum amount of blood required.

- Virtually pain-free testing
- Small, discreet pen-like design
- Eleven variable depth settings for maximum comfort
- Lancets available on prescription.

Accu-Chek Active Meter

- Small, sleek design
- Inserting test strip switches on meter automatically
- Two hundred test memory with date and time for automatic recording of results.

Active Glucose Test Strips

- Only a tiny drop of blood required
- Accurate results in just 5 seconds.

Running a Quality Control Test

For the quality control test, please have the following items ready (Fig. 17.2):

- Your Accu-Chek Active meter with the coding chip inserted
- The pack of Accu-Chek Active Glucose test strips you took the coding chip from
- The Accu-Chek Active Control solutions
- Carefully read the pack inserts that came with the test strips and the control solutions, and select a control solution

FIG. 17.2: Test strips and controls and the instrument

FIG. 17.3: Code key insertion

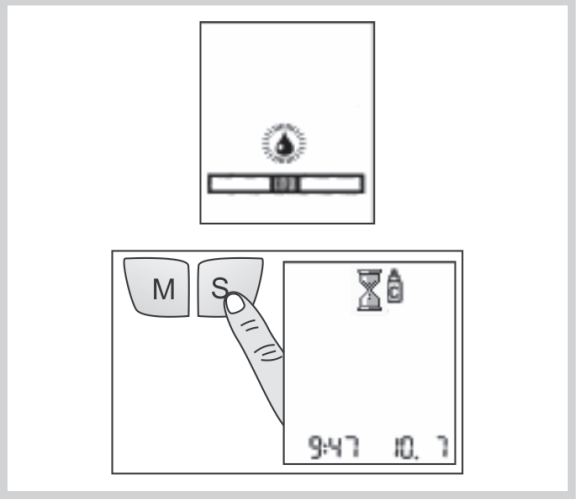


FIG. 17.4: Display with various symbols

- Remove a test strip from its container. Close the container immediately. The cap contains a drying agent which ceases to function if the container is left open, rendering the test strips unusable
- Check the round control window on the back of the test strip against the color scale printed on the test strip container. The color of the control window must match that of the color interval at the top (mg/dL). If the test strip shows a different color, do not use it
- Hold the test strip so that the application area and arrows are facing upwards. Gently push the test strip in the direction of the arrows into the test strip guide of your Accu-Chek Active meter, until you hear it click into place.

Inserting the test strip automatically puts the meter in Test Mode. Please remember that your Accu-Chek Active meter automatically turns off after about 1-2 minutes of non-use (i.e. when no button is pressed). If this happens, remove the test strip and repeat the procedure described above with a new test strip.

Now watch the display (Figs 17.3 and 17.4):

- The meter performs a display test lasting 2 seconds. Check that all the segments making up the numerals ("888" or "88.8") are properly displayed. If a segment is missing, test results may be displayed inaccurately (e.g. through 9 being confused with 3). If this happens, call your customer support and service center.
- The current code number then appears in the display. Is this the number printed on the test strip container? If not, check that your really did insert the coding chip from the new pack. If "code" is flashing and you see three horizontal bars (—) instead of a number, you have not inserted the coding chip. You can still do this now (while the display is flashing)
- Check that the correct date and time are displayed.

When the display test has been successfully completed and the code number matches, your Accu-Chek Active meter is ready for testing. The display screen that follows signals:

- That the test strip has been inserted
- The flashing drop symbol is your cue to apply the control solution (blood in the case of a real test).

To make quality control results stand out later from blood glucose test results, you can place what is known as a "flag" against them (Fig. 17.5)

- Press the S button once. In the display you see an hourglass symbol along with the control test flag (a bottle with the letter "C"). You can insert the flag at this point. If you pressed the S button inadvertently, you can press it again (before testing is complete) in order to remove the flag

- Apply one drop of the selected control solution to the application area of the test strip
- Your Accu-Chek Active meter beeps briefly to acknowledge application of solution and to announce the start of testing
- After 5 seconds a second beep signal indicates that testing is complete, and the result appears in the display. If you have not already flagged this quality control result, you may do so now.

The value displayed here is an example. The result shown on your Accu-Chek Active meter will not necessarily agree.

Now check that the displayed value is within the permitted range. Examine the test strip container and locate the "Accu-Chek Active Control" table (Fig. 17.6).

The table has two rows listed as "1" and "2", as well as two columns giving ranges in mg/dL and mmol/L.

- If you performed the test with Accu-Chek Active Control 1, see row 1 for the permitted range
- If you performed the test with Accu-Chek Active Control 2, see row 2 for the permitted range.

FIG. 17.5: Test value readout

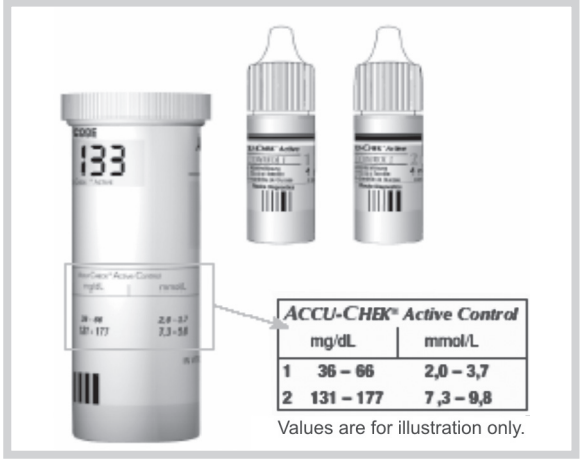


FIG. 17.6: Range reference values

If the result is within the stated range, all you need do still is carry out a visual plausibility test. It is important that this check be performed within 30-60 seconds after control solution was applied. Any later than this as comparison is no longer possible owing to excessive discoloration of the test strip.

If the result is outside the stated range, perform a second quality control test. If the second result is still outside the range, please call your customer support and service center.

- Pull the strip out of the meter. The result is saved as a control reading (which is ignored when the averages are calculated), and the meter switches off
- Turn over the test strip to reveal the circular control window on the back
- On the label of the test strip container is a color scale with blood glucose values printed alongside. Select the blood glucose value that best approximates the reading you obtained
- Compare the color of the control window with the color you selected on the label (Fig. 17.7).

The colors must be a fairly close match. If there is a great disparity, repeat the test. If you cannot obtain a close match even after several attempts at testing, please call your customer support and service center. If the colors are a close match, quality control testing of your Accu-Chek Active meter has been successfully concluded. The meter is now ready to perform further blood glucose tests.

If the measurement optics or any other part of your Accu-Chek active become soiled during testing, please clean the meter as instructed in 'Cleaning The Meter' section below.

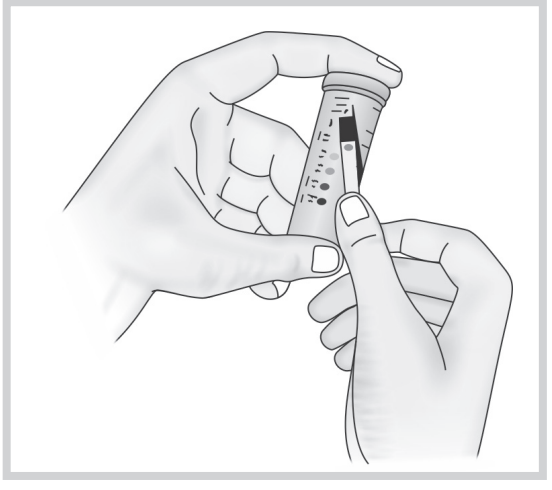


FIG. 17.7: Comparing colors

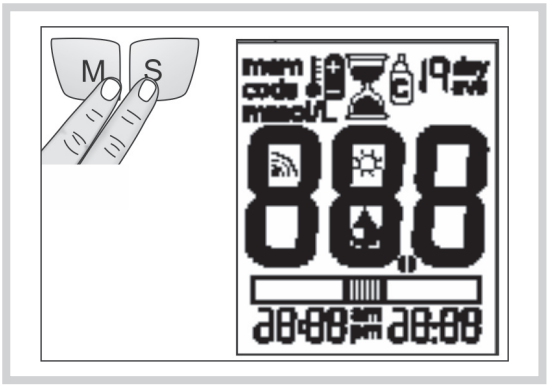


FIG. 17.8: Power on display of the instrument

The standard power-on display test checks the most important display elements. To verify that all of the elements are functioning correctly, you can carry out a full test (Fig. 17.8).

- Press and hold down the M and S buttons together for longer than 3 seconds
- All the elements of the liquid crystal display (LCD) are shown at once. Either "mmol/L" or "mg/dL" will be visible depending on the country-specific setting
- Press any key to terminate the display test and turn off your Accu-Chek Active meter. If you do not press a key, the meter will shut off automatically after about 2 minutes.

Cleaning the Meter

Your Accu-Chek Active meter has no moving parts and so will not suffer any mechanical wear and tear. As with any precision instrument, however, you will need to look after it carefully to keep it as its best.

A potential infection risk exists. Medical staff and other persons using Accu-Chek Active to test blood glucose from more than one patient must be aware that any item coming into contact with human blood is a potential source of infection. (Please see "Protection of Laboratory Workers from Infectious Diseases Transmitted by Blood, Body Fluids, and Tissues"; Second Edition, Tentative Guideline, 1991, Document M29-T2, National Committee for Clinical Laboratory Standards, US).

Accu-Chek Active utilizes an optical measuring method that relies heavily on all of its components being clean. Be sure to clean the meter, therefore:

- Whenever it is showing signs of soiling, however slight (especially on the test strip guide or the measurement optics located below it)
- Whenever you open a new pack of test strips
- Every 2 months at the latest.

FIG. 17.9: Maintenance—opening the cover

FIG. 17.10: Maintenance—cleaning the optics

Clean the measurement optics carefully with nothing other than cold water, soft lint-free cloths and cotton swabs. For disinfection you may use 70% alcohol. Any other cleaning agents may damage the meter or impair its measuring function (Figs 17.9 and 17.10).

- Slide off the test strip guide towards you (see illustration)
- After removal of the test strip guide from the meter, clean it with cold water
- Afterwards you may wipe the test strip guide with 70% alcohol

- Wipe of the alcohol immediately from the test strip guide and allow it to dry thoroughly
- Wipe the measurement optics components with a soft lint-free cloth and/or a cotton swab. The cloth/cotton swab may be slightly moistened. Make sure that no liquid enters the meter itself. Avoid scratching the measurement optics, as this will impair the measuring function
- When all of the components are thoroughly dry, you may slide the test strip guide back onto the meter. Ensure it clicks back into place. Then perform a quality control test.

Storing the Meter

Light Conditions

- Do not carry out a test where the meter and test strips are exposed to direct sunlight
- If the light is too bright; a symbol will appear in the display of your Accu-Chek Active meter
- If you see this symbol, find a shady location to carry out the test, or use your own body as a screen
- Avoid measuring in places where the light level is very changeable. Flash photography, for instance, can affect the result.

Atmospheric Humidity

- Relative humidity must be below 85%
- Sudden changes in temperature cause condensation within the meter. You may find that you are unable to turn on your Accu-Chek Active. Allow the meter to return slowly to room temperature, and never keep it in a room that is likely to harbor condensation (e.g. a bathroom).

Sources of Interference

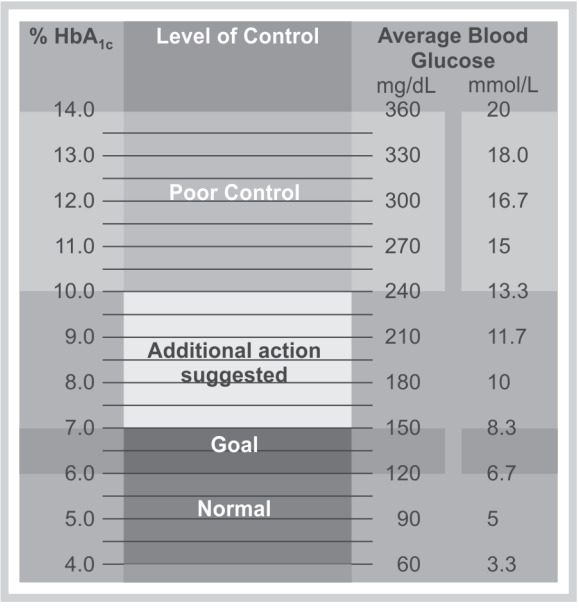
- Strong electromagnetic fields (found, for example, near mobile telephones, CB stations and microwave ovens) may affect the meter's performance. Accu-Chek Active detects this type of interference and displays an error message. Indoors stay at least 2 meters away from such sources of interference; if necessary, move somewhere else.

DCA 2000 Plus Analyzer (Figs 17.11A and B)

Accuracy, Precision, and Reproducibility with the Convenience of In-office Results

The DCA 2000+ analyzer is a point-of-care diabetes management platform that performs both hemoglobin A1c and microalbumin/creatinine tests in minutes.

A



B

FIGS 17.12A AND B: DCA 2000 plus analyzer

The DCA 2000+ allows healthcare professionals to make immediate diabetes management adjustments. Quantitative measurement of HBA1c in blood allows effective preventative treatment to reduce the risk of retinopathy, nephropathy and neuropathy in patients with diabetes. The system also measures low concentrations of albumin, creatinine and albumin/creatinine ratio in urine. The method permits decentralized testing using random urine samples, enabling early detection of complications associated with renal disease.

Easy Procedure

- Totally self-contained reagent cartridges-no reagent preparation, mixing.

Intensive Management Improves Glycemic Control

- Maintaining average blood glucose levels (120 mg/dL; 6.7 mmol/L; HbA1c 6%) lowers risk of complications
- Three to four times daily blood glucose monitoring is recommended
- Establish and follow a coherent approach of combined nutritional counseling, self-management training, and possible hospitalization for therapy initiation.

Reduce the Risk Monitor HbA1c Levels

- HbA1c results monitor glucose control over the preceding 90 to 120 days
- Complete normalization of glycemia levels may prevent complications
- Quarterly HbA1c determination recommended for all insulin-treated patients
- Recommendation also includes test for handling
- Sample collection capillary holder is an integral part of unique reagent cartridge
- No costly, time-consuming calibration-factory-calibrated instrument eliminates all wet calibrations
- Screen displays all instructions, calibration status, and testing information and results
- Up to 16 results stored in memory for convenient record keeping.

Laboratory-Accurate Results Just Minutes After Testing

- Review during patient visit, adjust blood glucose control regimen as appropriate
- Conforms with current guidelines for effective management.

HbA1c Results… in Minutes

- Guide and reinforce your patients to maintain target blood glucose levels
- Quantitative HbA1c value in 6 minutes from capillary blood
- Low cost per test
- Monoclonal antibody method provides outstanding accuracy and precision
- Correlation study shows 99% agreement with the HPLC method.

Microalbumin/Creatinine Ratio…in Minutes

- Achieving and maintaining near normoglycemic levels will delay onset of microalbuminuria and clinical albuminuria in IDDM patients
- One reagent cartridge provides results for both microalbumin and creatinine as well as an automatic calculation of the albumin to creatinine ratio
- Quantitative results and calculated ratio displayed within 7 minutes using random urine sample microalbuminuria and others, as indicated.

Detect Early Stages of Diabetic Nephropathy—Protect Your Patient from Complications

- Intensive diabetes management delays the onset of microalbuminuria—an early indicator of renal disease
- Microalbumin-to-creatinine ratio from a random urine sample is as valid an indicator of microalbuminuria as a timed 24 hours sampling
- Persistent microalbuminuria (30 to 300 mg/day) indicates the earliest stage of diabetic nephropathy
- May also signal presence of hypertension and the need to begin antihypertensive therapy
- Test for microalbuminuria should be performed yearly on postpubertal patients who have had diabetes for at least 5 years.

Specifications

Size
Depth: 10.7" (27.2 cm)
Width: 9.5" (24.1 cm)
Height: 9.4" (23.9 cm).

Weight
11.0 lb (5.0 kg).

Power
100 V to 240 V (0.4 A) 50/60 Hz.

Ambient Operating Temperature Range
15 to 32°C (59 to 90°F).

Ambient Operating Humidity Range
10 to 90% (noncondensing).

CHAPTER 18

Liver Function Tests

Liver Function Tests can be Classified as

a. Tests of excretion by the liver
b. Evaluation of synthesis in liver
c. Evaluation of enzyme activity.

Liver function tests are most often employed to determine: (i) the presence of liver disease, (ii) the type of liver disease, and (iii) the extent and progression of liver disease.

TESTS OF EXCRETION BY THE LIVER

Bile Pigment

Serum bilirubin concentration depends on the rate of removal of bilirubin from destruction of hemoglobin. Normal removal of bilirubin from the body is shown in Figure 18.1.

Normal
Bilirubin-Urobilinogen Cycle (Solid arrows = conjugated bilirubin; dotted arrows = urobilinogen)

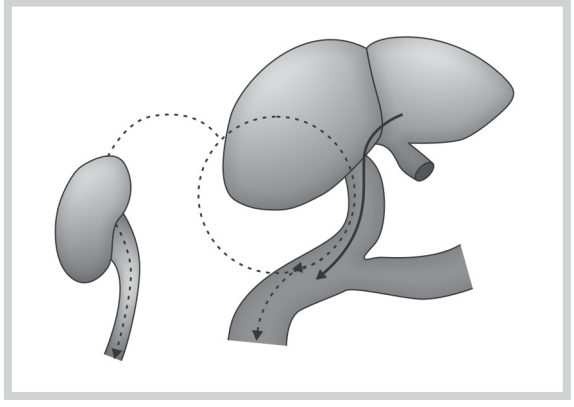


FIG. 18.1: Urine urobilinogen 0–4 mg/24 h; bilirubin absent; fecal urobilinogen 40–280 mg/24 h

Types of Bilirubin

In the plasma, bilirubin is present as 'indirect' reacting bilirubin, which is not water-soluble; and 'direct'-reacting esterified bilirubin (bilirubin glucuronide), which is water soluble. In the van den Bergh reaction, the water soluble ester reacts readily with diazo reagent (*'direct reaction'*), the addition of alcohol renders the unesterified bilirubin soluble so that diazotization may occur (*'indirect reaction'*). Jaundice: is a term used in clinical medicine to describe a visible yellow discoloration of the skin and sclera.

Classification of the Causes of Jaundice

Unconjugated Bilirubin

Prehepatic (hemolytic retention jaundice) (Fig. 18.2)

1. Excessive red cell hemolysis

Hemolytic jaundice
Bilirubin formation increased

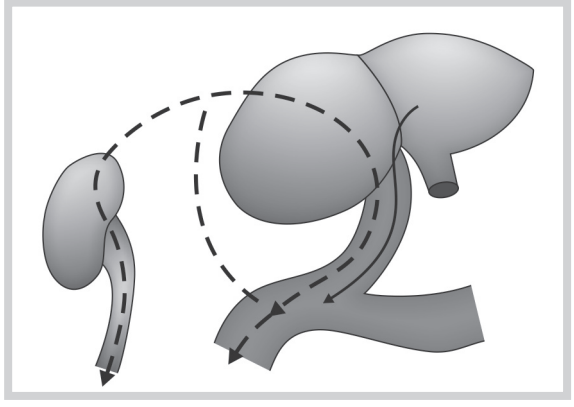


FIG. 18.2: Urine urobilinogen increased; bilirubin absent; fecal urobilinogen increased

Hepatitis
Bilirubin--Urobilinogen Cycle
(Solid arrows = Conjugated bilirubin; dotted arrows = urobilinogen)

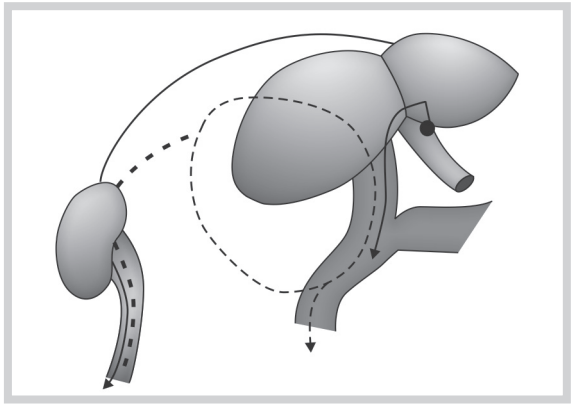


FIG. 18.3: Urine urobilinogen increased; bilirubin present; bilirubin excretion decreased; fecal urobilinogen decreased

Obstruction
Bilirubin present

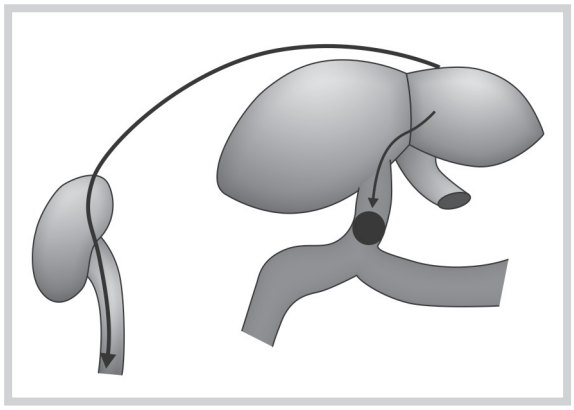


FIG. 18.4:

COMPLETE	Urine urobilinogen	Fecal urobilinogen
Tumor	Absent	Trace to Absent
Stricture		
Severe hepatitis (rarely)		
INTERMITTENT	Fluctuates	Fluctuates
Stone		

   a. *Familial:* e.g. spherocytosis, enzyme defects in red cell.
   b. *Acquired*
      - Traumatic, e.g. hematomas
      - Toxic, e.g. phenylhydrazine
      - Infective, e.g. malaria
      - Neoplastic, e.g. Hodgkin's disease.
2. Excessive "shunt" production.

Hepatic (Fig. 18.3)

1. Nonhemolytic retention jaundice (defect of transport into cell or microsomes).
   a. *Familial:*
      - UDP glucuronyl transferase deficiency (Types I and II)
      - Gilbert's disease
      - Crigler-Najjar syndrome.
   b. *Acquired or uncertain inheritance*
      Neonatal jaundice, e.g. physiological breast milk, or serum factor.

Conjugated Bilirubin

Intrahepatic Cholestasis (regurgitation jaundice)

A. Hepatocellular injury
   - *Toxic,* e.g. carbon tetrachloride necrosis
   - *Infective,* e.g. viral hepatitis
   - *Neoplastic,* e.g. primary or secondary carcinoma of liver
   - *Cirrhosis,* e.g. familial or acquired.

B. Bile duct injury
   - *Familial,* e.g. Dubin-Johnson syndrome. Rotor syndrome, recurrent familial cholestasis
   - *Toxic,* e.g. drugs (phenothiazines, steroids)
   - *Inflammatory,* e.g. sclerosing cholangitis
   - *Neoplastic,* e.g. cholangiocarcinoma
   - *Others,* e.g. primary biliary cirrhosis, pregnancy, intrahepatic atresias.

C. Posthepatic (extrahepatic cholestasis): Various causes are (Fig. 18.4):
   1. *Intramural:* e.g. stones, parasites.
   2. *Mural:*
      - *Congenital,* e.g. extrahepatic biliary atresia
      - *Inflammatory,* e.g. acute cholangitis
      - *Neoplastic,* e.g. cholangiocarcinoma, carcinoma of ampulla of Vater.
   3. *Extramural:*
      - *Inflammatory,* e.g. acute pancreatitis
      - *Neoplastic,* e.g. carcinoma of pancreas, lymphoma.

Hyperbilirubinemia

Various causes have been discussed, rise in conjugated or unconjugated bilirubin in blood/serum has been indicated.

Urine Urobilinogen

Urobilinogen is normally formed from bilirubin by bacterial action in the bowel. Normally, all urobilinogen

absorbed from the gut is excreted by the liver, only up to 4 mg appearing in urine in 24 hours.

Urine urobilinogen increased (> 4 mg/24 h);

A. Impaired liver function or partial duct obstruction.
B. 'Overloading' of the liver as a result of increased urobilinogen production following hemolytic disease.

Urine Urobilinogen Absent

If biliary duct obstruction is complete, no bilirubin enters the gut, no urobilinogen is formed, and none is found in the urine or feces.

Fecal Urobilinogen

If the hepatobiliary system is functioning, fecal urobilinogen varies directly with rate of red cell hemolysis.

Fecal urobilinogen increased occurs when blood destruction is increased and biliary obstruction is relieved.

In case of hemolysis, the daily excretion is related to the existing total body hemoglobin mass. If there is a reduced total body hemoglobin mass, accelerated rates of hemolysis may only yield an amount of urobilinogen that would be within normal limits for an individual with normal hemoglobin mass.

Fecal urobilinogen absent occurs with exclusion of bilirubin from the gut in complete biliary tract obstruction and in extreme cases of hepatocellular disease. Absence of urobilinogen in feces is important in indicating biliary tract obstruction, persistent absence is a strong indication of malignant obstructive disease. Decreased fecal urobilinogen excretion may occur when antibiotics which alter intestinal flora are used (tetracyclines, streptomycin, etc.).

Bromsulphalein (Sulfobromophthalein) Excretion Test

Sulfobromophthalein sodium (Bromsulphalein, BSP, is a dye which is bound avidly by albumin, the complex is picked up by the liver cell, and the BSP is transported into the microsomes, conjugated and excreted in the bile, in a manner analogous to bilirubin.

BSP is given intravenously and its amount present in the blood after 30, 45 or 60 minutes indicate hepatic function. The greater the liver function loss, greater the amount BSP present in blood (this test is of no significant value in differential diagnosis). BSP retention in blood conjugated/unconjugated runs parallel to bilirubin related disorders. In the presence of fevers, administration of anabolic steroids, Tele-paque, Dubin-Johnson syndrome and in Gilbert's disease, BSP retention is increased. In the latter two disorders the uptake by liver cells is normal (so normal retention at 45 minutes) but after getting conjugated it regurgitates back into blood (so BSP retention is marked at 3 hours period).

Method

1. Lipemic/jaundiced sera may cause interference. In any case one should not perform the test in the presence of acute liver/gallbladder disease.
2. Know the patient's weight, inject 5 mg BSP/kg body weight intravenously, over a period of 60 seconds. Having injected set the time to 45 minutes.
3. After 45 minutes withdraw 8–10 mL blood from the other arm, let it clot, remove serum after centrifuging.
4. Take 2 test tubes marked test and blank:

Test	*Blank*
1.0 mL serum +4.0 mL N/10 NaOH mix by gentle inversion	1.0 mL serum +4.0 mL N/10 HCl mix by gentle inversion

   The full reddish color develops in the alkaline solution, and the dye is colorless in acid solution.
5. Read the absorbance of test at 575 nm (or 590 nm) setting the zero with the blank.
6. Refer the absorbance reading to the calibration curve to obtain the percentage of the dye dose remaining in the blood after the 45 minutes interval. Report as percentage of dye retention afterminutes," giving the dose used. Normal retention is up to 4% (average 2.8%).

Calibration: Into a 1 liter volumetric flask pipette very accurately 2.0 mL of 5% BSP. Dilute to the mark with distilled water and mix well. Into 4 clean test tubes pipette accurately the following amounts of the diluted dye: 0.25, 0.50, 0.75, and 1.0 mL. Make each to 1.0 mL with distilled water. To each tube add exactly 4.0 mL of N/10 NaOH. Mix by inversion and read the absorbance at 575/590 nm. These standards correspond to values of 25, 50, 75 and 100% retention in the test. Plotted on graph paper, the readings should fall on a straight line passing through the origin.

Conditions Associated with Increased BSP Retention

Hepatobiliary System

- Jaundice from any cause except Gilbert's syndrome
- Viral hepatitis
- Toxic hepatitis
- Fatty liver
- Cirrhosis
- Bile duct obstruction
- Metastatic carcinoma
- Lymphomatous or leukemic infiltration
- Granulomatous inflammation

- Amyloidosis
- Dubin-Johnson syndrome.

Extrahepatic Conditions
- Congestive heart failure
- Fever above 39°C
- Oral contraceptive use
- Prolonged fasting or malnutrition
- Contrast media used for gallbladder examination.

Artefacts
- Obesity
  - Spuriously high retention because excessive weight results in excessive dose
- Hypoalbuminemia
  - Spuriously low retention because binding is reduced
- Ascites
  - Spuriously low retention because the dye enters the ascitic fluid
- Proteinuria
  - Spuriously low retention because albumin bound dye enters urine.

EVALUATION OF SYNTHESIS IN LIVER

Serum Proteins (Albumin Especially)

Since serum albumin and a small fraction of globulin are synthesized in liver, serum proteins are affected both quantitatively and qualitatively in liver disease. In any disease causing hepatocellular damage, the concentration of serum albumin decreases. In many liver disorders, serum globulins may rise to such a level so as to maintain normal or increased total protein concentration even when there is severe albumin depletion.

The changing levels of serum albumin thus provide valuable indices of severity, progress, and prognosis in hepatic disease. Decreased albumin and elevated globulins in serum indicate hepatocellular origin of jaundice or liver disease. In obstructive jaundice, serum protein changes occur late, after secondary hepatocellular damage has occurred. Cholangitis and biliary cirrhosis, however, result in liver damage which may not be accompanied by protein alteration. Furthermore, serum protein changes may return to normal before convalescence from hepatitis is complete. However, liver disease is not the only cause of serum protein alterations.

Chemical methods and electrophoretic methods are available for serum proteins estimation. Electrophoresis is most precise and specific way of assessing serum proteins. The flocculation and turbidity methods crudely estimate globulins and hence are not specific and obsolete in today's context.

Prothrombin Concentration

Deficiency of prothrombin may occur as a result of:
1. Inadequate absorption of bile from the intestinal tract, or
2. Inability of a damaged liver to convert vitamin K to prothrombin.

A normal prothrombin concentration does not rule out abnormal liver function.

Low Prothrombin in Presence of Jaundice

When a low prothrombin level is found in a jaundice patient, give 2–4 mg vitamin K, IV or IM, and measure prothrombin concentration later.
1. Return to normalcy of prothrombin concentration (85–100% of normal) indicates that the capacity of liver cells to synthesize prothrombin is good.
2. A poor response implies hepatocellular disease, either primary or following prolonged obstructive disease.

Low Prothrombin in the Absence of Jaundice

In the absence of jaundice, a low prothrombin level usually indicates serious liver damage, and no response to large doses (60–70 mg) of parenteral water-soluble vitamin K confirms it. This is true if jaundice is also present.

Cholesterol and its Esters

Decrease of Both Substances

Associated with extensive destruction of liver parenchyma is reduction in serum levels of cholesterol and cholesterol esters, extremely low concentration implies a poor prognosis. Persistently low cholesterol ester concentration or ester/total cholesterol ratio indicates continuing hepatocellular damage, a rise in cholesterol ester is considered as a good sign and heralds improvement.

Increase of Total but Decrease of Esters

Accompanying biliary obstruction is usually a rise in total cholesterol, but the cholesterol ester concentration is often unaffected. The determination of cholesterol ester, however, is not a fruitful exercise clinically.

Detoxification

The liver removes noxious materials or renders them harmless by conjugation of toxic substances with amino

acids, glucuronate and inorganic radicals (e.g. sulfate), by oxidation or reduction, by excretion, etc.

Hippuric Acid Test

This test depends upon conjugation by liver of sodium benzoate with glycine to produce hippuric acid, which is excreted in the urine. It is preferrable to give sodium benzoate-1.77 g-IV (instead of orally in which case the absorption may be irregular), one hour later at least 0.7 g of hippuric acid should be excreted in the urine. Consideration of low values is permissible only if impaired renal function is ruled out for retention of hippuric acid.

EVALUATION OF ENZYME ACTIVITY

Serum Transaminases

Liver and muscles are rich in enzymes of Kreb's cycle. Among such enzymes is a group responsible for transfer of NH_2 groups from amino acids to keto acids, thus, providing for metabolism of amino acids. Destruction of muscle or of liver cells releases the enzymes, with consequent rise in their values in plasma. In obstructive jaundice and more so in acute hepatitis, the serum levels of SGOT and SGPT rise to very high levels (300-1500 units, normal being 5-40 units), as does LDH concentration (normal concentration, 200-450 units). Chronic hepatitis may produce moderate elevations of serum transaminases. Liver cell destruction incident to neoplastic disease metastatic to the liver produces moderate elevation of transaminases concentration in the serum.

In many cases, there seems to be a correlation between the differences in the degree of elevation of SGOT and SGPT and the cause of jaundice. Rise of SGPT is greater than elevation of SGOT in extrahepatic obstruction, acute hepatitis and toxic hepatitis, the reverse is true in cirrhosis of liver, intrahepatic neoplasm, and hemolytic jaundice.

Serum Alkaline Phosphatase

The concentration of this enzyme often increases in the plasma of an icteric patient. It is normally present in the liver and excreted in the bile so that elevation of serum alkaline phosphatase may be a manifestation of retention; this is a convenient explanation for the observation that serum alkaline phosphatase concentration increases in obstructive jaundice. In acute and chronic hepatocellular disease, serum alkaline phosphatase is raised, but not to the extent typical of obstructive jaundice. In hemolytic jaundice, normal levels are the rule. In some cases of metastatic carcinoma of liver, serum alkaline phosphatase may rise in the absence of jaundice. It should be kept in mind that phosphatase levels may be normal early in obstructive disease and with relief of obstruction. Pregnancy and such diseases as Paget's disease of bone, hyperparathyroidism, and rickets/osteomalacia, are also associated with elevated serum alkaline phosphatase concentration and these must be ruled out.

SUGGESTED LIVER FUNCTION TESTS

A. Jaundice Absent

Urine bilirubin, urine urobilinogen, serum bilirubin, BSP excretion, transaminases.

B. Jaundice Present

As mentioned above (except BSP excretion), plus alkaline phosphatase, prothrombin response, serum proteins.

C. Possible Metastatic Cancer

Alkaline phosphatase, transaminases, and bilirubin. If alkaline phosphatase and transaminases are high in the presence of normal bilirubin and normal or mildly increased BSP retention, one should suspect metastatic cancer in the liver.

Serum protein changes in selected diseases

Disease	*Albumin*	*Globulin*	*β Globulin*	*γ Globulin*
Acute hepatitis	N or Sl ↓	N or Sl ↑	Sl ↑	↑ (IgG and M)
Laennec's cirrhosis	↓↓	N	Sl ↑	↑↑ (IgM and A)
Chronic active hepatitis	↓↓	N	↑	↑↑ (IgG)
Biliary cirrhosis	↓	↑	↑↑	↑ (IgM)
Extrahepatic biliary obstruction	N	N or Sl ↑	↑	N

N=normal Sl=slightly, ↓ = moderately depressed, ↓↓ = severely depressed, ↑ = moderately elevated, ↑↑ = markedly elevated

Liver Battery (Profile), Serum

Normal Values are Dependent Upon Methods/Kits/ Manufacturers

Normal values		*SI units*
Alanine Aminotransferase (ALT or SGPT)		
Adult female	4–35 U/L	
Adult male	7–46 U/L	
Children		
< 12 months	≤ 54 U/L	
Age 1–2 years	3–37 U/L	
Age 2–8 years	3–30 U/L	
Age 8–16 years	3–38 U/L	
Alkaline Phosphatase (ALP)		
Adults Age 20–60 years		
Bodansky	2–4 U/dL	10.7–21.5 IU/L
King-Armstrong	4–13 U/dL	25.0–92.3 IU/L
Bessey-Lowery-Brock	0.8–2.3 U/dL	13.3–38–3 IU/L
Elderly	Slightly Higher	
Newborn	1–4 times adult values	
Children	Values remain high until epiphyses close	
Female		
Age 2–10 years	100–350 U/L	
Age 10–13 years	110–400 U/L	
Males		
Age 2–13 years	100–350 U/L	
Age 13–15 years	125–500 U/L	
Aspartate Aminotransferase (AST or SGOT)		
Adult females		
≤ Age 60 years	8–20 U/L	8–20 IU/L
> Age 60 years	10–20 U/L	10–20 IU/L
Adult males		
≤ Age 60	8–20 U/L	8–20 IU/L
> Age 60	11–26 U/L	11–26 IU/L
Children		
Newborn	16–72 U/L	16–72 IU/L
Infant	15–60 U/L	15–60 IU/L
Age 1 year	16–35 U/L	16–35 IU/L
Age 5 years	19–28 U/L	19–28 IU/L

Contd...

Contd...

Bilirubin		
1 month–adult	< 1.5 mg/dL	1.7–20.5 µmol/L
Premature infant		
Cord	< 2.8 mg/dL	< 48 µmol/L
24 hours	1–6 mg/dL	17–103 µmol/L
48 hours	6–8 mg/dL	103–137 µmol/L
3–5 days	10–12 mg/dL	171–205 µmol/L
Full–term infant		
Cord	< 2.8 mg/dL	< 48 µmol/L
24 hours	2–6 mg/dL	34–103 µmol/L
48 hours	6–7 mg/dL	103–120 µmol/L
3–5 days	4–6 mg/dL	68–103 µmol/L
Gamma–Glutamyl Transferase/Transpeptidase (GGT/GGTP)		
Adult female	4–25 U	
	9–31 mU/mL	
	3.5–13 IU/L	
	3–33 U/L at 37°C	
Adult males	7–40 U	
	12–38 mU/mL	
	4–23 mU/mL	
	9–69 U/L at 37°C	
Children		
Cord blood	190–270 U/L at 37°C	
Premature infant	< 140 U/L at 37°C	
1–3 days	56–233 U/L at 37°C	
4–21 days	0–130 U/L at 37°C	
3–12 weeks	4–120 U/L at 37°C	
3–6 months, female	5–35 U/L at 37°C	
3–6 months, male	5–5 U/L at 37°C	
> 6 months, female	15–85 IU/L	
> 6 months, male	5–55 IU/L	
1–15 years	0–23 U/L > 37°C	
Hepatitis A, B, C, D, E Profile		
Negative		
Lactate Dehydrogenase (LD/LDH)		
Wroblewski method	150–450 U/L	72–217 IU/L
30°C		
Adults		
< Age 60	45–90 U/L	45–90 U/L
> Age 60	55–102 U/L	55–102 U/L

Contd...

Contd...

Children		
Newborn	160–500 U/L	160–500 U/L
Neonate	300–1500 U/L	300–1500 U/L
Infant	100–250 U/L	100–250 U/L
Child	60–170 U/L	60–170 U/L
Leucine Aminopeptidase (LAP)		
	12–33 IU/dL	< 50 IU/L
5'Nucleotidase (5'NT or 5'N)		
2–15 IU/L		
0–17 U/L		
0–1.6 U		
0.3–3.2 Bodansky units		
Protein Electrophoresis		
Normals are dependent on laboratory procedure.		
Percentage values are for the Agarose method and represent the percentage of total protein:		
Adult (Agarose method)		
Total protein		5.90–8.00
Albumin	58–74%	0.58–0.74
Alpha$_1$ globulin	2.0–3.5%	0.02–0.04
Alpha$_2$ globulin	5.4–10.6%	0.05–0.11
Beta globulin	7.0–14.0%	0.07–0.14
Gamma globulin	8.0–18.0%	0.08–0.18
Adult		
Total protein	6.0–8.0 g/dL	60–80 g/L
Albumin	3.3–5.0 g/dL	35–50 g/L
Alpha$_1$ globulin	0.1–0.4 g/dL	I–4 g/L
Alpha$_2$ globulin	0.5–1 g/dL	5–10 g/L
Beta globulin	0.7–1.2 g/dL	7–12 g/L
Gamma globulin	0.8–1.6 g/dL	8–16 g/L
Premature infant		
Total protein	4.4–6.3 g/dL	44–63 g/L
Albumin	3.0–4.2 g/dL	30–42 g/L
Alpha$_1$ globulin	0.11–0.5 g/dL	1.1–5 g/L
Alpha$_2$ globulin	0.3–0.7 g/dL	3–7 g/L
Beta globulin	0.3–1.2 g/dL	3–12 g/L
Gamma globulin	0.3–1.4 g/dL	3–14 g/L
Newborn		
Total protein	4.6–7.4 g/dL	46–74 g/L
Albumin	3.5–5.4 g/dL	35–54 g/L
Alpha$_1$ globulin	0.1–0.3 g/dL	1–3 g/L

Contd...

Contd...

Alpha$_2$ globulin	0.3–0.5 g/dL	3–5 g/L
Beta globulin	0.2–0.6 g/dL	2–6 g/L
Gamma globulin	0.2–1.2 g/dL	2–12 g/L
Infant		
Total protein	6.0–6.7 g/dL	60–67 g/L
Albumin	4.4–5.4 g/dL	44–54 g/L
Alpha$_1$ globulin	0.2–0.4 g/dL	2–4 g/L
Alpha$_2$ globulin	0.5–0.8 g/dL	5–8 g/L
Beta globulin	0.5–0.9 g/dL	5–9 g/L
Gamma globulin	0.3–0.8 g/dL	3–8 g/L
Child		
Total protein	6.2–8.0 g/dL	62–80 g/L
Albumin	4.0–5.8 g/dL	40–58 g/L
Alpha$_1$ globulin	0.1–0.4 g/dL	1–4 g/L
Alpha$_2$ globulin	0.4–1.0 g/dL	4–10 g/L
Beta globulin	0.5–1.0 g/dL	5–10 g/L
Gamma globulin	0.3–1.0 g/dL	3–10 g/L
Prothrombin time		
Adult		10–15 seconds
Newborn		< 17 seconds
Child		11–14 seconds

Usage

Work-up for liver disease, biliary disease, hepatoma, liver metastatic chronic active hepatitis, cirrhosis, including biliary cirrhosis, hepatic complications associated with medications or TPN.

Increased

See individual test listings.

Decreased

See individual test listings.

Description

Liver battery includes testing for several blood levels that reflect hepatic function. In general, a liver battery includes the following: Alanine Aminotransferase, Serum; Alkaline Phosphatase, Serum; Aspartate Aminotransferase, Serum; Bilirubin. Gamma-Glutamyl Transpeptidase, Blood; Hepatitis profile Antigen, Blood; Lactate Dehydrogenase. Blood: Leucine Aminopeptidase, Blood; 5' Nucleotidase. Blood: Protein, Electrophoresis, Serum; Prothrombin time, Blood. See individual test listings for specific descriptions.

CHAPTER 19

Clinical Chemistry

COLORIMETRY

Colorimetry is the science that deals with the measurement of the capacity of a chemical, colored system to absorb light. Since, it makes specific quantitative measurements, it is very useful and widely used in laboratories in the form of **colorimeter or spectrophotometer.** To understand colorimetry, it is essential to have some knowledge and understanding of what is meant by **Light, Color and Beer's Law.**

- **Light** is a form of energy (radiant energy)
- It moves in space in the form of waves like the electromagnetic waves.
- The peak of the wave is called the **CREST.**
- The lowest point of the wave is called the **Trough.**
- The distance between two identical points on a wave cycle is called the **wavelength.**
- The unit of measure for wavelength is **nanometer (nm).**
- Wavelengths are also expressed as **lambda (λ).**

The colors are the wavelength what we see. It is the wavelength that determines the color of the light. The human eye can only see the wavelengths of energy between about 400 and 750 nm. This is called the **visible spectrum.**

The total light spectrum can be divided into 3 distinct regions—the ultraviolet region, the visible region and the infrared region. The wavelengths of the various regions and colors are shown below.

Light whose wavelength is 400 nm is violet. Light with wavelength less than 400 nm is not visible to the human eye and is known as ultraviolet. Light with wavelengths of more than 700 is not visible and is known as infrared light. The visible spectrum occurs between the wavelengths of 400 and 700 nm. Here we have the colors of violet, blue, green, yellow, orange and red or the "rainbow". Thus, white light is seen colorless, it is composed of all colors of the visible spectrum.

The color of a substance will depend on the wavelength absorbed by the substance and which are transmitted to the observer's eye.

Beer's Law

When a colored solution is illuminated with monochromatic light, its absorbance is directly proportional to the concentration of the colored solution when the light path is constant.

Absorbance α Concentration
(when light path length is constant)

Lambert's Law

When a colored solution is illuminated with light, its absorbance is directly proportional to the light path when concentration of the solution is constant.

Absorbance α Length,
(when concentration is constant).

If we combine both we get the Beer Lambert's Law;

"When a colored solution illuminated with monochromatic light, it's absorbance is proportional to the concentration of the colored solution and the length of the light path."

Absorbance α Concentration X Length
(A) (C) (L)

$$A = \in CL$$

Where $\in$ is the molar absorption coefficient.

In all the colorimetric determinations, a reference standard of known concentration is used and its color intensity is compared with color intensity of the test sample,

$$A_t = \in C_t L$$

$$As = \in C_s L \text{ (t = test, s = standard)}$$

Since, the same cuvette is used for the test and standard, L is constant.

$$A_t = C_t$$

$$A_s = C_s$$

If concentration of the standard, i.e. C_s is known

$$\text{then } C_t = \frac{A_t \times Cs}{A_s}$$

Calculation of Absorbance (A)

Usually *colorimeters measure transmittance* rather than absorbance. *Transmittance and Absorbance has an inverse relationship.*

T is the ratio of intensity of emergent light (Ie) to the intensity of incident light (Io)

T = Ie / Io

A = –log T%

A = 1 / Log T% or A = – log T%

A = – log T%

= log 1/T%

= log 100–log T = 2.0–logT

In *clinical chemistry,* there are two ways of expressing the amount of light absorbed by a solution. These are:

Amount Transmission (%T)

Percent transmission is the amount of light, which passes through a colored solution compared to the amount of light, which passes through a blank or colorless solution. As the concentration of the colored solution increases the amount of light absorbed increases while the %T decreases.

Optical Density (OD)

The OD may be calculated from the %T and is the units preferred in clinical chemistries, the reason OD is usually preferred is that there is a *direct relationship* between the *concentration of a solution* and the OD, i.e. as the concentration of a solution increases, the absorbance or OD also increases.

By taking the unknown, we can use the derived formula from Beer's law to find the concentration of the unknown, which is:

$$\frac{\text{Concentration unknown}}{\text{Concentration standard}} = \frac{\text{OD unknown}}{\text{OD standard}}$$

$$\text{i.e. Concentration} = \frac{\text{OD unknown}}{\text{OD standard of standard}} \times \text{concentration of unknown}$$

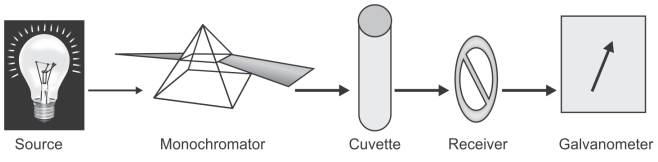
PHOTOMETER

The basic components of a photometer are as shown above. They are:

- Light source
- Filter or monochromator
- Cuvette
- Photodiode cell (receiving light signals)
- Galvanometer.

Light Source

A tungsten filament lamp is usually used as source for light (radiant energy). Its intensity varies upon the type of photometers. A uniform voltage supply is very important for a stable source of light. Ageing of the lamp or accumulation if dirt can result in range in absorbance reading.



Wavelength Selectors

In most instruments filters are used for this purpose. The filter chosen is usually complementary to the color of the solution to be measured (see table below).

Color of solution	Usual filter
Blue	Yellow
Bluish-green	Red
Purple	Green
Red	Bluish-green
Yellow	Blue
Yellowish-green	Violet

Filters are made of glass or dyed gelatine between glass plates and have a limited transmission band at which they transmit maximally. To understand the use of light filters consider a bluish-green solution which absorbs light in the red part of the spectrum. Such a solution when illuminated by white light absorbs red color wavelengths and emits bluish-green light together with a small amount of red. The greater the concentration of the solution the smaller the amount of red light transmitted. The most sensitive readings of the galvanometer will therefore be obtained by allowing only the transmitted red light to activate the photoelectric cell. The red filter achieves this by stopping the transmission of bluish-green light and allowing only the red light to pass through the solution.

In the more expensive type of equipment, a **diffraction grating** or a **prism** is used to obtain the required wavelength.

In **diffraction grating,** the white light is dispersed into a continuous spectrum. By turning a wavelength adjustment, the grating is rotated and different parts of the spectrum are allowed to fall onto the photocell.

In **glass prism** spectrophotometers, light is focused onto the prism. Light passes through and forms an extended spectrum. On adjusting the exit slit (wavelength adjustment) light can pass through the cuvette, and illuminate the photocell.

Cuvettes and Flow-through Cells

These are used to hold colored solutions and must be scrupulously clean, with no dirty finger marks or spillage of fluid on the outside of the optical side. Spillage of fluid or dirty finger marks will absorb light and interfere in the measurement of the color. Scratches on the glass must be avoided and if badly scratched it must be discarded.

In order to speed up laboratory work, a more recent development in colorimetry is the introduction of follow-through cells. These cells enable colorimetric readings to be speeded up considerably, since the cells or cuvettes can be drained without being removed from the colorimeters.

Photoelectric Cell

A photoelectric cell consists of photoelectric elements; light falling on these elements generates an electric current which deflects a galvanometer needle, the deflection being proportional to the light intensity.

Galvanometer

The galvanometer measures the output of the photosensitive element, and in good instruments a very sensitive instrument is used.

Requirements of Colorimetric Analysis

When colorimetric determinations are made, it is essential to ensure that the color being measured is only due to the substance under investigation and is not due to any of the reagents used. It is, therefore, essential to include the following solutions.

1. Test solution
This contains the unknown concentration of the substance together with the reagents used in the test.

2. Standard solution
This is usually identical to the test solution, except that it contains a known amount of the substance being determined and is approximately equal in concentration to that expected in the test.

3. Blank solution
This solution is identical to both the test and standard solution and it is carried through the complete test procedure and contains all the reagents used, but without any test or standard substance. Any color given by the reagents used in the analysis can be detected and eliminated.

In order to be sure that the absorbance is due solely to the substance under test, the reading given by the 'blank' solution must be considered with the reading obtained from the 'test' and 'standard' solutions. The photoelectric absorptiometer is set to read zero absorbance with distilled water. The blank, test and standard absorbance readings are recorded, rechecking the zero absorbance between each reading. The blank reading is then subtracted from the test and standard reading as follows:

$$\frac{\text{Test} - \text{Blank}}{\text{Standard} - \text{Blank}} \times \text{concentration of standard}$$

This procedure will usually ensure that only the substance under investigation is being measured. Satisfactory results are only obtained with absorbance ranging from 0.2 to 0.8, so that if possible the determination should be modified in order that the lower and upper limits of deflection fall within this range.

Sources of Error in Photometry

Errors in photometry can be attributed to three sources.

1. Inherent properties of the solution being measured
2. Instrument
3. Operator.

Inherent Properties of the Solution

The factors, which may be included in this group, influence the absorption of light by the solution and can be the cause of deviations from Beer's Law.

a. Chemical Nature of the Solvent and Solution

Deviations from Beer's law may occur either as a result of a shift in the shape of a given portion of the absorption curve as the concentration changes or because of the absence of a linear relationship between optical density and concentration. A shift in the shape of a portion of the absorption curve can indicate a chemical transformation of a portion of the colored component being analyzed into a second component of a different color. The production of a second colored component may also occur due to an impurity in the solvent in which the original colored

component is dissolved. For example, iodine dissolved in carbon tetrachloride is deep purple but dissolved in alcohol is brown. The presence of only 1% alcohol as an impurity in carbon tetrachloride is sufficient to change the color and hence, the shape of the absorption curve of iodine in carbon tetrachloride. Thus, the absolute purity of the solvent is very important in spectrophotometric work. This is particularly true for analysis carried out in the ultraviolet region. The breakdown of a linear relationship between optical density and concentration can be due to the dissociation of a colorless substance to give colored ions, or vice versa.

b. Exposure to Light

Certain compounds tend to bleach or discolor or get colored when exposed to light. Such photochemical reactions are likely to occur when the test sample is stored in a warm, brightly-lighted room. This may occur while the sample is in the photometer, if the intensity of illumination is too high, but in most instruments the intensity is below the danger level. Methods in which photochemical reactions are likely to occur usually mention the precautions to be taken against light exposure. For example, the reconstituted glucose reagent kit is recommended to be stored in a dark bottle, because on exposure to light a photochemical reaction takes place and the reagent gets oxidised and develops a pink color.

c. Color Instability

In some colorimetric reactions, the color may be stable for only a short period of time. It is then necessary to time the reaction carefully so that the readings of all samples and standards are made during the time that the color remains constant. Instability of color may be due to temperature or absorption by the walls of the container where these factors have an influence, they must be kept constant for test samples and standards.

d. Foreign Matter and Air Bubbles

Solutions in the cuvettes must be free of lint or other foreign matter, and air bubbles. A scrupulous cleaning of the cuvettes and other glassware used in the analysis should help to eliminate foreign matter from the solution. For example, in a flow through cuvette of most semi-automated analyzers an air bubble trapped in it, will lead to a decrease in optical density of the solution.

e. Errors of Weighing and Dilution

Simple errors of weighing and dilution in preparing reagents, sample and standards can affect photometric results appreciably. A good analytical balance and reliable volumetric glassware should be used. For example, while reconstituting a control serum care should be taken while dispensing the volume of distilled water to the control sera bulb. An error in dilution will result in change in the concentration of the constituents.

Instrument

Instruments are capable of considerable precision of measurement. The instrumental precision is of a higher order than that normally resulting from the development of color in the test solutions. Inherent errors of the solution as mentioned above actually cause greater deviation than do instrumental errors.

a. Light Source

Unless a double cell photometer is being used, the consistency and reproducibility of the light source is important. Fluctuations in voltage should be overcome by the use of a voltage stabilizer in line-operated instruments. Lamps should be allowed to warm up for at least 5 minutes before steady output can be expected.

b. Stray Light

Stray light from windows or overhead lighting striking the instrument can cause error since invisible particles suspended in solutions can reflect these rays. The covering of the cuvette compartment with a light-tight cover (as usually provided with the instrument) before taking readings will reduce this error.

c. Slit Width

In the prism spectrophotometer, the purity of the monochromatic band depends on the width of the entrance and exit slits. The use of a narrow slit width will produce more accurate results.

d. Moisture

Moisture can be the cause of fluctuating readings in spectrophotometric operation. In instruments employing desicant, it is advisable to change the freshly dried silica gel at regular intervals. This is particularly important in an environment of high humidity.

e. Linearity of Photocell Response

Reliable results depend upon the current output of the photocell being proportional to the light striking the photocell. This relationship can be disturbed if the photocell is not adequately protected from moisture and from overheating. Some instruments are fitted with heat absorbing filters in the optical system and provided with thermal insulation of the light source.

f. Cuvettes

An important source of error and one which requires constant checking is the cuvette. It is necessary that cuvettes be optically matched, so that readings will not be influenced by their individual variation when a series is used for making measurements. When it is necessary to reuse cuvettes that have not had adequate time to dry after cleaning, a rinse with alcohol and ether or acetone may be used to speed up the drying process. If cuvettes are dirty, etched, scratched or marked with fingerprints, erroneous readings will result.

g. Wavelength Calibration

A wavelength scale which is off calibration can be a source of error in spectrophotometers.

Operator Errors

Errors of the operator can be minimized by the practice of good technique in the laboratory preparation of the solutions and in the operation of the instrument. The operator should be guided in the latter instance by the instructions provided by the manufacturer. An awareness, of the sources of error in the preceding categories should tend to reduce these errors.

CLINICAL CHEMISTRY

Specimen Collection and Processing

With the exception of glucose, triglycerides and inorganic phosphorus, most blood chemical constituents reveal no significant change after a standard breakfast, so it is not essential for the patient to be in an absolute fasting state prior to blood specimen collection. However, lipemia (lactescence), caused by transient rise in triglycerides as chylomicrons following a meal containing fat may cause interference with a large number of chemical determinations because of turbidity. Therefore, blood is always collected from a patient in the post-absorptive state. This can be accomplished with an overnight fast (12–14 hours, especially for lipids), although a 4 to 6 hours fast will usually suffice.

Venipuncture should be performed for obtaining blood. Disposable needles eliminate the hazard of serum hepatitis transmission. Heparin is the most ideal anticoagulant for plasma determination. The cost is quite prohibitive so others EDTA, and trisodium citrate can be used without significant alteration in reading and results. For glucose, oxalate-fluoride mixture is used. Fluoride impairs glycolysis of the blood cells. Prompt separation of plasma/serum is essential to yield a proper specimen for most chemical estimations. Always collect a little more blood than required so as not to fall short of it subsequently. For 1 mL of serum about 2.5 mL of blood should be withdrawn. Labelling and identification is important.

Pipettes: For dispensing test materials or reagents, etc. exact quantities are needed. For volumes till 0.1 mL Borosil's pipettes can be used. Otherwise autopipettes for micro to macro sampling can be used. Dispensing exact amounts of test samples/reagents is the first step to accurate clinical chemistry.

Proper Specimen Collection

If the commercially available kits are being used, follow the manufacturers guidelines and collect the requisite amount of blood.

Specimen Collection

Chemistry (plain tube)

Amylase
Alcohol
Bilirubin
Barbiturate
Salicylate
BSP
Calcium
Cholesterol
Copper
Creatinine
CPK
SGOT
SGPT
Urea
T_4, T_3, TSH
Iron and iron binding capacity
LDH
Lipase
Lithium
LATS and TSH
Triglyceride
Electrolytes
BUN
Uric acid

Chemistry (Heparin)

pH
Ammonia
RBC Potassium
Renin
Plasma testosterone
Cholinesterase
Plasma cortisol
Methemoglobin
Plasma hemoglobin

Chemistry

Oxalate, fluoride tube
Glucose
Glucose tolerance test

Hematology (EDTA)

Complete blood counts
WBC, RBC, Hb, PCV,
MCV, MCH, MCHC
Differential count
Absolute eosinophil count

Hematology (EDTA)

Hb electrophoresis
G_6PD screening
Reticulocyte count
ESR
Sickling test
Platelet count.

Hematology (plain tube)

Haptoglobin,
LE preparation
Serum viscosity.

Hematology (Sodium citrate)

PTTK
Prothrombin time
Thrombin time
Fibrinogen titer
Fibrinogen level.

Blood bank (plain tube)

Crossmatch
Typing
Coombs' test
Antibody identification.

Serology (plain tube)

α_1 antitrypsin
Antinuclear antibody
Antistreptolysin-O
Antithyroid antibodies
Ceruloplasmin
C-reactive protein
Cold agglutinins
Paul Bunnel test
Immunoglobulins
Leptospira agglutination test
VDRL
Australia antigen.

Processing

Ideally all measurements should be performed within 1 hour after collection. Tests where proteins are first precipitated with tungstic acid, trichloroacetic acid or barium sulfate—samples for these tests can be stored in a refrigerator at 4–6°C if the interval before the analysis exceeds 30 minutes. In medical chemistry, plasma can be used for virtually all measurements (ideal anticoagulant being heparin), although a few require serum (serum enzymes and protein electrophoresis), while whole blood can for all practical purposes be eliminated. Whenever a delay of more than 1 hour is anticipated, refrigerate the sample at 4°C. For extracting serum—let the blood clot at room temperature (takes about 20–30 minutes), loosen the clot at the top by a stick. Centrifuge blood for 10 minutes at 3,000 rpm, serum can be removed with the use of a pasteur pipette. Label and store the serum in a refrigerator at 4–6°C until analyzed or freeze at –20°C, if the analysis is to be delayed by more than 4 hours.

Centrifuge

While centrifuging the principle of balance must always be observed. Tubes of equal weight, shape, and size should be placed in opposing positions in the centrifuge head (using water filled tubes whenever necessary). Tubes should be supported by appropriately shaped rubber cushions in the carrier of the centrifuge head. The speed of the centrifuge should be slowly accelerated.

Difficulties

1. All tubes should be chemically clean, i.e. free of actual or potential organic and/or inorganic constituents that may alter the result of a chemical analysis. They need not be sterile.
2. Hemolysis: It should always be avoided as release of RBC contents (e.g. LDH, acid phosphatase and potassium) or through color change (especially for photometric measurements using shorter wavelengths of the visible spectrum 400–500 nm) results may be falsely high. Hemoglobin interferes with specific chemical reactions (e.g. diazotization inhibition in bilirubin estimation).

Blood Collection, Precautions and Errors

1. Excessive venous stasis by prolonged application of tourniquet should be avoided. This would also raise concentration of certain constituents of blood hormones, calcium, K^+, Lactic acid, etc.).
2. The syringe, needle and the tube should be moisture free.
3. Blood should be withdrawn by needle of gauge less than 21.
4. Expelling blood through the needle into the container should be avoided.

5. Do not shake blood in container to mix with anticoagulants. Mix by gentle repetitive inversion—about 6 to 8 times.
6. Clotted specimens should not be disturbed for 20–30 minutes.
7. Prolonged contact of serum or plasma with blood cells should be avoided to minimize glycolysis and/or shift of constituents from cells to serum or plasma.
8. If the patient is already on an I/V drip, withdraw blood from the other arm.
9. Refrigeration of freshly collected blood specimen before clotting has occurred or freezing of whole blood before separation should be avoided.
10. Lactescence: Milky or lipemic plasma and serum are frequently obtained with blood samples collected 1 to 2 hours after a fatty meal or patients with hyperlipoproteinemia. It is associated with blood neutral fat levels. This interferes with certain photometric measurements, e.g. uric acid and enzymes. It also produces false elevations when serum is used in final test mixture but not in the reagent blanks.
11. *Concentration changes*: Changes in drawn blood samples from the original constituent concentrations occur through dilution or evaporation.
12. *Composition changes*: The major sources of composition alteration in blood specimens are bacterial and enzymatic effect, loss of volatile blood constituents by diffusion or evaporation and interchange of substance between the liquid and cellular components of blood. Protection from light is essential for certain constituents (e.g. bilirubin).
13. *Bacterial changes*: These include ammonia formation from urea and can be minimized by
    a. Sterile handling of the blood samples wherever possible
    b. Prompt separation of cells from plasma or serum
    c. Storage of the specimen at 4–6°C until analyzed or freezing at –20°C (minus 20°C) when possible.
14. *Enzymatic changes*: Glycolysis is minimized by the same measures as bacterial changes, except that sterility has no effect unless an enzymatic method is employed.

Clinical Chemistry and Drug Interference (See Appendix II)

Drug interference can occur in two ways:

Pharmacologic Interference

Whereupon some action of the drug or its metabolite can cause an alteration (in vivo) in the concentration of the substance being measured by the test, or

Chemical Interference

In which some physical or chemical property of the drug can alter the analysis directly.

Control Sera

Before one proceeds for knowing the unknown, one must have some known standards with which to compare and obtain the results of the unknown. Most kits do provide a standard solution of the substance to be measured but the following are outstanding and are extremely useful for preparation of calibration curves.

Normal Values Differ with Different Kits and Manufactures. Always Consult the Product Insert for Exact Method for a Particular Kit, Follow the Manufacturer's Instructions Strictly

(Kits of most tests mentioned in this and the next chapter can be obtained from Coral Clinical Systems, Goa).

Control Sera from Boehringer

	Product	*Standardises*
1.	Precibil	Bilirubin
2.	Preciflo (for Technicon systems)	23, chemical parameters
3.	Precilip (normal range)	26, chemical parameters
4.	Precilip EL (elevated range)	6 parameters (mainly lipids)
5.	Precinorm E (normal range)	16 enzyme parameters
6.	Precipath E (elevated range)	16 enzymic parameters
7.	Precinorm S (normal range)	5 chemical parameters
8.	Precipath S (elevated range)	15 chemical parameters
9.	Precinorm U (normal range)	38 chemical and enzymic parameters. Suitable for both automated and manual procedures
10.	Precipath U (abnormal range)	-do-
11.	Precinorm protein (normal range)	For IgG, IgM and transferrin values
12.	Special control serum for HDL cholesterol	
(For all the above mentioned controls the exact assay values are provided by the manufacturers)		

Blood Urea Nitrogen (BUN)

Normal Values

		SI units
Young adult < 40	5–18 mg/dL	1.8–6.5 mmol/L
Adult	5–20 mg/dL	1.8–7.1 mmol/L
Elderly > 60	8–21 mg/dL	2.9–7.5 mmol/L
Mild azotemia	20–50 mg/dL	7.1–17.7 mmol/L
Children		
Cord blood Premature	21–40 mg/dL	7.5–14.3 mmol/L
Infant, first 7 days	3–25 mg/dL	1.1–7.9 mmol/L
Full-term Newborn	4–18 mg/dL	1.4–6.4 mmol/L
Infant	5–18 mg/dL	1.8–6.4 mmol/L
Child	5–18 mg/dL	1.8–6.4 mmol/L
Panic level	> 100 mg/dL	> 35.7 mmol/L

Urea (DAM Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of urea in serum, plasma and urine (for in vitro diagnostic use only).

Summary

Urea is the end product of the protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy.

Principle

Urea in an acidic medium condenses with diacetyl monoxime at 100°C to form a red colored complex. Intensity of the color formed is directly proportional to the amount of urea present in the sample.

$$\text{Urea + Diacetyl monoxime} \xrightarrow{100°C} \text{Red colored complex}$$

Normal Reference Values

Serum, Plasma : 14–40 mg/dL
Urine : upto 20 g/L

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*25 Tests*	*50 Tests*
L1: Urea reagent	75 mL	150 mL
L2: Acid reagent	75 mL	150 mL
L3 : DAM reagent	75 mL	150 mL
S: Urea standard (40 mg/dL)	5 mL	5 mL

Storage/Stability

All reagents are stable at RT till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use. Do not pipette with mouth.

Sample Material

Serum, plasma or urine. Urine should be of 24 hours collection. Dilute the urine specimen 1:20 with distilled/deionised water before the assay. Urea is reported to be stable in serum for 5 days at 2–8°C.

Procedure

Wavelength/filter : 520 nm (Hg 546 nm)/Green
Temperature : 100°C
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Urea reagent (L1)	1.0	1.0	1.0
Acid reagent (L2)	1.0	1.0	1.0
DAM reagent (L3)	1.0	1.0	1.0
Distilled water	0.01	—	—
Urea standard (S)	—	0.01	—
Sample	—	—	0.01

Mix well and keep the test tubes in boiling water (100°C) for 10 minutes. Cool under running tap water and measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank.

Calculations

$$\text{Urea in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 40$$

$$\text{Urine Urea in g/L} = \frac{\text{Abs T}}{\text{Abs S}} \times 8$$

Urea nitrogen in mg/dL = Urea in mg/dL × 0.467.

Linearity

The procedure is linear upto 70 mg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay, Calculate the value using the proper dilution factor.

Note
The presence of ammonia does not interfere in this test.

System Parameters

Reaction	*End point*	*Interval*	
Wavelength	: 520 nm	Sample volume	: 0.01 mL
Zero Setting	: Reagent blank	Reagent volume	: 3.00 mL
Incub temp.	: 100°C	Standard	: 40 mg/dL
Incub time	: 10 minutes	Factor	:
Delay time	:	React slope	: Increasing
Read time	: —	Linearity	: 70 mg/dL
No. of read	: —	Units	: mg/dL

Urea (Mod Berthelot Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of urea in serum, plasma and urine (for in vitro diagnostic use only)

Summary

Urea is the end product of protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy.

Principle

Urease hydrolyzes urea to ammonia and CO_2. The ammonia formed further reacts with a phenolic chromogen and hypochlorite to form a green colored complex. Intensity of the color formed is directly proportional to the amount of urea present in the sample.

$$\textbf{Urea} + \textbf{H}_2\textbf{O} \xrightarrow{\text{Urease}} \textbf{Ammonia} + \textbf{CO}_2$$

Ammonia + Phenolic chromogen + Hypochlorite ⟶ Green colored complex

Normal Reference Values

Serum/plasma : 14–40 mg/dL
Urine : Upto 20 g/L

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*75 assays*	*3 × 75 assays*
L1: Buffer reagent	75 mL	3 × 75 mL
L2: Enzyme reagent	7.5 mL	3 × 7.5 mL
L3: Chromogen reagent	15 mL	45 mL
S: Urea standard (40 mg/dL)	5 mL	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use for the given procedure.

Working enzyme reagent: For the flexibility and convenience in performing large assay series, a working enzyme reagent may be made by pouring 1 bottle of L2 (Enzyme reagent) into 1 bottle of L1 (Buffer reagent). For smaller series combine 10 parts of L1 (Buffer reagent) and 1 part of L2 (Enzyme reagent). Use 1 mL of the working reagent per assay instead of 1 mL of L1 and 0.1 mL of L2 as given in the procedure. The working enzyme reagent is stable for at least 4 weeks when stored at 2–8°C.

Working chromogen reagent: For larger volume cuvettes, dilute 1 part of L3 (Chromogen reagent) with 4 parts of fresh ammonia free distilled/deionised water. Use 1 ml of working chromogen instead of 0.2 mL in the assay. The working chromogen reagent is stable for atleast 8 weeks when stored at 2–8°C in a tightly stoppered plastic bottle.

Sample Material

Serum, plasma, urine. Dilute urine 1 + 49 with distilled water before the assay (results × 50). Urea is reported to be stable in the serum for 5 days when stored 2–8°C.

Procedure

Wavelength/filter : 570 nm (Hg 578 nm)/yellow
Temperature : 37°C/RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Buffer reagent (L1)	1.0	1.0	1.0
Enzyme reagent (L2)	0.1	0.1	0.1
Distilled water	0.01	—	—
Urea standard (S)		0.01	—
Sample	—	—	0.01
Mix well and incubate for 5 minutes at 37°C or 10 minutes at RT (25°C)			
Chromogen reagent (L3)	0.2	0.2	0.2

Mix well and incubate for 5 minutes at 37°C or 10 minutes at RT (25°C). Measure the absorbance of the Standard (Abs S), and Test sample (Abs T) against the Blank, within 60 minutes.

Calculations

$$\text{Urea in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 40$$

Urea nitrogen in mg/dL = Urea in mg/dL × 0.467

Linearity

This procedure is linear upto 250 mg/dL. Using the working chromogen reagent (1 mL) the linearity is increased to 400 mg/dL. If values exceed this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note

Any contamination by ammonia or ammonium salts lead to erroneous results, hence plasma should not be collected with fluoride or heparin ammonium salts. The working enzyme reagent is not stable at elevated temperatures and should be stored back at 2–8°C immediately after use. The chromogen reagent contains chlorine. The bottle should be opened only when required and closed tightly after use to prevent the loss of active chlorine.

System Parameters

Reaction	: End point	No. of read	:
Wavelength	: 570 nm	Interval	:
Zero setting	: Reagent blank	Sample volume	: 0.01 mL
Incubation temperature	: 37°C/RT	Reagent volume	: 1.30 mL
Incubated time	: 5 min + 5 min or 10 min +10 min	Standard factor React slope	: 40 mg/dL : Increasing
Delay time	:	Linearity	: 250 mg/dL
Read time	:	Units	: mg/dL

Urea (GLDH Kinetic Method)

(*Courtesy:* Tulip Group of Companies)

For the determination of urea in serum or plasma (for in vitro diagnostic use only).

Summary

Urea is the end product of protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy.

Principle

Urease hydrolyzes urea to ammonia and CO_2. The ammonia formed further combines with a ketoglutarate and NADH to form glutamate and NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance in a fixed time which is proportional to the urea concentration in the sample.

$$\text{Urea} + H_2O + 2\,H^+ \xrightarrow[\text{GLDH}]{\text{Urease}} 2\,NH_4 + CO_2$$

$$2\,NH_4^+ + 2\,\alpha\text{ Ketoglutarate} + \downarrow$$

$$2\,\text{NADH} \longrightarrow 2\,\text{L-glutamate} + 2\,NAD^+ + 2\,H_2O$$

Normal Reference Values

Serum/plasma : 14–40 mg/dL
Urine : Upto 20 g/L

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*75 mL*	*2 ×75 mL*
L1: Enzyme reagent	60 mL	2 × 60 mL
L2: Starter reagent	15 mL	2 × 15 mL
S: Urea standard (40 mg/dL)	5 mL	5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: For sample start assays a single reagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottle of L1 (Enzyme reagent).

This working reagent is stable for at least 10 days when stored at 2–8°C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme reagent) and 1 part of L2 (Starter reagent). Alternatively 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum, plasma, urine. Dilute urine 1 + 49 with distilled water before the assay (results × 50). Urea is reported to be stable in the serum for 5 days when stored at 2–8°C.

Procedure

Wavelength/filter : 340 nm
Temperature : 37°C/30°C/25°C
Light path : 1 cm

Substrate Start Assay

Pipette into a clean dry test tube labeled standard (S) or test (T):

Addition Sequence	*(S)/(T) 37°C/ 30°C/25°C*
Enzyme reagent (L1)	0.8 mL
Urea standard/serum/diluted urine	0.01 mL
Incubate at the assay temperature for 1 minute and add	
Starter reagent (L2)	0.2 mL

Mix well and read the initial absorbance A for the standard and test after exactly 30 seconds. Read another absorbance A_2 of the standard and test exactly 60 seconds later. Calculate the change in absorbance ΔA for both the standard and test.

Sample Start Assay

Pipette into a clean dry test tube labelled Standard (S) or Test (T):

Addition	*(S)/(T)*
Sequence	37°C / 30°C / 25°C
Working reagent	1.0 mL
Bring to assay temperature and add	
Urea standard/serum/diluted urine	0.01 mL

Mix well and read the initial absorbance A_1 for the standard and test after exactly 30 seconds. Read another absorbance A_2 of the standard and test exactly 60 seconds later. Calculate the change in absorbance ΔA for both the standard and test.

For Standard $\Delta AS = A_2S - A_1S$
For Test $\Delta AT = A_2T - A_1T$

Calculations

$$\text{Urea in mg/dL} = \frac{\Delta A\ T}{\Delta A\ S} \times 40$$

Linearity

This procedure is linear upto 250 mg/dL. If values exceed this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note

Plasma should not be collected with fluoride or heparin salts as contamination by ammonia or ammonium salts lead to erroneous results.

System Parameters

Reaction	: Fixed time kin.	Interval	: 60 sec.
Wavelength	: 340 nm	Sample volume	: 0.01 mL
Zero setting	: Distilled Water	Reagent volume	: 1.00 mL
Incubation temperature	: 30°C/37°C	Standard	: 40 mg/dL
Incubated time	—	Factor	:
Delay time	: 30 seconds	React slope	: Decreasing
Read time	: 60 seconds	Linearity	: 250 mg/dL
No. of read	: 2	Units	: mg/dL

Normal Values (general reference)

Adults: BUN is 8–18 mg%, Urea = 15–40 mg%

Adults over 60 years: May have a little higher values normally. Low values may be found during pregnancy and in full-term infants, whereas premature infants may have slightly higher values than the adult range.

Clinical Relevance

Common Causes of Increased BUN or Uremia

Prerenal

- Reduced blood flow to kidney
- Shock, blood loss, dehydration
- Increased protein catabolism
- Crush injuries, burns, fever, hemorrhage into soft tissue or body cavities, hemolysis.

Renal

- Acute renal failure
- Glomerulonephritis, malignant hypertension, nephrotoxic drugs or metals, renal cortical necrosis
- Chronic renal disease
- Glomerulonephritis, pyelonephritis, diabetes mellitus, arteriosclerosis, renal tubular disease, collagen-vascular diseases.

Post-renal

- Ureteral destruction by stones, tumor, inflammation, surgical trauma, obstruction of bladder neck or urethra by prostate, stones, tumor, inflammation.

Decreased BUN is Associated with

a. Liver failure.
b. Negative nitrogen balance as may occur in malnutrition, excessive use of IV fluids and physiologic hydremia of pregnancy.
c. Impaired absorption as in celiac disease.
d. Occasionally in nephrotic syndrome.
e. Overhydration.

Interfering Factors

1. A combination of a low protein and a high carbohydrate diet cause a decreased BUN level.
2. The BUN is normally lower in children and women because they have a smaller muscle mass than adult men.
3. Increased BUN values occur in late pregnancy and infancy because of increased use of protein.
4. Older people may have an increased BUN when their kidneys are not able to concentrate urine adequately.
5. Decreased BUN values may normally occur earlier in pregnancy because of physiologic hydremia.
6. Many drugs can cause increased BUN levels.
7. Drugs that may cause decreased BUN levels include
   - Dextrose infusions
   - Phenothiazines
   - Thymol.

Comments

1. Ammonium oxalate should not be used as an anticoagulant. Plasma can be used if it is obtained from EDTA, citrate, potassium oxalate or heparin.
2. If the serum sample is very lipemic, prepare a special blank tube by adding the phenol color reagent to the urease before adding the serum. Set the zero absorbance for the particular sample with this blank.
3. For urgent test—the incubation time can be reduced to 5 minutes if the water bath temperature is raised to 55–56ºC.
4. Plasma or serum preserved with fluoride cannot be used as this inactivates the enzyme. Urea is stable in frozen serum for months.
5. Make sure that there is no contamination by ammonia or heavy metal ions.
6. For a small laboratory, commercially available multi/monostep kits can be used.

Plasma or Serum Creatinine

Normal Values

	Conventional units	*SI units*
Jaffe, manual method	0.8–1.5 mg/dL	70–133 µmol/day
Jaffe, kinetic or enzymatic method		
Adult		
Female	0.5–1.1 mg/dL	44–97 µmol/L
Males	0.6–1.2 mg/dL	53–106 µmol/L
Eldery	May be lower	May be lower
Children		
Cord blood	0.6–1.2 mg/dL	53–106 µmol/L
Newborn	0.8–1.4 mg/dL	71–124 µmol/L
Infant	0.7–1.7 mg/dL	62–150 µmol/L
Age 1 female	≤ 0.5 mg/dL	≤ 44 µmol/L
Age 1 male	≤ 0.6 mg/dL	≤ 53 µmol/L
Age 2–3 female	≤ 0.6 mg/dL	≤ 53 µmol/L
Age 2–3 male	≤ 0.7 mg/dL	≤ 62 µmol/L
Age 4–7 female	≤ 0.7 mg/dL	≤ 62 µmol/L
Age 4–7 male	≤ 0.8 mg/dL	≤ 71 µmol/L
Age 8–10 female	≤ 0.8 mg/dL	≤ 71 µmol/L
Age 8–10 male	≤ 0.9 mg/dL	≤ 80 µmol/L
Age 11–12 female	≤ 0.9 mg/dL	≤ 80 µmol/L
Age 11–12 male	≤ 1.0 mg/dL	≤ 88 µmol/L
Age 13–17 female	≤ 1.1 mg/dL	≤ 97 µmol/L
Age 13–17 male	≤ 1.2 mg/dL	≤ 106 µmol/L
Age 18–20 female	≤ 1.2 mg/dL	≤ 106 µmol/L
Age 18–20 male	≤ 1.3 mg/dL	≤ 115 µmol/L

Creatinine (Alkaline Picrate Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of creatinine in serum and urine (for in vitro diagnostic use only).

Summary

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy.

Principle

Picric acid in an alkaline medium reacts with creatinine to form a orange colored complex with the alkaline picrate. Intensity of the color formed is directly proportional to the amount of creatinine present in the sample.

Creatinine + Alkaline picrate→Orange colored complex

Reference Values

	Serum	*Urine, 24 hours collection*
Males	: 0.6–1.2 mg%	1.1–3.0 g
Females	: 0.5–1.1 mg%	1.0–1.8 g

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	15 Tests	35 Tests
L1: Picric acid reagent	60 mL	140 mL
L2: Buffer reagent	5 mL	12 mL
S: Creatinine standard (2 mg/dL)	5 mL	5 mL

Storage/Stability

All reagents are stable at RT till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use. Do not pipette with mouth.

Sample Material

Serum or Urine

Creatinine is stable in serum for 1 day at 2–8°C

Urine of 24 hours collection is preferred. Dilute the specimen 1:50 with distilled/deionised water before the assay.

Procedure

Wavelength/filter	520 nm (Hg 546 nm)/green
Temperature	RT
Light path	1 cm

Deproteinization of Specimen

Pipette into a clean dry test tube

Picric acid reagent (L1)	2.0 mL
Sample	0.2 mL

Mix well and centrifuge at 2500–3000 rpm for 10 minutes to obtain a clear supernatant.

Color Development

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Supernatant	-	-	1.1
Picric acid reagent (L1)	1.0	1.0	
Distilled water	0.1		
Creatinine standard (S)	-	0.1	
Buffer reagent (L2)	0.1	0.1	0.1

Mix well and keep the test tubes at RT for exactly 20 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank.

Calculations

$$\text{Creatinine in mg\%} = \frac{\text{Abs T}}{\text{Abs S}} \times 2.0$$

$$\text{Urine creatinine in g/L} = \frac{\text{Abs T}}{\text{Abs S}} \times 1.0$$

Urine creatinine g/24 h = Urine creatinine in g/L × Volume of urine in 24 hours in liters.

Linearity

The procedure is linear upto 8 mg% of creatinine.

If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note

Maintain the reaction time of 20 minutes as closely as possible since a longer incubation causes an increase in the values due to the reaction of pseudochromogens. The determination is not specific and may be affected by the presence of large quantities of reducing substances in the sample. The reaction is temperature sensitive and all the tubes should be maintained at a uniform temperature.

System Parameters

Reaction	: End point	Interval	:
Wavelength	: 520 nm	Sample Volume	: 0.20 mL
Zero setting	: Reagent blank	Reagent	: Volume 1.10 mL
Incubation temprature	: RT	Standard	: 2 mg/dL
Incubated time	: 20 minutes	Factor	:
Delay time	: —	React slope	: Increasing
Read time	: —	Linearity	: 8 mg/dL
No. of read	: —	Units	: mg/dL

Creatinine (Mod Jaffa's Kinetic Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of creatinine in serum and urine (for in vitro diagnostic use only).

Summary

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy.

Principle

Picric acid in an alkaline medium reacts with creatinine to form a orange colored complex with the alkaline picrate. Intensity of the color formed during the fixed time is directly proportional to the amount of creatinine present in the sample.
Creatinine + Alkaline Picrate → Orange colored complex.

Reference Values

	Serum	*Urine in 24 hours collection*
Males	: 0.6–1.2 mg%	1.1–3.0 g
Females	: 0.5–1.1 mg%	1.0–1.8 g

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*2 × 35 mL*	*2 × 75 mL*
L1: Picric acid reagent	35 mL	75 mL
L2: Buffer reagent	35 mL	75 mL
S: Creatinine standard (2 mg/dL)	5 mL	5 mL

Storage/Stability

All reagents are stable at RT till the expiry mentioned on the label.

Reagent Preparation

Reagents are ready to use. Do not pipette with mouth.

Working reagent: For larger assay series a working reagent may be prepared by mixing equal volumes of picric acid reagent and buffer reagent. The Working reagent is stable at RT (25–30°C) for at least one week.

Sample Material

Serum or Urine

Creatinine is stable in serum for 1 day at 2–8°C.

Urine of 24 hours collection is preferred. Dilute the specimen 1:50 with distilled/deionised water before the assay.

Procedure

Wavelength/filter : 520 nm (Hg 492 nm)/green
Temperature : 30°C/37°C
Light path : 1 cm

Pipette into a clean dry test tube labeled standard (S) or test (T):

Addition Sequence	*(S)/(T) 30°C/37°C*
Picric acid reagent (L1)	0.5 mL
Buffer reagent (L2)	0.5 mL
Bring reagents to the assay temperature and add	
Creatinine standard (S)/sample/diluted urine	0.1 mL

Mix well and read the initial absorbance A_1 for the standard and test after exactly 30 seconds. Read another absorbance A_2 of the standard and test exactly 60 seconds later. Calculate the change in absorbance ΔA for both the standard and test.

For standard $\Delta AS = A_2S - A_1S$
For test $\Delta AT = A_2T - A_1T$

Calculations

$$\text{Creatinine in mg/dL} = \frac{\Delta AT}{\Delta AS} \times 2.0$$

$$\text{Urine creatinine in g/L} = \frac{\Delta AT}{\Delta AS} \times 1.0$$

$$\text{Urine creatinine g/24 hours} = \frac{\text{Urine creatinine in g/L} \times \text{Volume of urine in liters 24 hours}}{}$$

Linearity

The procedure is linear upto 20 mg/dL of Creatinine. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note

The buffer reagent may turn milky or show white precipitates at cold temperatures. This is not a deterioration of the reagent. Dissolve/clear the same by warming the reagent to 37°C with gentle swirling before use. The determination is not specific and may be affected by the presence of large quantities of reducing substances.

As the test is temperature sensitive, it is essential to maintain the indicated reaction timings and temperatures meticulously during the test procedure.

System Parameters

Reaction	:	Fixed time kin	Interval	:	60 seconds
Wavelength	:	520 nm	Sample volume	:	0.10 mL
Zero setting	:	Distilled water	Reagent volume	:	1.00 mL
Incubation temperature	:	30°C/37°C	Standard	:	2 mg/dL
Incubated time	:	-	Factor	:	-
Delay time	:	30 seconds	React slope	:	Increasing
Read time	:	60 seconds	Linearity	:	20 mg/dL
No. of read	:	2	Units	:	mg/dL

Clinical Relevance

Causes of Raised Serum Creatinine Levels

All renal causes of uremia are usually associated with raised serum creatinine values. Elevated BUN levels in a patient with normal creatinine usually signal a nonrenal cause for the uremia. With severe, permanent renal damage, urea levels continue to climb, but creatinine values tend to plateau. At very high creatinine levels, some is excreted across the alimentary tract.

Decreased Creatinine Levels Occur in

Muscular dystrophy.

Interfering Factors

1. High levels of ascorbic acid can give a falsely increased level.
2. Drugs influencing kidney function (diuretics and dextran), chloral hydrate, marijuana, acetohexamide, guanethidine, furosemide, chloramphenicol, and sulfonamides can cause a change in blood creatinine.
3. A diet high is roast meat will cause increased levels.
4. Many drugs may cause a change in the blood creatinine. A normal blood serum creatinine does not always indicate unimpaired renal function. A normal value cannot be used as standard for a patient who is known to have existing renal disease.

Serum Bilirubin

Normal Values

		SI units
Total bilurubin		
1 month – adult	< 1.5 mg/dL	1.7–20.5 µmol/L
Premature infant		
Cord	< 2.8 mg/dL	< 48 µmol/L
24 hours	1–6 mg/dL	17–103 µmol/L
48 hours	6–8 mg/dL	103–137 µmol/L
3–5 days	10–12 mg/dL	171–205 µmol/L
Full-term infant		
Cord	< 2.8 mg/dL	< 48 µmol/L
24 hours	2–6 mg/dL	34–103 µmol/L
48 hours	6–7 mg/dL	103–120 µmol/L
3–5 days	4–6 mg/dL	68–103 µmol/L
Direct bilirubin	0.0–0.3 mg/dL	1.7–5.1 µmol/L
Indirect bilirubin	0.1–1.0 mg/dL	1.7–17.1 µmol/L

Bilirubin (Mod Jendrassik and Grof's Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of direct and total bilirubin in serum (for in vitro diagnostic use only).

Summary

Bilirubin is mainly formed from the heme portion of aged or damaged RBCs. It then combines with albumin to form a complex which is not water soluble. This is referred to as indirect or unconjugated bilirubin. In the liver, this bilirubin complex is combined with glucuronic acid into a water soluble conjugate. This is referred to as conjugated or direct bilirubin. Elevated levels of bilirubin are found in liver diseases (hepatitis, cirrhosis), excessive hemolysis/destruction of RBC (hemolytic jaundice) obstruction of the biliary tract (obstructive jaundice) and in drug induced reactions. The differentiation between the direct and indirect bilirubin is important in diagnosing the cause of hyperbilirubinemia.

Principle

Bilirubin reacts with diazotized sulfanilic acid to form a colored azobilirubin compound. The unconjugated bilirubin couples with the sulfanilic acid in the presence of a caffein-benzoate accelerator. The intensity of the color

formed is directly proportional to the amount of bilirubin present in the sample.

Bilirubin + Diazotized Sulfanilic acid→ Azobilirubin Compound

Normal Reference Values

Serum (Direct) : upto 0.2 mg/dL
(Total) : upto 1.0 mg/dL

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*30 tests*	*75 tests*
L1: Direct bilirubin reagent	75 mL	150 mL
L2: Direct nitrite reagent	4 mL	4 mL
L1: Total bilirubin reagent	75 mL	150 mL
L2: Total nitrite reagent	4 mL	4 mL
S : Artificial standard (10 mg/dL)	10 mL	10 mL

Storage/Stability

All reagents are stable at RT till the expiry mentioned on the label.

Reagent Preparation

Reagents are ready to use. Do not pipette with mouth.

Sample Material

Serum. Bilirubin is reported to be stable in the sample for 4 days at 2–8°C protected from light as it is photosensitive.

Procedure

Wavelength/filter : 546 nm/yellow-green
Temperature : RT
Light path : 1 cm

Direct Bilirubin Assay

Pipette into clean dry test tubes labeled as Blank (B), and Test (T):

Addition Sequence	*B (mL)*	*T (mL)*
Direct bilirubin reagent (L1)	1.0	1.0
Direct nitrite reagent (L2)	-	0.05
Sample	0.1	0.1

Mix well and incubate at RT for exactly 5 minutes. Measure the absorbance of the test samples (Abs T) immediately against their respective blanks.

Total Bilirubin Assay

Pipette into clean dry test tubes labeled as blank (B), and test (T):

Addition Sequence	*B (mL)*	*T (mL)*
Total bilirubin reagent (L1)	1.0	1.0
Total nitrite reagent (L2)	-	0.05
Sample	0.1	0.1

Mix well and incubate at RT for 10 min. Measure the absorbance of the test samples (Abs T) immediately against their respective blanks.

Calculations

Total or direct bilirubin in mg/dL = Abs T × 13 (13 being the factor).

Linearity

This procedure is linear upto 20 mg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note

In case, the exact wavelength is not available the artificial standard (S) may be used. Measure the absorbance of the artificial standard against distilled water with the appropriate filter and keep the same for future calculations by dividing the Abs T with the Abs. of the Std. × 10. Discard the artificial standard after use.

In case of neonates where the sample quantity is a limitation, and the samples have high bilirubin (above 3 mg/dL), only 0.05 mL/0.02 mL of the sample may be used for bilirubin estimation. The calculation factor in this case would be 24.9/60.5 respectively instead of 13. In case of using the standard the value of the same would be 19.1/46.5 mg/dL respectively instead of 10 mg/dL.

System Parameters

Reaction	: End point	Interval	:
Wavelength	: 546 nm	Sample volume	: 0.10 mL
Zero setting	: Sample blank	Reagent volume	: 1.05 mL
Incubation temperature	: RT	Standard	:
Incubated time	: 5 min/10 min	Factor	: 13
Delay time	: —	React slope	: Increasing
Read time	: —	Linearity	: 20 mg/dL
No. of read	: —	Units	: mg/dL

Causes of Hyperbilirubinemia

Unconjugated (Indirect) Hyperbilirubinemia

I. Overproduction of bilirubin
   A. Hemolytic disorders.
      1. Congenital (e.g. hemoglobinopathies)
      2. Acquired (e.g. Coombs' positive anemia)
      3. Liver disease (e.g. hepatitis and cirrhosis).
   B. Shunt hyperbilirubinemia

II. Defective uptake and storage of bilirubin
   A. Idiopathic unconjugated hyperbilirubinaemia.
      1. Hereditary-Gilbert's syndrome.
      2. Acquired
         – Post-viral hepatitis.
         – Post-portacaval shunt.
   B. Decreased availability of cytoplasmic binding proteins (Y and Z) in newborn and premature infants.
   C. Drugs (e.g. flavispidic acid).

III. Defective glucuronyl transferase activity.
   A. Deficiency.
      1. In newborn and premature infants
      2. Crigler-Najjar syndrome.
   B. Inhibition
      1. Abnormal steroids in breast milk or maternal plasma (Lucey-Driscoll type).
      2. Drugs (e.g. novobiocin).

Conjugated (Direct) Hyperbilirubinemia

Defective excretion of conjugated bilirubin

A. Hereditary
   1. Dubin-Johnson syndrome
   2. Rotor syndrome.
B. Obstructive
   1. Intrahepatic cholestasis
      a. Cirrhosis (occasionally)
      b. Hepatitis (often)
      c. Alcoholic liver disease (occasionally)
      d. Drugs (e.g. chlorpromazine and methyltestosterone).
      e. Primary biliary cirrhosis.
   2. Extrahepatic obstruction.
      a. Gallstones
      b. Carcinoma of the bile duct, pancreas, ampulla of Vater
      c. Bile duct stricture
      d. Biliary atresia.

Interfering Factors

1. A 1 hour exposure of the specimen to sunlight or high intensity artificial light at room temperature will reduce the bilirubin content.
2. Contrast media 24 hours before measurement may cause an altered reaction.
3. A high fat meal may cause decreased bilirubin levels by interfering with the clinical reactions.
4. Air bubbles and shaking of the specimen may cause decreased levels.
5. Foods (carrots, etc.) and drugs increase the yellowish hue in the serum.
6. Refer to the Many drugs can interfere with Bilirubin tests for a listing of the many drugs that may interfere with testing for bilirubin.
7. Hemolyzed blood will falsely elevate bilirubin level.

Comments

1. In severe obstructive jaundice with formation of biliverdin, low results for the degree of jaundice will be obtained since biliverdin does not react with the diazo reagent and cannot be determined.
2. An unusual source of otherwise unexplained elevated serum bilirubin has been described following 48 hours fasting. A normal bilirubin value from 0.68 mg% may rise to the abnormal range at 1.87 mg%.

Icterus Index

The icterus index is a measure of the degree of icterus (yellowish-green color) in a plasma or serum specimen in cases of jaundice. This is just a screening test for hyperbilirubinemia. Substances other than bilirubin in the serum (carotene, xanthophyll, hemoglobin, etc.) may contribute to the icterus index, therefore, limiting its clinical utility. The test is now considered to be obsolete.

Reagents

A. Potassium dichromate solution
   1. Stock solution (1%)
      Dissolve 1 g of potassium dichromate in 70 mL of water placed in a 100 mL volumetric flask. Add 2 drops of sulfuric acid and dilute to 100 mL mark with distilled water. Store in a glass-stoppered brown/amber colored bottle.
   2. Working standard solution (0.1%)
      Pipette 10 mL of the stock solution into a 100 mL volumetric flask and dilute to 100 mL mark with distilled water.
B. Saline (0.9% NaCL) isotonic.

Method

A. Dilute the serum specimen ten times with saline (1 mL of serum mixed with 9 mL of saline) in a test tube and mix.
B. Transfer the diluted serum into a cuvette and read absorbance at 420 to 460 nm. If too dark, dilute further

and multiply the final reading with the dilution factor utilized here.

C. Determine the icterus index from the calibration curve. Multiply the result by dilution factor. If the serum is diluted ten times, the dilution factor is 10.

Calibration Curve

A. Prepare three concentrations of the standard by diluting appropriate quantities of the stock solution of potassium dichromate in three 100 mL volumetric flasks
   1. Five mL stock mixed with 95 mL of water (1:20). This corresponds to 5 units.
   2. 25 mL of stock solution made to 100 mL with water (1:4 dilution). This corresponds to 25 units.
   3. 50 mL of stock solution made to 100 mL with water (1:2 dilution). This corresponds to 50 units.

B. Read the absorbance of each working standard solution corresponding to 5, 25 and 50 units at 420 to 460 nm using water as blank.

C. Tabulate the results with the units of icterus index and the corresponding absorbance values.

D. Plot a calibration curve and use this for the determination of icterus index.

TOTAL PROTEINS

Biuret Method

(*Courtesy*: Tulip Group of Companies)
For the determination of total proteins in serum and plasma (for in vitro diagnostic use only).

Summary

Proteins are constituents of muscle, enzymes, hormones and several other key functional and structural entities in the body. They are involved in the maintenance of the normal distribution of water between blood and the tissues. Consisting mainly of albumin and globulin the fractions vary independently and widely in diseases. Increased levels are found mainly in dehydration. Decreased levels are found mainly in malnutrition, impaired synthesis, protein losses as in hemorrhage or excessive protein catabolism.

Principle

Proteins, in an alkaline medium, bind with the cupric ions present in the biuret reagent to form a blue-violet colored complex. The intensity of the color formed is directly proportional to the amount of proteins present in the sample.

Proteins + $Cu^{++}\rightarrow$ Blue violet colored complex

Normal Reference Values

Serum and plasma : 6.0–8.0 g/dL

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*150 mL*	*2 × 150 mL*
Carton 1		
L1: Biuret reagent	150 mL	2 × 150 mL
Carton 2		
S: Protein standard (8 g/dL)	5 mL	5 mL

Storage/Stability

Carton 1 : Biuret reagent is stable at RT till the expiry mentioned on the label.

Carton 2 : Protein standard is stable at 2–8°C till the expiry mentioned on the label.

Reagent Preparation

Reagents are ready to use. Protect from bright light.

Sample Material

Serum or plasma. Proteins are reported to be stable in the sample for 6 days at 2–8°C.

Procedure

Wavelength/filter : 550 nm (Hg 546 nm)/yellow-green
Temperature : RT/37°C
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T)

Addition Sequence	B (mL)	S (mL)	T (mL)
Biuret reagent (L1)	1.0	1.0	1.0
Distilled water	0.02		
Protein standard (S)	-	0.02	
Sample	-	-	0.02

Mix well and incubate at 37°C for 10 minutes or at RT for 30 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 60 minutes.

Calculations

$$\text{Total proteins in g/dL} = \frac{\text{Abs T}}{\text{Abs S}} = \times 8$$

Linearity

This procedure is linear upto 15 g/dL. If values exceed this limit, dilute the sample with distilled water and

repeat the assay. Calculate the value using the proper dilution factor.

Note
Do not use if the reagent shows turbidity or black precipitates.

System Parameters

Reaction	:	*End point*	*Interval*	:	
Wavelength	:	550 nm	Sample vol	:	0.02 mL
Zero setting	:	Reagent blank	Reagent vol	:	1.00 mL
Incubation temperature	:	37°C/RT	Standard	:	8 g/dL
Incubated time	:	10 mm/30 min	Factor	:	
Delay time	:		React slope	:	Increasing
Read time	:		Linearity	:	15 g/dL
No. of read	:		Units	:	g/dL

SERUM ALBUMIN

Determination of Serum Albumin (BCG Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of albumin in serum or plasma (for in vitro diagnostic use only).

Summary

Albumin consists of approximately 60% of the total proteins in the body, the other major part being globulin. It is synthesized in the liver and maintains the osmotic pressure in blood. Albumin also helps in the transportation of drugs, hormones and enzymes. Elevated levels are rarely seen and are usually associated with dehydration. Decreased levels are seen in liver diseases (hepatitis, cirrhosis). Malnutrition, kidney disorders, increased fluid loss during extensive burns and decreased absorption in gastrointestinal diseases.

Principle

Albumin binds with the dye bromocresol green in a buffered medium to form a green colored complex. The intensity of the color formed is directly proportional to the amount of albumin present in the sample.

Albumin + Bromocresol green→ Green albumin BCG complex.

Normal Reference Values (Albumin)

Serum, plasma (albumin) : 3.7–5.3 g/dL
Globulin : 2.3–3.6 g/dL
A/G Ratio : 1.0–2.3

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*150 mL*	*2 × 150 mL*
Carton 1 L1: BCG reagent	150 mL	2 × 150 mL
Carton 2 S: Albumin standard (4 g/dL)	5 mL	5 mL

Storage/Stability

Carton 1 : BCG reagent is stable at RT till the expiry mentioned on the label.
Carton 2 : Albumin Standard is stable at 2–8°C till the expiry mentioned on the label.

Reagent Preparation

Reagents are ready to use. Protect from bright light.

Sample Material

Serum, EDTA plasma. Albumin is reported to be stable in the sample for 6 days at 2–8°C.

Procedure

Wavelength/filter : 630 nm (Hg 623 nm)/Red
Temperature : RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
BCG reagent (L1)	1.0	1.0	1.0
Distilled water	0.01	-	-
Albumin Standard (S)	-	0.01	-
Sample	-	-	0.01

Mix well and incubate at RT for 5 minutes. Measure absorbance of the standard (Abs S), and test sample (Abs T) against the blank.

Calculations

$$\text{Albumin in g/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 4$$

$$\text{Globulin in g/dL} = \underset{\text{(in g/dL)}}{\text{(Total proteins)}} - \underset{\text{(in g/dL)}}{\text{(Albumin)}}$$

$$\text{A/G Ratio} = \frac{\text{Albumin in g/dL}}{\text{Globulin in g/dL}}$$

Linearity

The procedure is linear upto 7 g/dL. If values exceed this limit, dilute the sample with distilled water and repeat

the assay. Calculate the value using the proper dilution factor.

Note
Gross hemolysis, ampicillin and heparin interfere with the results. Elevated bilirubin and lipemic samples may have a slight effect on accuracy. For grossly lipemic samples run a sample blank by adding 0.02 mL sample in 2 mL distilled water. Read the absorbance against DW and substract the blank absorbance from the test absorbance.

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	630 nm	Sample volume	:	0.01 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	RT	Standard	:	4 g/dL
Incubated time	:	5 minutes	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	7 g/dL
No. of read	:	—-	Units	:	g/dL

Normal Values

Total Proteins

		SI units
Adults	6.0–8.0 g/dL	60–80 g/L
Children		
Premature	4.3–7.6 g/dL	43–76 g/L
Newborn	4.6–7.4 g/dL	46–74 g/L
Infant	6.0–6.7 g/dL	60–67 g/L
Child	6.2–8.0 g/dL	62–80 g/L

Specimen Collection and storage

1. Serum is the specimen of choice.
2. Avoid excessive hemolysis since every 100 mg/dL of hemoglobin corresponds to about 100 mg/dL of albumin.
3. Albumin in serum is reported stable for one week at room temperature (18–30°C) and approximately one month when stored in the refrigerator (2–8°C) and protected against evaporation.

Clinical Relevance

Causes of Hypoalbuminemia

Reduced synthesis
- Malnutrition
- Malabsorption syndromes
- Chronic inflammatory diseases
- Acute hepatitis (lasting 14 days or more)
- Chronic liver disease
- Genetic abnormalities.

Increased Loss
- Nephrotic syndrome
- Massive burns
- Protein-losing enteropathy.

Increased catabolism
- Massive burns
- Widespread malignancy.

Multifactorial
- Cirrhosis
- Congestive heart failure
- Pregnancy.

Increased albumin levels are generally not observed (When albumin concentration decreases there is a relative increase in globulins. However, there is a definite rise in globulins in mono/polyclonal gammopathies).

Disorders Associated with Polyclonal Gammopathies

Chronic liver disease
- Nutritional cirrhosis
- Primary biliary cirrhosis
- Chronic active hepatitis
- Viral hepatitis.

Collagen diseases
- Rheumatoid arthritis
- Systemic lupus erythematosus
- Sjögren's syndrome
- Felty's syndrome
- Polymyositis
- Scleroderma.

Chronic Infections
- Tuberculosis
- Osteomyelitis
- Deep fungi
- Syphilis
- Bronchitis.

Miscellaneous
- Metastatic carcinoma
- Cystic fibrosis
- Recovery from trauma.

Causes of Monoclonal Gammopathies

- Multiple myeloma
- Waldenstrom's macroglobulinemia
- Benign idiopathic monoclonal gammopathy
- Heavy chain diseases
- Collagen disorders, autoimmune diseases
- Certain lymphomas
- Cirrhosis liver
- Neoplasms of colon, prostate, breast, female genital tract, stomach and lungs
- Myeloproliferative disorders-CML, polycythemia, myelofibrosis, erythrimic myelosis, erythroleukemia, other acute leukemias
- Aberrations in lipid metabolism
- Diabetes mellitus.

Interfering Factors

1. Low levels of albumin occur normally in all trimester's of pregnancy.
2. Bromosulfalein may cause a false elevation. Therefore, a serum protein test should not be done within 48 hours following a BSP test.
3. See appendix for complete listing of drugs that interfere with total protein levels.

SERUM CHOLESTEROL

Cholesterol (CHOD/PAP Method)

(*Courtesy*: Tulip Group of Companies)

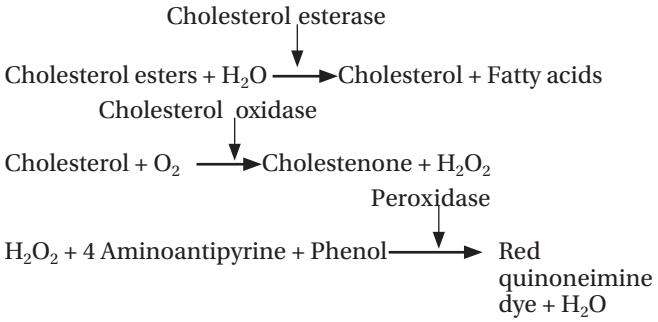
For the determination of cholesterol in serum or plasma (for in vitro diagnostic use only).

Summary

Cholesterol is the main lipid found in blood, bile and brain tissues. It is the main lipid associated with arteriosclerotic vascular diseases. It is required for the formation of steroids and cellular membranes. The liver metabolizes the cholesterol and it is transported in the blood stream by lipoproteins. Increased levels are found in hypercholesterolemia, hyperlipidemia, hypothyroidism, uncontrolled diabetes, nephrotic syndrome, and cirrhosis. Decreased levels are found in malabsorption, malnutrition, hyperthyroidism, anemias and liver diseases.

Principle

Cholesterol esterase hydrolyzes esterified cholesterols to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of cholesterol present in the sample.



Normal Reference Values

Serum/plasma (Suspicious) : 220 mg/dL and above
(Elevated) : 260 mg/dL and above

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	2 × 75 mL	2 × 150 mL
L1: Enzyme reagent 1	2 × 60 mL	2 × 120 mL
L2: Enzyme reagent 2	2 ×15 mL	2 × 30 mL
S: Cholesterol standard (200 mg/dL)	5 mL	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: Pour the contents of 1 bottle of L2 (Enzyme reagent 2) into 1 bottle of L1 (Enzyme reagent 1). This working reagent is stable for at least 8 weeks when stored at 2–8°C. Upon storage the working reagent may develop a slight pink color however, this does not affect the performance of the reagent. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme reagent 1) and 1 part of L2 (Enzyme reagent 2). Alternatively 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum, EDTA plasma. Cholesterol is reported to be stable in the sample for 7 days when stored at 2–8°C. The sample should preferably be of 12 to 14 hours fasting.

Procedure

Wavelength/filter : 505 nm (Hg 546 nm)/green
Temperature : 37°C/RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Working reagent	1.0	1.0	1.0
Distilled water	0.01	-	-
Cholesterol standard (S)	-	0.01	
Sample	-	-	0.01

Mix well and incubate at 37°C for 5 minutes or at RT (25°C) for 15 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 60 minutes.

Calculations

$$\text{Cholesterol in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 200$$

Linearity

This procedure is linear upto 750 mg/dL. If the value exceeds this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note
Anticoagulants such as fluorides and oxalates result in false low values. The test is not influenced by Hb values upto 20 mg/dL and bilirubin upto 10 mg/dL.

System Parameters

Reaction	:	End point	Interval	:	...
Wavelength	:	505 nm	Sample volume	:	0.01 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C / RT	Standard	:	200 mg/dL
Incubated time	:	5 min/15 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	750 mg/dL
No. of read	:	—	Units	:	mg/dL

Normal values

	Male		*Female*	
		SI units		*SI units*
Age	*mg/L*	*mmol/L*	*mg/dL*	*mmol/L*
Total cholesterol				
Adult				
20–24	124–218	3.21–5.64	122–216	3.16–5.59
25–29	133–244	3.44–6.32	128–222	3.32–5.75
30–34	138–254	3.57–6.58	130–230	3.37–5.96
35–39	146–270	3.78–6.99	140–242	3.63–6.27
40–44	151–268	3.91–6.94	147–252	3.81–6.53
45–49	158–276	4.09–7.15	152–265	3.94–6.86
50–54	158–277	4.09–7.17	162–285	4.20–7.38
55–59	156–276	4.04–7.15	172–300	4.45–7.77
60–64	159–276	4.12–7.15	172–297	4.45–7.69
65–69	158–274	4.09–7.10	171–303	4.43–7.85
> 70	144–265	3.73–6.86	173–280	4.48–7.25
Child				
Cord blood	44–103	1.14–2.66	50–108	1.29–2.79
< 4	114–203	2.95–5.25	112–200	2.90–5.18
5–9	121–203	3.13–5.25	126–205	3.26–5.30
10–14	119–202	3.08–5.23	124–201	3.21–5.20
15–19	113–197	2.93–5.10	119–200	3.08–5.18
High-density lipoprotein cholesterol (HDL)				
Adult				
20–24	30–63	0.78–1.63	33–79	0.85–2.04
25–29	31–63	0.80–1.63	37–83	0.96–2.15
30–34	28–63	0.72–1.63	36–77	0.93–1.99
35–39	29–62	0.75–1.60	34–82	0.88–2.12
40–44	27–67	0.70–1.73	34–88	0.88–2.28
45–49	30–64	0.78–1.66	34–87	0.88–2.25
50–54	28–63	0.72–1.63	37–92	0.96–2.38
55–59	28–71	0.72–1.84	37–91	0.96–2.35
60–64	30–74	0.78–1.91	38–92	0.98–2.38
65–69	30–75	0.78–1.94	35–96	0.91–2.48
> 70	31–75	0.80–1.94	33–92	0.85–2.38
Child				
Cord blood	6–53	0.16–1.37	13–56	0.34–1.45
5–9	38–75	0.98–1.94	36–73	0.93–1.89
10–14	37–74	0.96–1.91	37–70	0.96–1.81
15–19	30–63	0.78–1.63	35–74	0.91–1.91

Contd...

Contd...

Low-Density lipoprotein Cholesterol (LDL)				
Adult				
20–24	66–147	1.71–3.81	57–159	1.48–4.12
25–29	70–165	1.81–4.27	71–164	1.84–4.25
30–34	78–185	2.02–4.79	70–156	1.81–4.04
35–39	81–189	2.10–4.90	75–172	1.94–4.45
40–44	87–186	2.25–4.92	74–174	1.92–4.51
45–49	97–202	2.51–5.23	79–186	2.05–4.82
50–54	89–197	2.31–5.10	88–201	2.28–5.21
55–59	88–203	2.28–5.26	89–210	2.31–5.44
60–64	83–210	2.15–5.44	100–224	2.59–5.80
65–69	98–210	2.54–5.44	92–221	2.38–5.72
> 70	88–186	2.28–4.82	96–206	2.49–5.34
Child				
Cord				
blood	20–56	0.52–1.45	21–58	0.54–1.50
5–9	63–129	1.63–3.34	68–140	1.76–3.63
10–14	64–133	1.66–3.44	68–136	1.76–3.52
15–19	62–130	1.61–3.37	59–137	1.53–3.55
			SI Units	
Cholesterol esters	60–75% of total or		0.60–0.75	
	< 210 mg/dL		< 5.43 mmol/L	
Free cholesterol	< 50 mg/dL		< 1.29 mmol/L	
LDL:HDL ratio	< 3	< 3		

Clinical Relevance

1. Increased levels of cholesterol
   a. Levels above 250 mg/dL are considered elevated and call for a triglyceride test.
   b. Conditions related to elevated cholesterol
      1. Cardiovascular disease and atherosclerosis
      2. Type II, familial hypercholesterolemia
      3. Obstructive jaundice (also an increase in bilirubin)
      4. Hypothyroidism (decreased in hyperthyroidism)
      5. Nephrosis
      6. Xanthomatosis
      7. Uncontrolled diabetes
      8. Nephrotic syndrome
      9. Obesity.
   c. Free versus esterified cholesterol. There is a markedly abnormal ratio of free to esterified cholesterol in disease of the liver biliary tract, infectious disease, and extreme cholesterolemia.
2. Decreased levels of cholesterol
   a. Conditions where cholesterol is not absorbed from the gastrointestinal tract
      1. Malabsorption
      2. Liver disease
      3. Hyperthyroidism
      4. Anemia
      5. Sepsis
      6. Stress
      7. Drug therapy such as antibiotics.
   b. Other disorders related to decreased cholesterol levels
      1. Pernicious anemia
      2. Hemolytic jaundice
      3. Hyperthyroidism
      4. Severe infections
      5. Terminal stages of debilitating diseases such as cancer
      6. Hypolipoproteinemias.
   c. Esterol fraction decreases in liver diseases, liver cell injury, malabsorption syndrome, and malnutrition.
3. Increased levels of cholesterol esters are associated with familial deficiency of Lecithin—cholesterol acyltransferase (LCAT).
4. Decreased levels of cholesterol are associated with liver disease. This is because persons with liver diseases may have impaired formation of LCAT with a resulting deficiency of the enzyme.
5. Cholesterol ester storage disease causes accumulation of cholesterol esters in the tissues, but it has no effect on the percentage of esterified cholesterol in the blood.
6. The higher the cholesterol phospholipid ratio, the greater the possible risk of developing atherosclerosis.

Interfering Factors

1. Cholesterol is normally slightly elevated in pregnancy.
2. Estrogen decreases plasma cholesterol and oophorectomy increases it.
3. Many drugs may cause a change in the blood cholesterol

Patient Preparation

1. Advise patient about fasting for a night for 12 hours before the test.
2. Water is permitted.
3. Before fasting, the patient should be on a normal diet for 7 days before testing.

4. No alcohol should be consumed 24 hours before testing.
5. Lipid lowering drugs such as estrogen, oral contraceptives, and salicylates should be withheld.

HDL CHOLESTEROL

PEG/CHOD-PAP Method

(*Courtesy*: Tulip Group of Companies)
For the determination of HDL cholesterol in serum or plasma (for in vitro diagnostic use only).

Summary

Lipoproteins are the proteins which mainly transport fats in the bloodstream. They can be grouped into chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Chylomicrons and VLDL transport mainly triglycerides, though VLDLs also transport some amount of cholesterol. LDL carries cholesterol to the peripheral tissues where it can be deposited and increase the risk of arteriosclerotic heart and peripherial vascular disease. Hence, high levels of LDL are atherogenic. HDL transports cholesterol from the peripherial tissues to the liver for excretion, hence, HDL has a protective effect. The measurement of total and HDL cholesterol and triglycerides provide valuable information for the risk assessment of coronary heart diseases.

Principle

When the serum is reacted with the polyethylene glycol contained in the precipitating reagent, all the VLDL and LDL are precipitated. The HDL remains in the supernatant and is then assayed as a sample for cholesterol using the cholesterol (CHOD/PAP) reagent.

Normal Reference Values

		Prognostically favorable	*Standard risk level*	*Risk indicator*
HDL cholesterol (mg/dL)	Males	> 55	35–55	< 35
	Females	> 65	45–65	< 45
LDL cholesterol (mg/dL)	Males Females	< 150	150–190	> 190
Total cholesterol HDL cholesterol	Males	> 3.8	3.8–5.9	< 5.9
	Females	> 3.1	3.1–4.6	< 4.6

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*75 mL*
L1 : Enzyme reagent 1	60 mL
L2 : Enzyme reagent 2	15 mL
L3 : Pricipitating reagent	2.5 mL
S : HDL cholesterol standard (25 mg/dL)	5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: Pour the contents of 1 bottle of L2 (Enzyme reagent 2) into 1 bottle of L1 (Enzyme reagent 1). This working reagent is stable for at least 8 weeks when stored at 2–8°C. Upon storage the working reagent may develop a slight pink color however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme reagent 1) and 1 part of L2 (Enzyme reagent 2). Alternatively 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum, EDTA plasma. Cholesterol and HDL cholesterol are reported to be stable in serum for 7 days when stored at 2–8°C. The sample should preferably be of 12 to 14 hours fasting.

Procedure

Wavelength/filter : 505 nm (Hg 546 nm)/green
Temperature : 37°C/RT
Light path : 1 cm

Precipitation of VLDL and LDL

Pipette into a clean dry test tube :

Precipitating reagent (L3)	0.1 mL
Sample	0.1 mL

Mix well and incubate at RT for 5 minutes. Centrifuge at 2500-3000 rpm to obtain a clear supernatant.

Cholesterol Assay

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Working reagent	1.0	1.0	1.0
Distilled water	0.05	-	-
HDL standard (S)	-	0.05	-
Supernatant *	-	-	0.05

Mix well and incubate at 37°C for 5 minutes or at RT (25°C) for 15 minutes. Measure the absorbance of the standard (Abs. S), and test sample (Abs. T) against the blank, within 60 minutes.

* If only total cholesterol is to be determined use only 0.01 mL of DW/cholesterol Std/sample directly in the cholesterol assay.

Calculations

$$\text{HDL cholesterol in mg/dL} = \frac{\text{Abs. T}}{\text{Abs. S}} \times 25 \times 2$$

(Where 2 is the dilution factor due to the deproteinization step)

Calculation of LDL Cholesterol (mg/dL)

(Friedewald's Formula)

$$= \text{Total cholesterol} - \left(\frac{\text{Triglycerides}}{5}\right) - \text{HDL cholesterol}$$

Friedewald's formula is reliable provided that:

1. No chylomicrons are present, i.e. it is a fasting sample.
2. Triglyceride values are below 400 mg/dL.
3. Type III hyperlipoproteinemia is absent.

Linearity

This procedure is linear upto 150 mg/dL of HDL cholesterol. If values exceed this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note

The supernatant should be clear. If it is hazy or cloudy, the sample should be diluted 1 + 1 with normal saline (NaCL 0.9%) and the precipitation step should be repeated (Results × 2)

Anticoagulants such as fluoride, oxalates and hemolyzed serums should not be used.

System Parameters

Reaction	:	End Point	Interval	:	-
Wavelength	:	505 nm	Sample volume	:	0.05 mL

Contd...

Contd...

Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C/RT	Standard	:	25 mg/dL × 2
Incubated time	:	5 min/15 min	Factor	:	-
Delay time	:	-	Reaction slope	:	Increasing
Read time	:	-	Linearity	:	150 mg/dL
No. of read	:	-	Units	:	mg/dL

Risk Factor

Coronary heart disease (CHD) risk factor can be calculated using total lipid profile, as suggested by Castelli, et al. The risk factor gives a most accurate and definite assessment of heart disease risk.

The factors are calculated by the ratio of total cholesterol to HDL—cholesterol and by the ratio of LDL—cholesterol (Low density lipoproteins—cholesterol) to HDL—cholesterol.

Risk	*Ratio: Total/ HDL—Cholesterol*		*Ratio: LDL/ HDL—Cholesterol*	
	Men	*Women*	*Men*	*Women*
½ Average	3.43	3.27	1.00	1.47
Average	4.97	4.44	3.55	3.22
2 × Average	9.55	7.05	6.25	5.03
3 × Average	23.99	11.04	7.99	6.14

HDL Cholesterol ppt Set (PEG Precipitation Method)

(*Courtesy*: Tulip Group of Companies)

For the determination of HDL cholesterol in serum or plasma (for in vitro diagnostic use only).

Summary

Lipoproteins are the proteins which mainly transport fats in the bloodstream. They can be grouped into chylomicrons, very low density lipoproteins VLDL, low density lipoproteins (LDL) and high density lipoproteins (HDL). Chylomicrons and VLDL transport mainly triglycerides, though VLDLs also transport some amount of cholesterol. LDL carries cholesterol to the peripheral tissues where it can be deposited and increase the risk of arteriosclerotic heart and peripheral vascular disease. Hence, high levels of LDL are atherogenic. HDL transports cholesterol from the peripheral tissues to the liver for excretion, hence HDL has a protective effect. The

measurement of total and HDL cholesterol and triglycerides provide valuable information for the risk assessment of coronary heart diseases.

Principle

When the serum is reacted with the polyethylene glycol contained in the precipitating reagent, all the VLDL and LDL are precipitated. The HDL remains in the supernatant and is then assayed as a sample for cholesterol using the cholesterol (CHOD/PAP) reagent.

Normal Reference Values

		Prognostically standard		*Risk*
		favourable	*risk level*	*indicator*
HDL cholesterol (mg/dL)	Males Females	> 55 > 65	35–55 45–65	< 35 < 45
LDL cholesterol	Males	< 150 Females	150–190	> 190
Total cholesterol_ HDL cholesterol	Males	> 3.8	3.8–5.9	< 5.9
	Females	> 3.1	3.1–4.6	< 4.6

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*10 mL*
L1: Precipitating reagent	10 mL
S: HDL cholesterol standard (25 mg/dL)	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

After the precipitation step cholesterol reagent is required additionally for conducting the cholesterol assay.

Sample Material

Serum, EDTA plasma. HDL cholesterol is reported to be stable in serum for 7 days when stored at 2–8°C. The sample should preferably be of 12 to 14 hours fasting.

Procedure

Precipitation of VLDL and LDL:

Pipette into a clean dry test tube

Precipitating reagent (L1) 0.1 mL

Sample 0.1 mL

Mix well and incubate at RT for 5 minutes. Centrifuge at 2500–3000 rpm to obtain a clear supernatant.

Procedure for the Cholesterol Assay

Wavelength/filter : 505 nm (Hg 546 nm)/green

Temperature : 37°C/RT

Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition	*B*	*S*	*T*
Sequence	*(mL)*	*(mL)*	*(mL)*
Working reagent	1.0	1.0	1.0
Distilled water	0.05	-	-
HDL standard (S)	-	0.05	-
Supernatant	-	-	0.05

Mix well and incubate at 37°C for 5 mm. or at RT (25°C) for 15 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 60 minutes.

Calculations

$$\text{HDL cholesterol in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 25 \times 2$$

(Where 2 is the dilution factor due to the deproteinization step)

Calculation of LDL cholesterol (mg/dL):

(Friedewald's formula)

$$= (\text{Total cholesterol}) - \frac{\text{Triglycerides}}{5} - (\text{HDL cholesterol})$$

Freidewald's formula is reliable provided that:

1. No chylomicrons are present, i.e. it is a fasting sample.
2. Triglyceride values are below 400 mg/dL.
3. Type III hyperlipoproteinemia is absent.

Linearity

This procedure is linear upto 150 mg/dL of HDL cholesterol. If values exceed this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note

The supernatant should be clear. If it is hazy or cloudy, the sample should be diluted 1 + 1 with normal saline (NaCL 0.9%) and the precipitation step should be repeated (results × 2).

Anticoagulants such as fluoride, oxalates and hemolyzed serums should not be used.

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	505 nm	Sample volume	:	0.05 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C / RT	Standard	:	25 mg/dL × 2
Incubated time	:	5 min/15 min	Factor		
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	150 mg/dL
No. of read	:	—	Units	:	mg/dL

Clinical Relevance

1. Increased values are associated with a chronic liver disorder.
2. Decreased values are associated with
   a. Increased risk of coronary heart disease when HDL—Cholesterol is less than 45 mg/dL in men and less men 55 mg/dL in women.
   b. Inheritance and chronic physical inactivity (25–35 mg/dL). Long level distance runners have higher levels of HDL.
3. Levels can be either high or low in primary biliary cirrhosis, chronic hepatitis, or alcoholism.

Interfering Factors

1. Decreased HDL is associated with smokers.
2. Increased HDL is associated with moderate intake of alcohol.
3. Iodine contrast substances interfere with test results.
4. Recent weight gains or losses can interfere with the test results.

Patient Preparation

1. Overnight fasting is required. Water is permitted.
2. If possible, all medication should be withheld for 24 to 48 hours before testing—confer with attending physician regarding this.
3. Ask patient if there has been any drastic change in weight in last few weeks before testing.

Patient Aftercare

Person with decreased HDL can be counseled to take measures to increase levels by losing weight, cutting down on calories consumption, eating less red meat, and taking lecithin supplements. Moderate alcohol consumption is believed by some to be a factor in increased HDL.

Cholesterol: LDL and VLDL

Very low density lipoproteins (VLDL) and low-density lipoproteins (LDL).

Normal Values

VLDL cholesterol: 25–50%
LDL cholesterol: 62–185 mg/dL.

VLDL is a major carrier of triglyceride (60-70% triglyceride, 10–15% cholesterol). Degradation of VLDL leads to a major source of LDL. Circulating fatty acids are vitalized by the liver to form triglycerides that are packaged with apoprotein and cholesterol and exported into the blood as very low density lipoproteins.

LDLs are the cholesterol rich remnants of the lipid transport vehicle, VLDL. Since, LDL has a longer half-life (3–4 days) than its precursor, VLDL, LDL is more prevalent in the blood. It is finally catabolized in the liver and possibly in nonhepatic cells as well.

LDL-Cholesterol Fully Enzymatic, Colorimetric Test

(*Courtesy*: Randox)

Principle

Low-density lipoproteins (LDL) are precipitated by heparin at their isoelectric point (pH 5.04). After centrifugation the high-density lipoproteins (HDL) and the very low-density lipoproteins (VLDL) remain in the supernatant. These can then be determined by enzymatic methods.

LDL-cholesterol = Total cholesterol–cholesterol in the supernatent.

Sample

Serum

Reagents

Contents *Initial Concentration of Solution*

1. Precipitation Reagent
   Heparin 50,000 IU/L
   Sodium citrate 0.064 mol/L, pH 5.04.

Preparation of Reagents

1. Precipitation reagent
   Ready for use
   Stable up to expiry date when stored at + 2 to + 8°C.
2. Reagent solution for cholesterol determination.

Procedure

Wavelength 500 nm Hg 546
Cuvette 1 cm light path

Temperature	+ 20 to + 25°C, 37°C
Pipette into centrifuge tube	
Serum	100 µL
Precipitation reagent (1)	1000 µL

Mix well, have to stand for 10 minutes at + 15 to + 25°C and centrifuge for 15 minutes at approx. 4000 rpm. Determine the cholesterol concentration of the supernatant within 1 hour after centrifugation.

Pipette into test tubes:

	Reagent blank	*Standard*	*Sample*
Distilled water	50 µL		
Standard		50 µL	
Supernatant			50 µL
Reagent (2)	1000 µL	1000 µL	1000 µL
(Cholesterol reagent)			

Mix well, incubate for 10 minutes at + 20 to + 25°C or for 5 min at 37°C and measure the absorbance of the sample (A_{sample}) against the reagent blank.

Calculation

Using a standard

Concentration of cholesterol in the supernatant:

$$\frac{A_{sample}}{A_{standard}} \times \text{concentration of std.}$$

Calculation of the LDL-cholesterol

LDL-cholesterol = Total cholesterol—cholesterol in the supernatant.

Using a Factor

Cholesterol concentration of the supernatant

$= A_{sample} \times F$

Factor (F) is given in table below:

mmol/L	*mg/dL*	
Hg 546	49.63	1920
500 nm	32.70	1265

Calculation the LDL-Cholesterol

LDL–cholesterol = total cholesterol— cholesterol in the supernatant

Clinical Interpretation

	mg/dL	*mmol/L*
No treatment required	< 150	3.9
Suspect range	150–190	3.9–4.9
Treatment required	> 190	> 4.9

Note

Low-density lipoproteins and the very rare atherogenic Lp(a) are precipitated qualitatively. There is a slight coprecipitation of the VLDL but as the cholesterol content of these is low, the LDL cholesterol values are not increased significantly and the estimation of cardiovascular risk is not affected.

Test Significance

This test is specifically done to determine the risk of coronary heart disease. The low-density lipoproteins are closely correlated with an increased incidence of atherosclerosis and coronary heart disease. One on the other hand, a decreased incidence of coronary heart disease is seen in persons with high levels of HDL.

The VLDL cholesterol concentration is expressed as percent of total cholesterol.

Triglycerides (GPO/PAP Method)

(*Courtesy*: Tulip Group of Companies)

For the determination of triglycerides in serum or plasma (for in vitro diagnostic use only).

Summary

Triglycerides are a form of fatty acid esters. They are produced in the liver by binding glycerol and other fatty acids. They are transported by VLDL and LDL and act as a storage source for energy. Increased levels are found in hyperlipidemias, diabetes, nephrotic syndrome, hypothyroidism. Increased levels are risk factor for arteriosclerotic coronary disease and peripheral vascular disease. Decreased levels are found in malnutrition and hyperthyroidism.

Principle

Lipoprotein lipase hydrolyzes triglycerides to glycerol and free fatty acids. The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidized by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of triglycerides present in the sample.

$$\text{Triglycerides} \xrightarrow{\textit{Lipoprotein Lipase}} \text{Glycerol + Free fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\textit{Glycerol Kinase}} \text{Glycerol 3 Phosphate + ADP}$$

$$\text{Glycerol 3 Phosphate + } O_2 \xrightarrow{\textit{Glycerol 3 PO}} \text{Dihydroxyacetone phosphate + } H_2O_2$$

$$H_2O_2 + 4\ \text{Aminoantipyrine} \xrightarrow{\textit{Peroxidase}} \text{Red + Phenol Quinoneimine dye} + H_2O$$

Normal Reference Values

Serum/plasma (Suspicious) : 150 mg/dL and above
(Elevated) : 200 mg/dL and above

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*25 mL*	*2 × 75 mL*
L1: Enzyme Reagent 1	20 mL	2 × 60 mL
L2: Enzyme Reagent 2	5 mL	2 × 15 mL
S: Triglycerides Standard (200 mg/dL)	5 mL	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: Pour the contents of 1 bottle of L2 (Enzyme reagent 2) into 1 bottle of L1 (Enzyme reagent 1). This working reagent is stable for at least 8 weeks when stored at 2–8°C. Upon storage the working reagent may develop a slight pink color however, this does not affect the performance of the reagent. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme reagent 1) and 1 part of L2 (Enzyme reagent 2). Alternatively 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum, plasma. Triglycerides is reported to be stable in the sample for 5 days when stored at 2–8°C.

Procedure

Wavelength/filter : 505 nm (Hg 546 nm)/green
Temperature : 37°C/RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Working reagent	1.0	1.0	1.0
Distilled water	0.01		
Triglycerides standard (S)	-	0.01	
Sample	-	-	0.01

Mix well and incubate at 37°C for 5 minutes. or at RT (25°C) for 15 minutes. Measure the absorbance of the standard (Abs. S), and test sample (Abs. T) against the blank, within 60 minutes.

Calculations

$$\text{Triglycerides in mg/dL} = -\frac{\text{Abs T}}{\text{Abs S}} \times 200$$

Linearity

This procedure is linear upto 1000 mg/dL. If values exceed this limit, dilute the serum with normal saline (NaCI 0.9%) and repeat the assay.

Note

Fasting samples of 12 to 14 hours are preferred. Fatty meals and alcohol may cause elevated results. Patient should not drink alcohol for 24 hours before the test.

System Parameters

Reaction	:	End point	Interval	:	—
Wavelength	:	505 nm	Sample volume	:	0.01 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C/RT	Standard	:	200 mg/dL
Incubated time	:	5 mm/15 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	1000 mg/dL
No. of read	:	—	Units		mg/dL

Normal Values

		SI units
Adult females		
Age 20–29	10–100 mg/dL	0.11–1.13 mmol/L
Age 30–39	10–110 mg/dL	0.11–1.24 mmol/L
Age 40–49	10–122 mg/dL	0.11–1.38 mmol/L
Age 50–59	10–134 mg/dL	0.11–1.51 mmol/L
Age > 59	10–147 mg/dL	0.11–1.66 mmol/L
Adult males		
Age 20–29	10–157 mg/dL	0.11–1.77 mmol/L
Age 30–39	10–182 mg/dL	0.11–2.05 mmol/L
Age 40–49	10–193 mg/dL	0.11–2.18 mmol/L
Age 50–59	10–197 mg/dL	0.11–2.22 mmol/L

Contd...

Contd...

Age > 59	10–199 mg/dL	0.11–2.24 mmol/L
Children		
Female,		
age 1–19	10–121 mg/dL	0.11–1.36 mmol/L
Male,		
age 1–19	10–103 mg/dL	0.11–1.16 mmol/L
Note: Plasma values are lower by about 3%.		

Classification of Triglyceride Levels

Borderline	200–400 mg/dL	2.26–4.5 mmol/L high
High	400–1000 mg/dL	4.5–11.3 mmol/L
Very high	>1000 mg/dL	> 11.3 mmol/L

Clinical Implications

1. Increased triglyceride levels are believed to be a factor in increased risk for atherosclerosis
   A. Increased levels occur in
      1. Types I, IIb, III, IV, and V hyperlipoproteinemias
      2. Liver disease
      3. Nephrotic syndrome
      4. Hypothyroidism
      5. Poorly controlled diabetes
      6. Pancreatitis
      7. Glycogen storage disease
      8. Myocardial infarction (increases may last one year)
      9. Metabolic disorders related to endocrinopathies.
   B. Many of the clinical conditions that cause an increase in cholesterol levels also cause increase in triglycerides
      1. Nephrotic syndrome
      2. Pancreatic dysfunction
      3. Toxemia
      4. Hypothyroidism.
2. Decreased levels occur in malnutrition and congenital alpha-beta lipoproteinemia.

BLOOD GLUCOSE

Glucose (GOD/POD Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of glucose in serum, plasma, and CSF (for in vitro diagnostic use only).

Summary

Glucose is the major carbohydrate present in blood. Its oxidation in the cells is the source of energy for the body. Increased levels of glucose are found in diabetes mellitus, hyperparathryroidism, pancreatitis, renal failure. Decreased levels are found in insulinoma, hypothyroidism, hypopituitarism and extensive liver disease.

Principle

Glucose is oxidised to gluconic acid and hydrogen peroxide in the presence of glucoseoxidase. Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of glucose present in the sample.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + 4\ \text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red + Phenol quinoneimine dye} + H_2O$$

Normal Reference Values

Serum/plasma (fasting)	:	70–110 mg/dL
(2 hours PP)	:	upto 140 mg/dL
CSF	:	50–80 mg/dL

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*2 × 150 mL*	*1000 mL*
L1: Glucose Reagent	2 × 150 mL	1000 mL
S: Glucose Standard (100 mg/dL)	5 mL	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels. Upon storage the glucose reagent may develop a slight pink color. This does not affect the performance of the test.

Reagent Preparation

Reagents are ready to use.

Sample Material

Serum, plasma, CSF. Glucose is reported to be stable in the sample for 7 days when stored at 2–8°C.

Procedure

Wavelength/filter	:	505 nm (Hg 546 nm)/green
Temperature	:	37°C/RT
Light path	:	1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition	*B*	*S*	*T*
Sequence	*(mL)*	*(mL)*	*(mL)*
Glucose reagent (L1)	1.0	1.0	1.0
Distilled water	0.01		
Glucose standard(s)	-	0.01	
Sample	-	-	0.01

Mix well and incubate at 37°C for 10 minutes or at RT (25°C) for 30 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 60 minutes.

Calculations

$$\text{Total glucose in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 100$$

Linearity

This procedure is linear upto 500 mg/dL. If values exceed this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note

To avoid glycolysis the serum should be separated from the clot as soon as possible, and plasma should be collected in an EDTA + fluoride bulb (0.5 mg + 1 mg per ml of blood).

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	505 nm	Sample volume	:	0.01 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C/RT	Standard	:	100 mg/dL
Incubated time	:	10 min/30 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	500 mg/dL
No. of read	:	—	Units	:	mg/dL

Body Fluid, Glucose

Normal Values

		SI units
Cerebrospinal fluid lags behind blood glucose levels by 2–4 hours. Fasting to 4 hours postpradially 50–80% of serum glucose.		
Adult	40–80 mg/dL	2.2–4.4 mmol/L
Premature infant	24–63 mg/dL	1.3–3.5 mmol/L
Full-term infant	34–119 mg/dL	1.9–6.6 mmol/L
Child	35–75 mg/dL	1.9–4.1 mmol/L
Peritoneal fluid	70–100 mg/dL	3.8–5.5 mmol/L
Pleural fluid	Same as blood glucose level, with a time lag of 2–4 hours or no less than 40 mg/dL (2.2 mmol/L) below blood glucose	
Fasting blood	60–110 mg/dL	3.3–6.1 mmol/L
Synovial fluid	No more than 10 mg/dL (0.6 mmol/L SI units) lower than good glucose level	

Clinical Relevance

Persistent Hyperglycemia

- Diabetes mellitus
- Adrenal cortical hyperactivity (Cushing's syndrome)
- Acromegaly
- Obesity.

Transient Hyperglycemia

- Pheochromocytoma
- Severe liver disease
- Acute stress reaction (physical or emotional)
- Shock
- Convulsions.

Persistent Hypoglycemia

- Insulinoma
- Adrenal cortical insufficiency
- Hypopituitarism
- Galactosemia
- Ectopic insulin production from tumors.

Transient Hypoglycemia

- Acute alcohol ingestion
- Drugs: Salicylates, anti-tuberculosis agents
- Severe liver disease
- Several glycogen storage diseases
- "Functional" hypoglycemia
- Hereditary fructose intolerance.

URIC ACID

Uricase/PAP Method

(*Courtesy*: Tulip Group of Companies)
For the determination of uric acid in serum or plasma (for in vitro diagnostic use only).

Summary

Uric acid is the end product of purine metabolism. Uric acid is excreted to a large degree by the kidneys and to a smaller degree in the intestinal tract by microbial degradation. Increased levels are found in gout, arthritis, impaired renal functions and starvation. Decreased levels are found in Wilson's disease, Fanconi's syndrome and yellow atrophy of the liver.

Principle

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of uric acid present in the sample.

$$\text{Uric acid} + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + H_2O_2$$

$$H_2O_2 + 4\ \text{Aminoantipyrine} + \text{Phenolic Compound} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye} + H_2O$$

Normal Reference Values

Serum/plasma (Males) : 3.4–7.0 mg/dL
(Females) : 2.5–6.0 mg/dL

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*25 mL*	*75 mL*
L1: Buffer reagent	20 mL	60 mL
L2: Enzyme reagent	5 mL	15 mL
S: Uric acid standard (8 mg/dL)	5 mL	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: Pour the contents of 1 bottle of L2 (Enzyme reagent) into 1 bottle of L1 (Buffer reagent). This working reagent is stable for at least 4 weeks when stored at 2–8°C. Upon storage the working reagent may develop a slight pink color however, this does not affect the performance of the reagent. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme reagent 1) and 1 part of L2 (Enzyme reagent 2). Alternatively 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum, plasma. Uric acid is reported to be stable in the sample for 3 to 5 days when stored at 2–8°C.

Procedure

Wavelength/filter : 520 nm (Hg 546 nm)/yellow-green
Temperature : 37°C/RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Working reagent	1.0	1.0	1.0
Distilled water	0.02		
Uric acid standard (s)	-	0.02	-
Sample	-	-	0.02

Mix well and incubate at 37°C for 5 minutes or at RT (25°C) for 15 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 30 minutes.

Calculations

$$\text{Uric Acid in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 8$$

Linearity

This procedure is linear upto 20 mg/dL. If values exceed this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	520 nm	Sample volume	:	0.02 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL

Contd...

Contd...

Incubation temperature	: 37°C / RT	Standard	: 8 mg/dL
Incubated time	: 5 min/ 15 min	Factor	:
Delay time	: —	React slope	: Increasing
Read time	: —	Linearity	: 20 mg/dL
No. of read	: —	Units	: mg/dL

Normal Values

		SI units
Adult females	2.5–6.0 mg/dL	143–357 µmol/L
Adult males	3.4–7.0 mg/dL	202–416 µmol/L
Children	2.5–5.5 mg/dL	119–327 µmol/L
Panic level	> 12 mg/dL	> 714 µmol/L

Clinical Relevance

Factors Affecting Serum Uric Acid levels

Increased Production, Raised Serum Levels

- Idiopathic mechanisms associated with primary gout
- Excessive dietary purines (organ meats, legumes, anchovies, etc.)
- Cytolytic treatment of malignancies, especially leukemias and lymphomas
- Polycythemia
- Myeloid metaplasia
- Psoriasis
- Sickle cell anemia.

Decreased Excretion, Raised Serum Levels

- Alcohol ingestion
- Thiazide diuretics
- Lactic acidosis
- Aspirin doses < 2 g/day
- Ketoacidosis especially diabetes or starvation.
- Renal failure due to any cause.

Increased Excretion, Lowered Serum Levels

- Probenecid, sulfinpyrazone, aspirin doses above 4 g/day
- Corticosteroids and ACTH
- Coumarin anticoagulants.
- Estrogens.

Decreased Production, Lowered Serum Levels

- Allopurinol.

CALCIUM

OCPC Method

(*Courtesy*: Tulip Group of Companies)

For the determination of calcium in serum or plasma (for in vitro diagnostic use only).

Summary

Calcium, in the body, is found mainly in the bones (approximately 99%). In serum calcium exists equally in a free ionised form and in a bound form (with albumin). Hence, a decrease in albumin causes lower calcium levels and vice versa. The levels of calcium in serum depend on the parathyroid hormone. Increased calcium levels are found in bone tumors, hyperparathyroidism. Decreased levels are found in hypoparathyroidism, renal failure, rickets, vitamin D deficiency and pancreatitis.

Principle

Calcium in an alkaline medium combines with O-Cresolphthalein complexone to form a purple colored complex. Intensity of the color formed is directly proportional to the amount of calcium present in the sample.

$$\text{Calcium} + \text{OCPC} \longrightarrow \text{Purple colored complex}$$

Normal Reference Values

Serum/plasma : 8.7–11.0 mg/dL (general)

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*2 × 35 mL*	*2 × 75 mL*
L1: Buffer reagent	35 mL	75 mL
L2: Color reagent	35 mL	75 mL
S: Calcium standard (10 mg/dL)	5 mL	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use. Protect from bright light.

Working reagent: For convenience a single working reagent may be prepared by mixing equal parts of the buffer reagent and color reagent. This combined reagent is stable for 7 days at 2–8°C.

Sample Material

Serum/heparinized plasma. Calcium is reported to be stable in serum for 7 days at 2–8°C.

Procedure

Wavelength/filter : 570 nm (Hg 578 nm)/yellow
Temperature : RT
Light path : 1 cm
Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Buffer reagent (L1)	0.5	0.5	0.5
Color reagent (L2)	0.5	0.5	0.5
Distilled water	0.02	-	-
Calcium standard (s)	-	0.02	
Sample	-	-	0.02

Mix well and incubate at RT (25°C) for 5 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 60 minutes.

Calculations

$$\text{Calcium in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 10$$

Linearity

This procedure is linear upto 18 mg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution.

Note

As calcium is a very widely distributed ion, care should be taken to avoid any contamination. All glassware being used for the test should first be rinsed with 1% or 0.1 N HCI and then with good quality deionized water before use.

It is suggested that after the rinsing of the tubes with HCI the reagent be pipetted in their respective tubes and the tubes be rinsed with the reagent. The reagent then should be pooled together in the 'blank' tube and repipetted out into the 'standard' and 'test' test tubes. This will ensure that any remaining contamination will be carried over equally in all the tubes. For flow cell cuvettes, it is suggested that some reagent be aspirated before the blank to take away any contamination in the flow through tubing or cuvette which may cause a higher than the actual blank of the reagent.

Chelating agents such as EDTA, present even in traces, prevent the formation of the color complex, hence necessary care should be taken during the assay.

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	570 nm	Sample volume	:	0.02 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	RT	Standard	:	10 mg/dL
Incubated time	:	5 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	18 mg/dL
No. of read	:	—	Units	:	mg/dL

Calcium (Arsenazo III Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of calcium in serum or plasma (for in vitro diagnostic use only).

Summary

Calcium, in the body, is found mainly in the bones (approximately 99%). In serum calcium exists equally in a free ionized form and in a bound form (with albumin). Hence, a decrease in albumin causes lower calcium levels and vice versa. The levels of calcium in serum depend on the parathyroid hormone. Increased calcium levels are found in bone tumors, hyperparathyroidism. Decreased levels are found in hypoparathyroidism, renal failure, rickets, vitamin D deficiency and pancreatitis.

Principle

Calcium combines specifically with arsenazo III at a neutral pH to form a blue purple colored complex. Intensity of the color formed is directly proportional to the amount of calcium present in the sample.

$$\text{Calcium + Arsenazo III} \longrightarrow \text{Blue Purple colored complex}$$

Normal Reference Values

Serum/plasma : 8.7–11.0 mg/dL

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	75 mL	3 × 75 mL
L1: Calcium reagent	75 mL	3 × 75 mL
S: Calcium standard (10 mg/dL)	5 mL	5 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use. Protect from bright light.

Sample Material

Serum/heparinized plasma. Calcium is reported to be stable in serum for 7 days at 2–8°C.

Procedure

Wavelength/filter : 650 nm (Hg 623 nm)/red
Temperature : RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Calcium reagent (L1)	1.0	1.0	1.0
Distilled water	0.01		
Calcium standard(s)	-	0.01	
Sample	-	-	0.01

Mix well and incubate at RT (25°C) for 5 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 60 minutes.

Calculations

$$\text{Calcium in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 10$$

Linearity

This procedure is linear upto 15 mg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note

As calcium is a very widely distributed ion, care should be taken to avoid any contamination. All glassware being used for the test should first be rinsed with 1% or 0.1 N HCI and then with good quality deionized water before use.

It is suggested that after the rinsing of the tubes with HCI the reagent be pipetted in their respective tubes and the tubes be rinsed with the reagent. The reagent then should be pooled together in the 'blank' tube and repipetted out into the 'standard' and 'test' test tubes. This will ensure that any remaining contamination will be carried over equally in all the tubes. For flow cell cuvettes it is suggested that some reagent be aspirated before the blank to take away any contamination in the flow through tubing or cuvette which may cause a higer than the actual blank of the reagent.

Chelating agents such as EDTA, present even in traces, prevent the formation of the color complex, hence necessary care should be taken during the assay.

System Parameters

Reaction	:	End point		Interval	:		
Wavelength	:	650 nm		Sample volume	:	0.01 mL	
Zero setting	:	Reagent blank		Reagent volume	:	1.00 mL	
Incubation temperature	:	RT		Standard	:	10 mg/dL	
Incubated time	:	5 min		Factor	:		
Delay time	:	—		React slope	:	Increasing	
Read time	:	—		Linearity	:	15 mg/dL	
No. of read	:	—		Units	:	mg/dL	

Normal Values

Adults		
18–60 years	8.6–10.5 mg/dL	2.15–2.62 mmol/L
60–90 years	8.8–10.7 mg/dL	2.20–2.67 mmol/L
> 90 years	8.2–9.6 mg/dL	2.05–2.40 mmol/L
Children		
Cord blood	8.2–11.2 mg/dL	2.05–2.80 mmol/L
Premature infant	6.2–11.0 mg/dL	1.55–2.75 mmol/L
< 10 days	7.6–10.4 mg/dL	1.90–2.60 mmol/L
10 days–2 years	9.0–11.0 mg/dL	2.25–2.75 mmol/L
2–12 years	8.8–10.8 mg/dL	2.20–2.70 mmol/L
12–18 years	8.4–10.5 mg/dL	2.10–2.62 mmol/L
Panic levels		
Tetany	< 7 mg/dL	< 1.75 mmol/L
Coma	> 12 mg/dL	> 2.99 mmol/L
Possible death	< 6 mg/dL	< 1.50 mmol/L

Specimen Collection and Storage

1. Fresh, unhemolyzed serum is the preferred specimen.
2. Heparinized plasma may also be used.
3. Anticoagulants other than heparin should not be used.
4. Remove serum from clot as soon as possible since red cells can absorb calcium.
5. Older serum specimens containing visible precipitate should not be used.
6. Serum calcium is stable for 24 hours at room temperature, one week at 2–8°C, and up to 5 months frozen (–15 to –25°C.) and protected from evaporation. Specimens should not be thawed and refrozen.

Clinical Relevance

Normal levels of total calcium combined with other findings

1. Normal calcium levels with overall normal findings in other tests indicate that there are no problems with calcium metabolism.
2. Normal calcium and abnormal phosphorus indicate impaired calcium absorption due to alteration of parathyroid hormone activity or secretion. In rickets, the calcium level may be normal or slightly lowered and the phosphorus level is depressed.
3. Normal calcium and elevated BUN indicates
   a. Possible secondary hyperparathyroidism. Initially lowered serum calcium results from uremia and acidosis. The lower calcium level stimulates the parathyroid to release parathyroid hormone, which acts on bone to release more calcium.
   b. Possible primary hyperparathyroidism. Excessive amounts of parathormone cause elevation in calcium levels, but secondary kidney disease would cause retention of phosphate and concomitant lower calcium.
4. Normal calcium and decreased serum albumin. This is indicative of hypercalcemia, since, there should be a decrease in calcium when there is a decrease in albumin because of the 50% of serum calcium that is protein-bound.

Hypercalcemia (Increased Total Calcium)

Hypercalcemia is associated with many disorders, but its greatest clinical importance rests in its association with cancer, including multiple myeloma, parathyroid tumors, nonendocrine tumors producing a parathormone-like substance, and cancers metastasizing to the bone. Increased calcium levels are caused by or associated with.

1. Hyperparathyroidism due to
   a. Parathyroid adenoma associated with hypophosphatemia
   b. Hyperplasia of parathyroid glands associated with hypophosphatemia.
2. Cancer
   a. Metastatic cancers involving bone cancers of lung, breast, thyroid, kidney, and testes may metastasize to bone
   b. Hodgkin's disease other lymphomas
   c. Multiple myeloma in which there is extensive bone destruction
   d. Lung and renal cancers may produce parathormone resulting in symptoms of hypercalcemia
   e. Sarcoidosis due to increased IgG or IgA
   f. Leukemia.
3. Addison's disease
4. Hyperthyroidism
5. Paget's disease of bone (also accompanied by high levels of alkaline phosphatase)
6. Prolonged immobilization
7. Bone fractures combined with bed rest
8. Excessive intake of vitamin D
9. Prolonged use of diuretics, thiazides
10. Respiratory alkalosis
11. Milk alkali syndrome (history of peptic ulcer could indicate excessive intake of milk and antacids).

Hypocalcemia (Decreased Total Calcium Levels)

Commonly caused by/associated with

1. Pseudohypocalcemia (hyperproteinemia). Actually, what looks like hypocalcemia is really a reflection of diminished albumin (as revealed by a serum protein electrophoresis). It is the reduced protein that is responsible for the low calcium, since 50% of the calcium total is protein-bound. (Excessive use of IV fluids will decrease albumin levels and thus, decrease the amount of calcium).
2. Hypoparathyroidism (primary is very rare) may be due to accidental removal of parathyroid glands during a thyroidectomy, irradiation, hypomagnesemia, GI disorders, renal wasting.
3. Hyperphosphatemia
   Due to renal failure, laxatives, cytotoxic drugs
4. Malabsorption
   Due to sprue, celiac disease, pancreatic dysfunction (fatty acids combine with calcium and are precipitated and excreted in the feces).
5. Acute pancreatitis
6. Alkalosis (calcium ions become bound to protein)
7. Osteomalacia
8. Diarrhea
9. Rickets.

Increased Ionized Calcium

1. Primary hyperparathyroidism
2. Ectopic parathyroid hormone producing tumors
3. Excess intake of vitamin D
4. Various malignancies.

Decreased Ionized Calcium

Primary hypoparathyroidism is associated with low ionized calcium level and low total calcium level.

Be Careful

1. Thiazide diuretics may lead to impairment of urinary calcium excretions and consequent hypercalcemia.

2. Patients with renal insufficiency who are undergoing dialysis, a calcium-ion exchange resin is sometimes used for hyperkalemia. The use of this resin may lead to increased calcium levels.
3. Increased intake of magnesium and phosphates and the excessive use of laxatives may lower the blood calcium level. This occurs because of the increased intestinal loss of calcium produced by these elements.
4. When decreased calcium levels are due to magnesium deficiency, as in poor absorption from the bowel, the administration of magnesium will correct the calcium deficiency.
5. If a patient is known to have or suspected of having a pH abnormality, a concurrent pH should be requested with ionized calcium.

Interfering Factors

Many drugs can alter blood calcium levels for the list of drugs that may alter blood calcium level.

PHOSPHORUS

Molybdate UV Method

(*Courtesy*: Tulip Group of Companies)
For the determination of inorganic phosphorus in serum, plasma and urine (for in vitro diagnostic use only).

Summary

Phosphorus is mainly combined with calcium and is found in the bones. Approximately 15% exists as inorganic phosphorus or phosphate esters. It is involved in the carbohydrate metabolism and is a component of many other substances. Increased levels are found in hypoparathyroidism, renal failure, bone metastatis and liver diseases. Decreased levels are found in hyperparathyroidism, rickets and vitamin D deficiency.

Principle

Phosphate ions in an acidic medium react with ammonium molybdate to form a phosphomolybdate complex. This complex has an absorbance in the ultraviolet range and is measured at 340 nm. Intensity of the complex formed is directly proportional to the amount of inorganic phosphorus present in the sample.

Phosphorus + Ammonium molybdate ⟶ Phosphomolybdate complex

Normal Reference Values

Serum	(Adults)	:	2.5–5.0 mg/dL
	(Children)	:	4.0–6.5 mg/dL
Urine		:	0.3–1.0 g/24 hours

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	75 mL	2 × 75 mL
L1: Acid ragent	60 mL	2 × 60 mL
L2: Molybdate reagent	15 mL	2 × 15 mL
S: Phosphorus standard 5 mg/dL	5 mL	5 mL

Storage/Stability

Reagents are stable at RT (25–30°C) till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: Pour the contents of 1 bottle of L2 (molybdate reagent) into 1 bottle of L1 (Acid reagent). This working reagent is stable for at least 6 months when stored at 2–8°C. Upon storage the working reagent may develop a slight blue color, however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (acid reagent) and 1 part of L2 (molybdate reagent 2). Alternatively 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum heparinized/EDTA plasma or urine. Acidify the urine with a few drops of conc. Hydrochloric acid and dilute 1 + 19 before the assay, (results × 20). Inorganic phosphorus is reported to be stable in serum for 7 days at 2–8°C.

Procedure

Wavelength/filter : 340 nm (Hg 365 nm)
Temperature : RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Working reagent	1.0	1.0	1.0
Distilled water	0.01		
Phosphorus standard(s)	-	0.01	
Sample	-	-	0.01

Mix well and incubate at RT for 5 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the Blank, within 60 minutes.

Calculations

$$\text{Phosphorus in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 5$$

Linearity

This procedure is linear upto 20 mg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note

Hemolysis interferes with the test.

Use clean glassware washed with N/10 HCI as detergents may contain phosphate ions.

System Parameters

Reaction	:	UV end point	Interval	:	
Wavelength	:	340 nm	Sample volume	:	0.01 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation tempeerature	:	RT	Standard	:	5 mg/dL
Incubated time	:	5 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	20 mg/dL
No. of read	:	—	Units	:	mg/dL

Phosphorus
Mod Gomorri's Method

(*Courtesy*: Tulip Group of Companies)

For the determination of inorganic phosphorus in serum, plasma and urine (for in vitro diagnostic use only).

Summary

Phosphorus is mainly combined with calcium and is found in the bones. Approximately, 15% exists as inorganic phosphorus or phosphate esters. It is involved in the carbohydrate metabolism and is a component of many other substances. Increased levels are found in hypoparathyroidism, renal failure, bone metastasis and liver diseases. Decreased levels are found in hyperparathyroidism, rickets and vitamin D deficiency.

Principle

Phosphate ions in an acidic medium react with ammonium molybdate to form a phosphomolybdate complex. This complex reacts with metol and is reduced to a molybdinum blue complex. Intensity of the molybdinum blue complex formed is directly proportional to the amount of inorganic phosphorus present in the sample.

Phosphorus + Ammonium Molybdate→Phosphomolybdate complex
Phosphomolybdate complex + Metol→Molybdinum Blue Complex

Normal Reference Values

Serum	(Adults)	:	2.5–5.0 mg/dL
	(Children)	:	4.0–6.5 mg/dL
Urine		:	0.3–1.0 g/24 h

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	10 Tests	25 Tests
L1: Acid reagent	30 mL	75 mL
L2: Molybdate reagent	30 mL	75 mL
L3: Color reagent	30 mL	75 mL
S: Phosphorus standard (5 mg/dL)	5 mL	5 mL

Storage/Stability

Reagents are stable at RT (25–30°C) till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Sample Material

Serum, heparinized/EDTA plasma or urine. Acidify the urine with a few drops of cone. Hydrochloric acid and dilute 1 + 19 before the assay, (results x 20). Inorganic phosphorus is reported to be stable in serum for 7 days at 2–8°C.

Procedure

Wavelength/filter	:	650 nm (Hg 623 nm)/Red
Temperature	:	RT
Light path	:	1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Acid reagent	1.0	1.0	1.0
Molybdate reagent	1.0	1.0	1.0
Distilled water	0.1		
Phosphorus standard(s)	-	0.1	
Sample	-	-	0.1
Color reagent	1.0	1.0	1.0

Mix well and incubate at RT for 5 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 30 minutes.

Calculations

$$\text{Phosphorus in mg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times \text{L5}$$

Linearity

This procedure is linear upto 15 mg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Multiply the value obtained with an appropriate dilution factor.

Notes

Hemolysis interferes with the test.

Use clean glassware washed with N/10 HCI as many detergents contain phosphate ions.

The addition sequence of the reagents and the sample is important and should not be changed.

System Parameters

Reaction	: End point	Interval	:
Wavelength	: 650 nm	Sample volume	: 0.1 mL
Zero setting	: Reagent blank	Reagent volume	: 3.00 mL
Incubation temperature	: RT	Standard	: 5 mg/dL
Incubated time	: 5 min	Factor	:
Delay time	: —	React slope	: Increasing
Read time	: —	Linearity	: 15 mg/dL
No. of read	: —	Units	: mg/dL

Normal Values

SI units		
Adults < age 60	2.7–4.5 mg/dL	0.87–1.45 mmol/L
Females > age 60	2.8–4.1 mg/dL	0.90–1.30 mmol/L
Males > age 60	2.3–3.7 mg/dL	0.74–1.20 mmol/L
Cord blood	3.7–8.1 mg/dL	1.20–62 mmol/L
Premature infant	5.4–10.9 mg/dL	1.74–3.52 mmol/L
Newborn	4.5–9 mg/dL	1.45–2.91 mmol/L
Infant		
(10 days–24 months)	4.5–6.7 mg/dL	1.45–2.16 mmol/L
Child		
(24 months–12 years)	4.5–5.5 mg/dL	1.45–1.78 mmol/L

Clinical Relevance

Hyperphosphatemia (Increased Phosphorus Levels)

The most common causes of elevated blood phosphate levels are found in association with kidney dysfunction and uremia. This is because phosphate is so closely regulated by the kidneys.

Increased phosphorus levels are associated with

a. Renal insufficiency and severe nephritis accompanied by elevated BUN and creatinine.
b. Hypoparathyroidism (accompanied by elevated phosphorus, decreased calcium, and normal renal function).
c. Hypocalcemia
d. Excessive intake of alkali (possible history of peptic ulcer)
e. Excessive intake of vitamin D
f. Fractures in the healing stage
g. Bone tumors
h. Addison's disease
i. Acromegaly.

Hypophosphatemia (Decreased Phosphorus Levels)

Decreased phosphorus levels may be associated with

a. Hyperparathyroidism (accompanied by increased calcium, no renal disease)
b. Rickets (childhood), osteomalacia (adults)
c. Diabetic coma because of increased carbohydrate metabolism
d. Hyperinsulinism
e. Continuous administration of intravenous glucose in a non-diabetic patient.

Interfering Factors

1. Normally high in children
2. Falsely increased by hemolysis of blood
3. Drugs causing possible elevation
   a. Diphenylhydantoin (phenytoin)
   b. Heparin
   c. Pituitrin
   d. Vitamin D
   e. Methicillin
   f. Tetracyclines
   g. Alkaline antacids
   h. Lipomol.
4. The use of laxatives or enemas containing large amounts of sodium phosphate will cause increased phosphorus levels.
5. Drugs causing possible decreases
   a. Aluminum hydroxide
   b. Epinephrine (adrenaline)
   c. Insulin

d. Mannitol
e. Mithramycin
f. Parathyroid injection.

CHLORIDE

Thiocyanate Method

(*Courtesy*: Tulip Group of Companies)

Chloride is a major extracellular anion and maintains the cation/anion balance between intra and extracellular fluids, mostly as a salt with sodium. Increased levels are usually found in dehydration, kidney dysfunction, and anemia. Decreased levels are found in extensive burns, vomiting, diarrhea, intestinal obstructions, and salt losing nephritis.

Principle

Chloride ions combine with free mercury ions and release thiocyanate from mercuric thiocyanate. The thiocyanate released combines with the ferric ions to form a red brown ferric thiocyanate complex. Intensity of the color formed is directly proportional to the amount of chloride present in the sample.

$$2\ Cl^- + Hg(SCN)_2 \longrightarrow HgCl_2 + 2\ (SCN)^-$$

$$3\ (SCN) + Fe^{3+} \longrightarrow Fe(SCN)_3$$

Normal Reference Values

Serum/plasma chloride : 96–106 mmol/L
Urine chloride : 170–250 mmol/24 h
CSF chloride : 120–135 mmol/L

It is recommended that each laboratory establish its own normal range representing its patient population.

Chloride Kit

L1 : Chloride reagent 75 mL
S : Chloride standard (100 mmol/L) 5 mL

Storage/Stability

All reagents are stable at RT till the expiry mentioned.

Reagent Preparation

Reagents are ready to use.

For chloride: Serum, plasma, urine, and CSF. Dilute urine samples 1 + 1 with distilled water before the assay. Chloride is reported to be stable in serum for 7 days at 2–8°C.

Procedure

Wavelength/filter : 505 nm (Hg 546/green)
Temperature : RT
Light path : 1 cm

Chloride Assay

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T).

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Chloride reagent (L1)	1.0	1.0	1.0
Deionised water	0.01	–	–
Chloride standard (s)	–	0.01	
Sample	–	–	0.01

Mix well and incubate at RT for 2 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against blank, within 60 minutes.

Linearity

The Chloride assay is linear between 70–140 mmol/L. If values exceed this limit, dilute the sample with deionized water (free from $Na^+/K^+/Cl^-$ ions) and repeat the assay. Calculate the value using the proper dilution factor.

Notes

Bring all reagents to RT before use.

Turbid or icteric samples may produce falsely elevated results.

The procedure for chloride measures total halides such as bromides, iodides, and fluorides in addition to chlorides hence, their contamination should be avoided. Since the test is temperature sensitive, so a constant temperature should be maintained during incubation and reading.

System Parameters, Cl^-

Reaction	:	End point	Interval	:	
Wavelength	:	505 nm	Sample volume	:	0.01 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	RT	Standard	:	100 mmol/L
Incubated time	:	2 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	70–140 mmol/L
No. of read	:	—	Units	:	mmol/L

Calculation

$$\text{Chloride in mmol/L} = \frac{\text{Abs. T}}{\text{Abs. S}} \times 100$$

Normal Values

		SI units
Children and adults	97–106 mEq/L	97–107 mmol/L
Premature infants	95–110 mEq/L	95–110 mmol/L
Full-term infants	96–106 mEq/L	96–106 mmol/L
Panic levels	< 80 mEq/L	< 80 mmol/L
	> 115 mEq/L	> 115 mmol/L

Clinical Relevance

1. Whenever, the serum level is much lower than 100 mEq/L, the urinary excretion of chloride falls to a very low level.
2. The reason why decreased chloride levels often occur in acute infections is not clear.
3. Chloride measurements are of limited value in renal diseases for the reason that plasma chloride can be maintained near normal limits even when a considerable degree of renal failure is present.
4. Increased chloride levels occur in
   a. Cushing's syndrome
   b. Dehydration
   c. Hyperventilation
   d. Eclampsia
   e. Anemia
   f. Cardiac decompensation
   g. Some renal disorders.
5. Decreased chloride levels occur in
   a. Severe vomiting
   b. Severe diarrhea
   c. Ulcerative colitis
   d. Pyloric obstruction
   e. Severe burns
   f. Heat exhaustion
   g. Diabetic acidosis
   h. Addison's disease
   i. Fever
   j. Acute infections such as pneumonia
   k. Use of drugs such as mercurial and chlorothiazide diuretics.

Interfering Factors

1. The plasma chloride concentration of infants is usually higher than that of children and adults.
2. Many drugs may cause a change in chloride levels.

Be Careful

1. In intravenous therapy, if the solution contains 100 mEq/L, there is ample chloride present for the correction of urine metabolic acidosis.
2. If an electrolyte disorder is suspected, daily weight and accurate intake and output should be recorded.

SERUM IRON AND TIBC

Ferrozine Method

(*Courtesy*: Tulip Group of Companies)
For the determination of iron and total iron binding capacity in serum (laboratory reagent for professional use only).

summary

Iron found in blood is mainly present in the hemoglobin of the RBCs. Its role in the body is mainly in the transportation of oxygen and cellular oxidation. Iron is absorbed in the small intestine, and bound to a globulin in the plasma, called transferrin and transported to the bone marrow for the formation of hemoglobin. Increased serum levels are found in hemolytic anemias, hepatitis, lead and iron poisoning. Decreased serum levels are found in anemias caused by iron deficiency due to insufficient intake or absorption of iron, chronic blood loss, late pregnancy and cancer. Increase in TIBC is found in iron defecient anemias and pregnancy. Decrease in TIBC is found in hypoproteinemia, hemolytic/pernicious/sickle cell anemias, inflammatory diseases and cirrhosis.

Principle

Iron, bound to transferrin, is released in an acidic medium and the ferric ions are reduced to ferrous ions. The Fe (II) ions react with ferrozine to form a violet colored complex. Intensity of the complex formed is directly proportional to the amount of iron present in the sample. For TIBC, the serum is treated with excess of Fe (II) to saturate the iron binding sites on transferrin. The excess Fe (II) is adsorbed and precipitated and the Iron content in the supernatant is measured to give the TIBC.

$$\text{Fe (III)} \xrightarrow{\text{Acidic Medium}} \text{Fe (II)}$$

$$\text{Fe (II)} + \text{Ferrozine} \longrightarrow \text{Violet colored complex}$$

Normal Reference Values

Serum Iron	(Males)	: 60–160 µg/dL
	(Females)	: 35–145 µg/dL
	(Neonates)	: 150–220 µg/dL
TIBC		: 250–400 µg/dL
UIBC		: 160–360 µg/dL.

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	35 mL	75 mL
Iron reagents		
L1: Iron cutter reagent	35 mL	75 mL
L2: Iron color reagent	2 mL	4 mL
S: Iron standard (100 µg/dL)	2 mL	2 mL
TIBC reagents		
L1: TIBC saturating reagent	10 mL	20 mL
L2: TIBC precipitating reagent	1 g	2 g

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Sample Material

Serum, free from hemolysis. Iron is reported to be stable in serum for 7 days at 2–8°C.

Procedure

Wavelength/filter : 570 nm (Hg 578 nm)/yellow
Temperature : RT
Light path : 1 cm

Iron Assay

Pipette into clean dry test tubes labeled as blank (B), standard (S), sample blank (SB) and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*SB (mL)*	*T (mL)*
Iron buffer reagent (L1)	1.0	1.0	1.0	1.0
Distilled water	0.2	-	-	-
Iron standard (S)	-	0.2	-	-
Sample	-	-	0.2	0.2
Iron color reagent (L2)	0.05	0.05	-	0.05

Mix well and incubate at RT for 5 minutes. Measure the absorbances of the blank (Abs B), standard (Abs S), sample blank (Abs SB) and test sample (Abs T) against DW.

TIBC Assay

Pipette into a clean dry test tube

Serum TIBC saturating reagent (L1)	0.5 mL 1.0 mL
Mix well and allow to stand at RT for 10 min and add	
TIBC precipitating reagent (L2)	Approx 50 mg

Mix well and allow to stand at RT for 10 minutes. Centrifuge at 2500–3000 rpm for 10 minutes to obtain a clear supernatant. Determine the iron content in the supernatant as above mentioned iron assay.

Calculations

$$\text{Iron in µg/dL} = \frac{\text{Abs T- (Abs SB + Abs B)}}{\text{Abs S – Abs B}} \times 100$$

$$\text{TIBC in µg/dL} = \frac{\text{Abs T – (Abs SB + Abs B)}}{\text{Abs S – Abs B}} \times 300$$

UIBC in µg/dL = TIBC in µg/dL – Iron in µg/dL

Linearity

This procedure is linear upto 1000 µg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Notes

Hemolysis interferes with the test as the hemoglobin present in the RBCs has a very high iron content.

All glassware being used for the test should first be rinsed with 1% or 0.1 N HCI and then with good quality deionized water before use.

System Parameters

Reaction	: End point +	Interval	: SB
Wavelength	: 578 nm	Sample volume	: 0.2 mL
Zero setting	: Deionized	Reagent volume	: 1.05 mL water
Incubation temperature	: RT	Standard	: 100 ng/dL
Incubated time	: 5 min	Factor	:
Delay time	: —	React slope	: Increasing
Read time	: —	Linearity	: 1000 µg/dL
No. of read	: —	Units	: µg/dL

Clinical Relevance

1. TIBC is raised in
   a. Inadequate dietary iron
   b. Iron deficiency anemia due to hemorrhage
   c. Acute hepatitis
   d. Polycythemia
   e. Oral contraceptive use.
2. Decreased levels of TIBC are caused by
   a. Pernicious anemia
   b. Thalassemia
   c. Sickle cell anemia

d. Chronic infection
e. Cancer
f. Hepatic disease
g. Uremia
h. Rheumatoid arthritis.

Interfering Factors

1. Transferrin is elevated in
   a. Children 2½ to 10 years of age
   b. Pregnant women during the third trimester
2. Drugs that may cause increased TIBC are
   a. Chloramphenicol
   b. Fluorides.

TRACE ELEMENTS

The term trace elements refers to inorganic substances which occur in concentration < 0.01% of the body mass, i.e. in amounts < 10^{-6} g/g of body weight. They are divided into essential and nonessential trace elements. In humans, Cr, Co, Cu, Fe, l, Mn, Mo, Ni, Se, Zn belong to the former category; Al, Ag, As, Au, Ba, Bi, Cs, Cd, Pb, Ti, and V belong to the group of nonessential trace elements. The latter also include elements without physiological functions as well as toxic heavy metals. Magnesium, in a strict sense, is not a trace element but is customarily considered to be one. In this issue three trace elements are considered.

ZINC

Oxidation state + 2,
Atomic number 30,
Atomic symbol Zn,
Atomic weight 65.38,
Electron configuration— 8-18-2.

ZINC (COLORIMETRIC METHOD)

(*Courtesy*: Tulip Group of Companies)
For the determination of zinc in serum and urine (laboratory reagent for professional use only).

Summary

Zinc is important in man for growth and sexual development. It is present in various organs and is a component of many enzymes. Zinc found in serum is totally bound to protein with over 60% being bound to albumin. Increased levels are found in patients associated with gastrointestinal disorders accompanied with nausea, vomiting, high fever and a metallic taste. Decreased levels are found in cirrhosis, lung carcinomas, sickle cell anemia, acute myocardial infarction, renal failure, corticosteroid and oral contraceptive therapy.

Principle

Zinc in an alkaline medium reacts with nitro-PAPS to form a purple colored complex. Intensity of the complex formed is directly proportional to the amount of zinc present in the sample.

$$\text{Zinc + Nitro-PAPS} \xrightarrow[\text{Medium}]{\text{Alkaline}} \text{Purple colored complex}$$

Normal Reference Values

Serum : 60–120 µg/dL
Urine : 100–1000 µg/24 h

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*25 mL*	*75 mL*
L1: Buffer reagent	20 mL	60 mL
L2: Color reagent	5 mL	15
S: Zinc standard (200 µg/dL)	2 mL	2 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: Pour the contents of 1 bottle of L2 (Enzyme reagent 2) into 1 bottle of L1 (Enzyme reagent 1). This working reagent is stable for at least 2 weeks when stored at 2–8°C.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme reagent 1) and 1 part of L2 (Enzyme reagent 2). Alternatively 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum (Free from hemolysis) or urine.

Zinc is reported to be stable in serum for 7 days at 2–8°C.

Procedure

Wavelength/filter : 570 nm (Hg 578 nm)/yellow
Temperature : RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Working reagent	1.0	1.0	1.0
Distilled water	0.05		
Zinc standard (S)	-	0.05	
Sample	-	-	0.05

Mix well and incubate at RT (25°C) for 5 minutes. Measure the absorbance of the standard (Abs S), and Test sample (Abs T) against the blank, within 20 minutes.

Calculations

$$\text{Zinc in } \mu g/dL = \frac{\text{Abs T}}{\text{Abs S}} \times 200$$

Linearity

This procedure is linear upto 700 µg/dL. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Notes

Chelating agents such as EDTA, oxalate and citrate, present even in traces, prevent the formation of the color complex, hence necessary care should be taken during the assay.

Highly lipemic samples could interfere and should be cleared by centrifugation of filtration before use.

For a seminal fluid assay, centrifuge the sample for 10 min at 3000 RPM. Dilute the supernatant 1 + 99 with normal saline before use.

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	578 nm	Sample volume	:	0.05 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	RT	Standard	:	200 µg/dL
Incubated time	:	5 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	700 µg/dL
No. of read	:	—	Units	:	µg/dL

Normal Values

Serum : 60–120 µg/dL or 9.18–18.4 µmol/L
Urine : 100–1000 µg/24 h

Less than 60 µg/dL is considered as deficiency state.

Clinical Relevance

Toxic Level Symptoms

Cough, chest discomfort, tachycardia, hypertension, gastrointestinal discomfort, nausea, vomiting, diarrhea, metallic taste in the mouth. Treatment includes removal of intake and peritoneal dialysis.

Deficiency Symptoms

May progress from decreased weight, low sperm count, and impaired wound healing to alopecia, hypogonadism, ataxia, tremors, and impaired resistance to infection. Treatment includes dietary replenishment, medication or hyperalimentation.

Values are Increased in

Anemia, arteriosclerosis, coronary heart disease, dietary intake of acidic food or beverages from galvanized containers, industrial exposure to zinc (welding), and primary osteosarcoma of bone. Drugs include cisplatin, corticosteroids, estrogens, interferon, oral contraceptives (containing estrogen), phenytoin, and thiazides.

Values are Decreased in

Acrodermatitis enteropathica, alopecia, alcoholism, anemia (hemolytic), celiac sprue, cirrhosis, diarrhea, gallbladder disease, hepatic metastases, hypoalbuminemia, hypogonadal dwarfism, acute infections, leukemias, lymphomas, malabsorption, myocardial infarction, dietary deficiency, pregnancy (especially third trimester), receiving parenteral nutrition, chronic renal failure, acute stress, thalassemia major, enteric fever, and pulmonary tuberculosis. Drugs include antimetabolites, chlorthalidone, cisplatin, diuretics, estrogens, histidine, and penicillamine.

COPPER

Oxidation state + 1 + 2,
Atomic number 29,
Atomic symbol Cu,
Atomic weight 63.546,
Electron configuration—8-18-1.

Colorimetric Method

(*Courtesy*: Tulip Group of Companies)

For the determination of copper in serum. (Laboratory reagent for professional use only).

Summary

Copper is widely distributed in the various organs of the body. The highest concentration is found in the liver

followed by the brain and kidneys. It plays an important part in the iron metabolism by converting the ferrous ions to a ferric state. Over 90% of the copper in plasma is bound to the protein ceruloplasmin. Increased levels are found in chronic/malignant diseases, e.g. leukemia, cirrhosis, various infections and in patients on oral contraceptives and estrogens. Decreased levels are found in Wilson's disease, decreased synthesis of ceruloplasmin, malabsorption, malnutrition, and nephrotic syndrome.

Principle

Copper, released from ceruloplasmin, in an acidic medium, reacts with Di-Br-PAESA to form a colored complex. Intensity of the complex formed is directly proportional to the amount of copper present in the sample.

$$\text{Copper} + \text{Di-Br-PAESA} \xrightarrow[\text{Medium}]{\text{Acidic}} \text{Colored complex}$$

Normal Reference Values

Serum (males) : 80–140 µg/dL
(females) : 80–155 µg/dL
(newborns) : 12–67 µg/dL
(children upto 10 years) : 30–150 µg/dL

It is recommended that each laboratory establish its own normal range representing.

Contents	25 mL	75 mL
L1: Buffer reagent	12.5 mL	37.5 mL
L2: Color reagent	12.5 mL	37.5 mL
S: Copper standard (200 µg/dL)	2 mL	2 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use. Protect from bright light.

The cold Buffer (L1) when retrieved from 2–8°C may have a particulate suspension. The suspension clears up once the Buffer attains a temperature over 25°C.

Working Reagent

For larger assay series a working reagent may be prepared by mixing equal volumes of L1 (Buffer reagent) and L2 (Color reagent). The Working reagent is stable at 2–8°C for at least 3 weeks. Keep tightly closed.

Sample Material

Serum, free from hemolysis. Copper is reported to be stable in the sample for 6 days when stored at 2–8°C.

Procedure

Wavelength/filter : 580 nm (Hg 578 nm)/yellow
Temperature : RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Buffer reagent (L1)	0.5	0.5	0.5
Color reagent (L2)	0.5	0.5	0.5
Distilled water	0.05		
Copper standard (s)	-	0.05	
Sample	-	-	0.05

Mix well and incubate at RT (25°C) for 10 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 30 minutes.

Calculations

$$\text{Copper in µg/dL} = \frac{\text{Abs T}}{\text{Abs S}} \times 200$$

Linearity

This procedure is linear upto 500 µg/dL. If the value exceeds this limit, dilute the serum with normal saline (NaCL 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Notes

Chelating agents such as EDTA, oxalate and citrate, present even in traces, prevent the formation of the color complex, hence necessary care should be taken during the assay.

Highly lipemic samples could interfere and should be cleared by centrifugation or filtration before use.

The assay can be run at 600 nm, however the absorbances would be approx. 30% lower as compared to 570 nm.

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	578 nm	Sample volume	:	0.05 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	RT	Standard	:	200 µg/dL
Incubated time	:	10 minutes	Factor	:	

Contd...

Contd...

Delay time	: —	React slope	: Increasing
Read time	: —	Linearity	: 500 µg/dL
No. of read	: —	Units	: µg/dL

Clinical Relevance

Toxic Level Symptoms

Jaundice, hepatic injury, headache, vomiting, and may lead to hemolytic shock.

Deficiency Symptoms

Impaired erythrocyte production and survival time and lowered catabolism by copper-containing enzymes.

Values are Increased in

Alzheimer's disease, anemia (aplastic, pernicious, megaloblastic anemia of pregnancy; iron deficiency), cirrohosis (biliary), elevated CRP, glomerulonephritis, hemochromatosis, Hodgkin's disease, hyperestrogenemia, hypothyroidism, hyperthyroidism, infections, leukemia, lymphoma, Lofgren syndrome, myocardial infarction, pellagra, pregnancy (especially third trimester), rheumatic fever, rheumatoid arthritis, sarcoidosis, and systemic lupus erythematosus. Drugs include carbamazepine, estrogens and oral contraceptives, henobarbital, and phenytoin sodium.

Copper Urine

Normal Values

All ages	0–60 µg/24 h
Wilson's disease	> 100 µg/24 h

Values are Increased in

Alzheimer's disease, aminoaciduria, cirrhosis (biliary, Indian childhood), hepatitis (chronic active), hyperceruloplasminemia, nephritic syndrome, pellagra, proteinuria, and Wilson's disease.

Values are Decreased in

Burns, hypoproteinemia, Kwashiorkor, malabsorption, Menkes' Hair syndrome, nephrosis, and Wilson's disease.

MAGNESIUM

Oxidation + 2,
Atomic number 12,
Atomic symbol Mg,
Atomic weight 24.305,
Electron configuration—2-8-2.

Calmagite Method

(*Courtesy*: Tulip Group of Companies)
For the determination of magnesium in serum, urine and CSF (Laboratory reagent for professional use only).

Summary

Magnesium, along with potassium, is a major intracellular cation. It is an activator of various enzymes. It is also involved in amino acid activation and protein synthesis. Increased levels are found in dehydration, Addison's disease and uremia. Decreased levels are found in malabsorption, during treatment of diabetic coma, chronic renal disease, chronic alcoholism, pancreatitis and hyperthyroidism.

Principle

Magnesium combines with Calmagite in an alkaline medium to form a red colored complex. Interference of calcium and proteins is eliminated by the addition of specific chelating agents and detergents. Intensity of the color formed is directly proportional to the amount of magnesium present in the sample.

$$\text{Magnesium + Calmagite} \xrightarrow[\text{Medium}]{\text{Alkaline}} \text{Red colored complex}$$

Normal Reference Values

Serum (Children)	: 1.5–2.0 mEq/L
(Adults)	: 1.3–2.5 mEq/L
CSF	: 2.0–3.0 mEq/L
Urine	: 6.0–8.5 mEq/24 h

It is recommended that each laboratory establish its own normal range representing its patient population.

Note : 2 mEq/L = 1 mmol/L = 2.44 mg/dL

Contents	*25 mL*	*75 mL*
L1: Buffer reagent	12.5 mL	37.5 mL
L2: Color reagent	12.5 mL	37.5 mL
S: Magnesium standard (2.0 mEq/L)	2 mL	2 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use. Protect from bright light.

Working reagent: For larger assay series a working reagent may be prepared by mixing equal volumes of L1 (Buffer reagent) and L2 (Color reagent). The working reagent is stable at 2–8°C for at least one month. Keep tightly closed.

Sample Material

Serum (Free from hemolysis), urine and CSF.

24 hour collected urine should be acidified to a pH of 2–3 by the addition of approx. 10 to 15 mL of HCI and diluted 1 + 3 with deionized. Water before use. Multiply results by 4. Magnesium is reported to be stable in serum/plasma for 7 days at 2–8°C.

Procedure

Wavelength/filter : 510 nm (Hg 546 nm)/green
Temperature : RT
Light path : 1 cm

Pipette into clean dry test tubes labeled as blank (B), standard (S), and test (T):

Addition Sequence	*B (mL)*	*S (mL)*	*T (mL)*
Buffer reagent (L1)	0.5	0.5	0.5
Color reagent (L2)	0.5	0.5	0.5
Distilled water	0.01		
Magnesium standard (s)	-	0.01	
Sample	-	-	0.01

Mix well and incubate at RT (25°C) for 5 minutes. Measure the absorbance of the standard (Abs S), and test sample (Abs T) against the blank, within 30 minutes.

Calculations

$$\text{Magnesium in mEq/L} = \frac{\text{Abs T}}{\text{Abs S}} \times 2$$

Linearity

This procedure is linear upto 10 mEq/L. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using an appropriate dilution factor.

Notes

All glassware being used for the test should first be rinsed with 1% or 0.1 N HCI and then with good quality deionized water before use.

Chelating agents such as EDTA, oxalate and citrate, present even in traces, prevent the formation of the color complex, hence necessary care should be taken during the assay.

RBCs have double the magnesium content compared to serum, and hence hemolyzed samples should not be used.

System Parameters

Reaction	:	End point	Interval	:	
Wavelength	:	510 nm	Sample volume	:	0.01 mL
Zero setting	:	Reagent blank	Reagent volume	:	1.00 mL
Incubation temperature	:	RT	Standard	:	2.0 mEq/L
Incubated time	:	5 min	Factor	:	
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linearity	:	10 mEq/L
No. of read	:	—	Units	:	mEq/L

Normal Values

Serum (children)	1.5–2.0 mEq/L
(adults)	1.3–2.5 mEq/L
CSF	2.0–3.0 mEq/L
Urine	6.0–8.5 mE1/24 h
Panic level	> 3.0 mEq/L or < 0.5 mEq/L (serum)
Toxic level (serum)	> 12 mEq/L

Clinical Relevance

Toxic Level Symptoms

Lethargy, drowsiness, flushing, nausea, vomiting, slurred speech, hypotension, weak or absent deep tendon reflexes, ECG changes (prolonged PR and QT intervals, widened QRS, bradycardia), respiratory depression (Treatment includes stop magnesium intake, promote excretion, give calcium salts, hemodialysis).

Deficiency Symptoms

Muscle tremors, twitching, tetany, hypocalcemia, hyperactive deep tendon reflexes, ECG changes (prolonged PR and QT intervals, broad flat T-waves, premature ventricular contractions, ventricular tachycardia, fibrillation), anorexia, nausea, vomiting, lethargy, insomnia.

Values are Increased in

Addison's disease, adrenalectomy, ataxia, dehydration (severe), diabetes (uncontrolled diabetes, diabetic acidosis before treatment, controlled diabetes in older patients), dysarrhythmias, hypercalcemia, hypothyroidism, hypophosphatemia, renal lithiasis, leukemias (lymphocytic and myelocytic), renal insufficiency and failure. Drugs

include antacids containing magnesium, calcium containing medications, cathartics, ethacrynic acid, laxatives (epsom salt and magnesium citrate), loop diuretics, and thyroid medications.

Values are Decreased in

Acute tubular necrosis (diuretic phase), alcholism (chronic), Bartter's syndrome, bowel resection complications, convulsions, diabetic ketoacidosis, diarrhea (chronic), dysarrythmias, excessive lactation, excessive sweating, hepatitis, hepatic cirrhosis, hepatic insufficiency, hungry bone syndrome, hypokalemia, hypercalcemia, hyperthyroidism, hypoparathyroidism, hypocalcemia, IV solutions without magnesium, ketoacidosis, Kwashiorkor (severe malnutrition), laxative abuse, magnesium deficiency tetany syndrome, pancreatitis (acute and chronic), phosphate depletion, postobstructive diuresis, postoperartively, primary hyperaldosteronism, prolonged gastric drainage, reduced magnesium intake, reduced magnesium absorption (specific magnesium malabsorption, generalized malabsorption syndrome, excessive bowel resection, diffuse bowel disease or injury), renal tubular acidosis, stress states with catecholamine excess, tetany, toxemia of pregnancy, ulcerative colitis, volume expansion (extracellular fluid). Drugs include alcohol, amphotericin B, some antibiotics (neomycin, aminoglycosides), calcium gluconate, corticosteroids, cyclosporine, diuretics (e.g. mercurial, ethacrynic acid), glucose, insulin, mannitol, and urea.

Magnesium in Urine

Values are Increased in

Alcoholism, Bartter's syndrome, hypermagnesemia, and nephrolithiasis. Drugs include aldosterone, cisplastin, corticosteroids, diuretics (ethacrynic acid), and thiazide.

Values are Decreased in

Renal disease, magnesium deficit, osteoporosis, and syndrome of inappropriate antidiuretic hormone secretion (SIADHS).

AUTOMATION IN CLINICAL CHEMISTRY

Instrumentation

Till very recently the only machine one could find in a pathologist's biochemistry laboratory was the centrifuge. That added by a simple colorimeter, a microscope, some chemicals and test tubes were typical exhibits in a laboratory. This picture is changing very rapidly. New instruments are entering the laboratory. With the advancements made in the field of computer sciences more and more automation is entrusted to instrumentation.

Automation, in general is expected to bring in convenience, speed and reliability without compromising accuracy. Let us see how these aspects are served by clinical chemistry analyzers for analyzing blood samples. Analyzers system consists of two parts: (i) chemical reagents which are mixed with blood samples whereby the particular substance to be estimated is reacted with the specific reagent and (ii) optoelectronic analyzer to process/monitor/estimate the above reaction and thereby establish the exact amount of the substance.

Present Status

For convenience, there are three broad categories of automatic analyzers presently available:

1. Random access auto analyzer
2. Batch analyzer
3. Semiauto analyzer.

Category (1) is very popular in most of the advanced countries. In India, such machines are found only in very large hospitals and in big towns, their price is approximately ₹ 15 lacs and more. Batch analyzers are available at a range of ₹ 4 to 9 lacs, whereas semiauto analyzers are in the range of around ₹ 2.5 lacs. There are a large number of semiautomatic analyzer installations in India and newer models are coming in the market regularly.

Semiautomatic analyzers offer a big leap in a laboratory's capability. It forms a major breakthrough, since it offers enhancement of speed and convenience. Let us first see what should normally be the expectations of a laboratory from an analyzer and how would a semi-automatic analyzer answer them.

Benefits of a Semi-automatic Analyzer

Economy

As compared with a colorimeter the analyzer is a considerably more expensive instrument. The first and foremost expectation from it therefore, is that it should justify the higher investment.

Semi-automatic analyzers work on in general 1.0 mL of reagent volume per test. There are some which work on as low a volume as 0.5 mL. The colorimeter requires 5.0 mL of reagent though some are now available for use with 2.5 mL or 3 mL. The main pay back comes from the savings on recurring costs of reagents.

Other benefits such as savings on time costs (employing less number of persons) glassware work space, etc. are

also available, but the amount of savings on reagents costs form the major part of economy of operation.

Wide Spectrum of Tests

All tests based on the principle of colorimetry can be performed on analyzers. The analyzers have high quality filters or a spectrophotometer for selection of wavelengths. More important, kinetic and end point mode chemistries can be performed on analyzers. An ultraviolet wavelength of 340 nm is available and the combination 340 nm and kinetic and end point mode opens out a very wide range of chemistries. A particular chemistry may possibly be done at a different wavelength on a colorimeter, but analyzer will make available a quicker and accurate method of latest technology.

Entire range of organ functions like cardiac, hepatic and renal profiles can be performed by analyzers.

Speed

A typical workload in a laboratory is expected to be finished in a couple of hours. Samples collected during the morning hours are analyzed in the afternoon. Before the evening, results are compiled and patient reports prepared to be delivered in the evening. A medium size laboratory can meet its work load through a semiautomatic analyzer (about 100 tests per day). As the number of tests per day increases, a batch analyzer or large random access analyzer is called for.

Accuracy and Reliability

The chances of manual errors are reduced in analyzers because of automatic settings of wavelength, temperature, factors and other variable parameters which have to be set-up on machines. Pipetting errors are also eliminated by use of automatic micropipettes. The analyzers are computer-based instruments and have self-monitoring features. They monitor the progress of chemical reactions and indicate any abnormality. Manual steps at various stages are reduced and the results obtained are accurate and reliable.

Convenience

The convenience achieved through the use of the analyzer is manifold. Three main features are: (i) elimination of manual calculations, (ii) digital display and print out of results, (iii) emergency requests can be handled with speed and accuracy. For example, a colorimeter based test for SGOT/SGPT would take 1½ to 2 hours whereas on an analyzer the results are available within minutes.

Non-dependence on Technicians

An analyzer is easy to operate as most of the steps are automatic. A technician requires only a few days to learn operations of the machine. Besides, in absence of the technician it is not very difficult for the senior person/doctor to handle the workload in case of a small/medium laboratory.

Business Growth

With an automatic analyzer installed in the laboratory, physicians as well as patients develop a great amount of confidence in the results. This added by wider range of tests make the business of the laboratory grow fast.

Which Lab Needs Automation?

Given above are some of the expectations that can be fulfilled by an analyzer. The importance given to a particular factor would depend on the choice of an individual lab. Even if the analyzer is purchased primarily for any one of the above mentioned reasons, all of the remaining purposes are served.

Earlier, the main criterion used to be speed, keeping in mind the requirement of finishing the lab workload in 2 to 3 hours. The ideas have changed. There are laboratories which have purchased it for handling the 'night' (emergency) workload—by keeping the laboratories open all 24 hours. There are laboratories which have started with the purchase of a semi-automatic analyzer and established themselves in the group of reputable laboratories in the towns in a span of one year. There are quality conscious buyers wanting to give adequate confidence to their referring physicians and patients. There are nursing homes who have started laboratories with an analyzer not limiting the facilities only to their in-house and OPD patients but making them available to any patient.

In short, presently there is no predictable trend of particular laboratories buying analyzers. It is quite understandable looking to the varied purposes an analyzer can serve. Any laboratory is fully justified in its purchase of an analyzer unit.

Selection of A Model

While selecting a particular model the primary objective of the laboratory for purchase has to be well-defined. It could be anyone or a combination of the above mentioned points. A particular model may have an advantage in one area (say economy), whereas another will be a better choice for some other features (say, an elaborate display or print out). Quicker pay back of the analyzer's investment is the next point that one must consider. This should be looked at from different angles. One model may be available at a price, say, ₹ 20,000 less but if its recurrent costs on reagents, etc. are comparatively more that would not turn out to be the best alternative. The cost analysis should be done for a period of 5 years minimum.

After-sales service forms an important part for all instrumentation systems. The company selling it should be of repute and should have a network of field service engineers. Failure rate is normally more in a country with tropical weather conditions. As far as possible the system should be proven to be working reliably in various places and be tropicalized. A modular system can be of advantage.

The selling company should also have a good reagent support. It is better to have the total system from one company. This makes sure that the company personnel also understand the exact application of the unit rather than only the engineering aspects.

Analyzer Classification

Broad based classification of analyzers can fall under the following three categories.

Semi-autoanalyzers

Serum sample-reagent mixture is done manually outside the unit and fed manually. The unit performs/monitors/evaluates the chemical reaction and gives the result on display and/or printer. The setting of the unit is normally automatic as regards wavelength, temperature, etc.

Batch Analyzers

Serum sample-reagent mixing is normally automatic and the mix is fed automatically to the analyzer. The analyzer performs the same function as a semiauto analyzer unit. Here a batch of a particular chemistry test (say, glucose, urea) can be set up and performed automatically. One reagent is stored on the machine at a time.

Random Access Autoanalyzer

On the machine more than one reagent is stored. Patient samples are kept on the machine. The computer is programed to carry out selected tests on individual samples. Any particular sample and reagent can be selected, mixed, incubated and monitored as per the program. The patient report is fully compiled by the unit and printed out.

Clin Check Plus

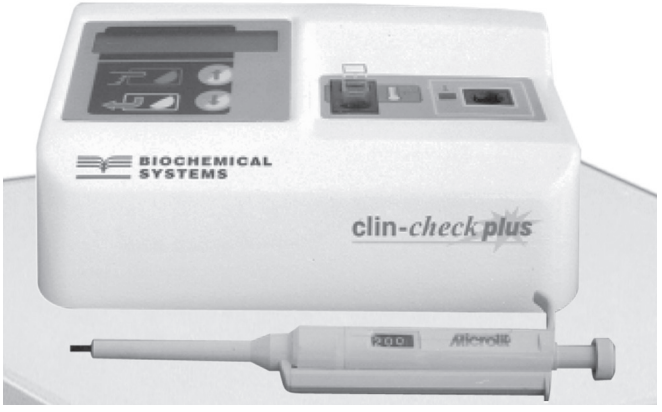
(*Courtesy*: Tulip Group of Companies)

The clin check plus is a clinical chemistry/biochemistry analyzer, designed for a precise and fast execution of almost all-clinical chemistry analysis. Automatic selection of filters and temperature controller is all managed by the microprocessor. The CCP is a fully open machine with 40 locations. Following arrays can be performed on the machine:

EP/KIN/FXT/MSD and ABS mode

Excellent features; with only 4 operating keys; very simple to handle; reads OD up to 2.0 A; In-built incubation (2 positions); RS 232 port makes compatible with PC Communication; which can also handle data from machine to PC. It is a lightweight and sturdy machine with less breakdown and low maintenance cost.

The machine is ideally suited for growing or nascent laboratories.



CCP picture

Features

- User friendly, fully open system with 40 programable locations
- Only 4 operating keys- very simple to handle
- Reads OD up to 2.0 A large working range
- Two inbuilt incubator positions
- RS 232 software support for external serial port printer
- Data handling software available
- Lightweight, only 3.8 kg.

Technical Features CCP

Photometric Systems:

Light source	2 W long-life tungsten incandescent lamp
Spectral field	340 to 700 nm
Filter change	automatic
Filters	340, 405, 505, 546, 630 nm; 8 nm pass-band
Detector	solid state device

Thermostating

Heating element	semiconductor
Temperature	37°C
Temperature accuracy	± 0.2 ° C
Stabilisation period	10–20 minutes
Thermostatic unit	Two places for square or cylindrical cuvettes

Measuring System

Reset	Automatic
Measuring range	0 to + 2000 OD
Photometric linearity	± 2% from 0 to 1700 OD
Photometric accuracy	± 2% from 0 to 1700 OD
Precision	± 2 digit
Drift	Lower than 0.005 OD per hour
Reagent volume	1 mL minimum.
Measure method	End point, kinetics, fixed-time, differential

Data Display and Programing:

Keyboard	With 4 function keys
Display	32 characters, liquid crystals, Alpha-numeric
Serial Output	Rs. 232 standard
Power Supply	220 V, 50 Hz., 117V, 60Hz., 40 VA
Size	cm. 27.5 × 21 × 9
Weight	3.8 kg.

Screenmaster 3000

(*Courtesy*: Tulip Group of Companies)

A typical clinical chemistry semi-autoanalyzer with unmatched features designed for Indian laboratories. Quality at par and performance as per international standards which can perform. EP/KIN/FXT/MSD with ABS mode.

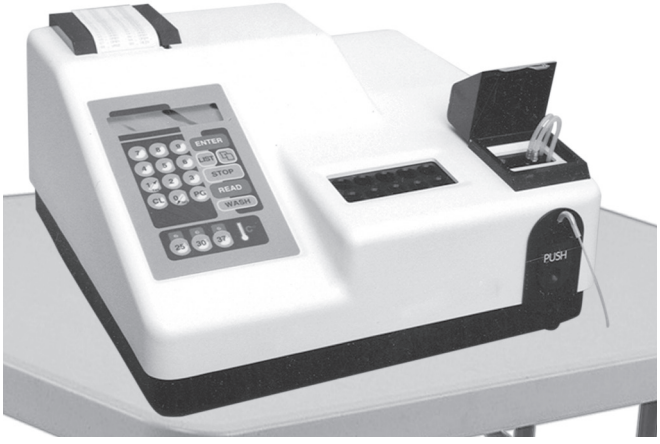
The machine uses both flow cell (18 µL) and cuvettes (semi-micro cuvettes) also ensuring less carry over effect and less breakdown period of the machine. It covers the entire range of spectrum for measurements (340–700 nm). Automatic selection of filters and is managed by microprocessors with in-built 10 position incubator which is regulated by Peltier heat pumps.

It is very simple to handle and is user friendly as its operation and programing of machine is done by keyboard and set of instruction displayed on the display. All results are interpreted by units and also graphs are available for KIN/MSD. Also has dynamic display (real time display of OD) for KIN and also reagent blank OD (save)-ensures minimum consumption of reagents. Any errors or malfunction in the machine is denoted on the display or printout.

The machine is capable of storing 500 results in memory and is able to retrieve also for later use. Also has inbuilt 20 column thermal printer (optional). A built in QC program which validates the overall performance of system. Gives mean, SD, CV and Levey-Jennings chart. Computer compatibility with RS 232 port, which also supports data handling software.

Features

- User friendly, fully open system with 90 programable locations
- 18 µL flowcell minimizes reagent consumption and avoids carry over
- Reads OD up to 2.5 A-large working range
- Built in 24 column graphic thermal printer
- 10 position incubator at 25°, 30°, 37°C
- Incubated flowcell resting chamber
- Stores 500 test results in memory
- Built in software for QC program with mean, SD, CV and Levey-Jennings chart.



Screenmaster 3000

Technical Features of Screenmaster 3000

Operating modes	Absorbance, end point, fixed-time, kinetic and multi-standard and tests
For flow cell	
Flowcell volume	18 µL
Typical working volume	500 µL
Minimum working volume	350 µL
Aspiration	Peristaltic pump with programable intake volume
For cuvette	
Wavelength range	340-700 nm
Filters	340-405-505-546-578-630 nm
Reagents volumes in cuvette	1 mL (min) for macro cuvette, 0.3 for micro cuvette
Measuring system	
Reset	Automatic
Measuring range	from – 200 to + 2500 OD units

Photometric accuracy	± 2% from 0 to 2000 OD units
Photometric linearity	± 2% from 0 to 2000 OD units
Reproducibility	± 1% digit

General

Incubator	10 positions at 25/30/37°C + 0.2°C
Printer	built-in graphic printer with 20 columns
Power supply	220 V, 50 Hz or 110 V, 60 Hz
Dimensions	40 × 40 ×17 cm
Weight	13.0 K

Maplab Plus

(*Courtesy*: Tulip Group of Companies)
Unique and the only semi-autoanalyzer with True Elisa reader available in Indian market.

The machine is capable of performing routine biochemistry test as well as ELISA tests in a single machine, which can perform the following operations: EP/KIN/FXT/MSD/latex/QLT and ELISA.

Uses both cuvettes and flowcell for measurement ensures low carry over effect and less breakdown period of the machine.

Machine has 2 separate set of optical system (Lamps as well as Filters): 6 (biochemistry) and 4 (ELISA).

Covers entire range of spectrum for biochemistry (340–700 nm), and also for ELISA: 405, 450, 492, 630.

Selection of filters is automatically managed by microprocessors. This minimizes errors in the selection of filters. Separate lamp source is used 20W and 2W (biochemistry and ELISA). Also has 10 positions for in-built incubator fixed at 37°C with the help of semiconductor (less power consumption).

In-built thermal printer with graphical printouts and results made interpretation much easier. The analytical results are directly displayed or printed with preset units as programed.

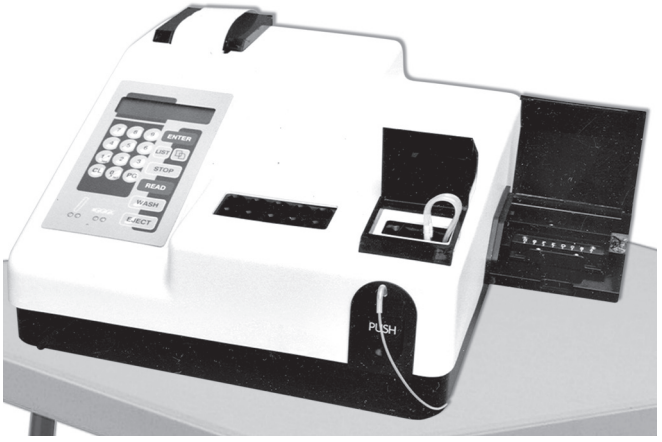
Dynamic display (real time display of OD) in KIN gives extra check for chemistry being performed.

Especially 40 locations are available for MSD mode, which cover entire ElA con.; EIA, QLT, latex, etc. can take 7 standards to plot a curve. With the help of special algorithm the machine can recalibrate the graph with the help of 2 stands. Partial calibration.

PC compatible with RS 232 ports; helps to communicate with machines, data and PC. Also supports data handling software and also support data management software provided with machine for ELISA section only.

Machine has built in QC program which validates the overall performance of system. Gives mean, SD, CV and Levey-Jennings chart.

Ideally suitable for any lab with workload of biochemistry plus ELISA (MSD).



Maplab plus

Features

- User friendly, fully open system with 90 programable locations
- Separate optical systems for clinical chemistry and ELISA
- 18 µL flowcell minimizes reagent consumption and avoids carry over
- Reads OD up to 3.2 A-large working range
- Dynamic display for kinetics
- Built in 24 column graphic thermal printer
- 10 position Incubator
- Incubated flowcell resting chamber
- Built in QC with mean, SD, CV and Levey-Jennings chart
- RS 232 software support with DMS and DHS
- Service support by instruments division, Tulip Diagnostics Pvt Ltd.

Technical Features of Maplab Plus

Operating modes	Absorbance, end point, fixed-time, kinetic, multistandard and qualitative tests

For flow cell

Flowcell volume	18 µL
Typical working volume	500 µL
Minimum working volume	350 µL
Aspiration	Peristaltic pump with programable intake volume

For cuvette	
Wavelength range	340–700 nm
Filters	340–405–505–546–578–630 nm
Reagents volumes in cuvette	1 mL (min) for macrocuvette, 0.3 for microcuvette
For microwell strip	
Wavelength range	405-700 nm
Filters	405-450-492-630 nm
Microwell strips type	All kind of 8 micro well strips
Measuring system	
Reset	Automatic
Measuring range	from –200 to + 3200 OD units
Photometric accuracy	± 2% from 0 to 2500 OD units
Photometric linearity	± 2% from 0 to 2500 OD units
Reproducibility	± 1% digit
General	
Incubator	10 positions at 37 ± 0.2°C
Printer	built-in graphic printer with 20 columns
Power supply	220 V, 50 Hz or 110 V, 60 Hz
Dimensions	40 × 40 × 17 cm
Weight	13.5 kg

Fully

(*Courtesy*: Tulip Group of Companies)

Innovative technology for user friendly meeting all requirement simplicity, reliability, accuracy and competitive. Today's laboratory environment demands an expert system with an economic advantage. The revolutionary design makes Fully a compact and complete clinical chemistry analyzer. Fully machine is uniquely designed to answer the real needs of laboratories low acquisition and operating costs, simple and flexible use minimum maintenance.

System Overview

- The special design of the reagent bottle has been developed in order to avoid reagent loss
- 20 position reagent tray to maximize the number of tests per sample at a time
- Flexibility to run both single and two reagent chemistries
- Open system that enables the programing of an unlimited number of techniques.

Modern

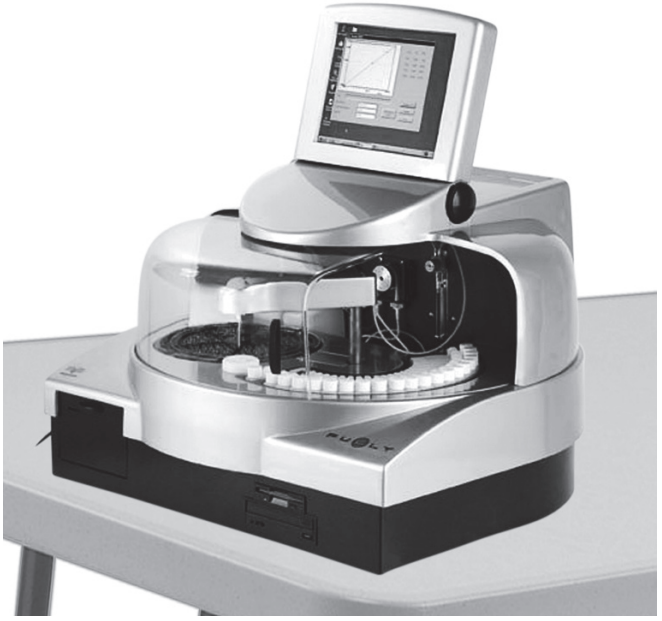
- Common shape of reaction wells and cups is easily available in local market
- Programable washing cycle between samples and tests for minimizing carry over
- Fully walk away system capable of running 54 samples at a time
- Auto rerun with pre dilution.

Compact

- Proven and high technique will assure accurate measuring system and easy maintenance
- 18 µL flow cell volume for economical running cost
- Peltier controlled flow cell temperature
- Washing and waste tanks level sensors
- Auto level sensing
- Built in high performance PC.

Software

- Discover the simplicity of operations with windows environment
- Very user friendly, enables skilled laboratory staff to run fully within short time
- Easy selection of menus by icons
- Reagent positions are automatically determined by the workplan with volumes required once the sample and reagent have been programed
- Nine profiles of up to unlimited chemistries each to simplify your task of profile management
- Continuous working status monitoring
- QC program with Levey-Jennings plot
- Intelligent error management
- Multilanguage support
- Network integration facilities
- Built in thermal printer and additional output for external connection.



Fully

Technical Specifications of Fully Measuring System

Operating modes	Absorbance, end-point, fixed time, kinetic, multistandard
Throughput	Up to 120 tests/h
Light source	Halogen lamp 12 V 20 W
Spectral range	320–690 nm
Filter wavelength	340, 405, 492, 505, 546, 578, 630 nm, one free position
Measuring range	from –0.200 to 2.500 OD
Flow cell	18 µL
Incubation temperature	37°C ± 0.2°C
Minimum reading volume	350 mL
Typical reading volume	500 mL
Sample Handling	
Sample number	54 positions including samples, calibrators and controls/tray
Sample container	Cup (1.2 mL)
Sample volume	2-200 µL (l µL steps)
Automatic dilution	Pre and postdilution ration: 1:2–1:20
Reagent Handling	
Reagent tray	20 bottles + 1 bottle for diluent
Reagent bottle	45 mL
Reagent volume	Reagent 1 volume range: 30 to 1000 µL (1 µL steps) Reagent 2 volume range: 0 to 1000 µL (1 µL steps)
Liquid detection	Sensor
Reagent warming	Pre-heated ARM
Reagent identification	Position ID
Tray exchange	Possible with reagent bottles
Reaction	
Number of wells	144 wells (12 wells × 12 strips)
Well volume	1 mL
Well temperature	37 ± 2°C
Diluter	
Syringe	1000 µL, 1 µL steps
Accuracy	± 1% at 5 µL
Computer	
Computer	Built-in
Computer description	Intel® Processor, 20 GB HD, 128 MB RAM
Operating system	Microsoft Windows® display TFT 12", 800 × 600
Network adapter	Built-in
Printer	Built-in thermal printer, 120 mm
Output	RS 232, LPT
Drive	CD ROM, Floppy disk 3.5" 1.44 M bytes
General	
Power Supply	AC 115–230 V 50–60 Hz full range
Dimensions	720 (W) × 680 (D) × 750 (H) open or 530 (H) close mm
Weight	55 kg

PRINCIPLES OF QUALITY ASSURANCE AND STANDARDS FOR CLINICAL CHEMISTRY

Preanalytical Factors Important in Clinical Chemistry

A. Specimen Collection, Handling, and Transport to the Laboratory

Samples should be appropriately collected, handled and transported to the laboratory in a timely manner, dependent on the type of specimen and its stability. For any assay performed in the laboratory, information concerning sample requirements, proper collection, handling, and delivery or shipping procedures should be available to clients in a laboratory services manual, special information sheets, journal or newsletter articles, other written material, or by personal or telephone conversation.

B. Specimen Identification

Specimens should be identified with pertinent information as determined by the laboratory, name of clinic or doctor, address, telephone and fax numbers, e-mail address, location from which the specimen was collected, etc.) on the submission container and submission form.

C. Test Identification

The requested test(s) should be clearly stated on the submission form.

D. Specimen Accessioning

The specimen should be correctly entered into the laboratory system. Test request entry, delivery of the specimen to the correct location, and specimen aliquoting (if necessary) or sharing between laboratories or departments (i.e. pharmacology, endocrinology, and clinical chemistry) should be coordinated.

E. Client Communication and Education

Communication between laboratory personnel and clients should be timely and courteous regarding preanalytical factors influencing laboratory test results (e.g. incomplete submission forms, inappropriate sample or sample handling

or poor sample quality). Clients should be informed of the expected time for receipt of preliminary and final reports.

F. Personnel Safety

Personal protective equipment should be appropriate for handling specimens and equipment used for clinical chemistry. Safety procedures and disposal of all samples and supplies should be appropriate for the type of specimen. Personnel should receive safety and biohazard training and information about exposure to potentially hazardous chemicals or infectious agents. All training should be documented.

G. Laboratory Environment

The laboratory space should be clean, well lit, and organized to ensure proper achievement of the above goals.

H. Personnel Requirements

Laboratory personnel should have training in specimen handling and sample preparation. Documentation of training, continuing education and periodic proficiency assessment should be at the discretion of the laboratory director.

Analytical Factors Important in Clinical Chemistry

A. Monitoring

1. Internal monitoring should include the following

a. Quality of water (as specified by instrumentation and essays)
b. Stability of electrical power (as specified by instrumentation)
c. Temperatures of water bath, refrigerator, and freezer (recommended at least monthly)
d. Regular calibration of analytical balances and pipettes (recommended annually)
e. Maintenance of up-to-date procedure manuals with clearly stated dates when procedures are first implemented and when any changes are made and implemented
f. Maintenance of adequate inventory, with proper storage and handling
g. Maintenance of a log of changes in any procedures, problems or other factors affecting methods, as well as actions that resolved the problem. All entries should be clearly dated and signed by laboratory personnel.

2. External monitoring should include participation in an external proficiency program

a. All participating laboratories should analyze the same materials
b. Results should be tabulated regularly (monthly, quarterly) and distributed to participants with statistical summaries and comparison of participating laboratories with mean indices expressing the closeness of individual laboratory results to the group mean
c. Means should be calculated and analyzed based on identification of the method (same methods compared)
d. Each laboratory should carefully assess the validity of their reported performance and consider any changes indicated by the proficiency program

B. Method Validation

Method validation should be performed before a test procedure is placed into routine use. Validation may be accomplished by thoroughly testing reference materials or by comparison of results of tests performed by an alternative method. For each method, the laboratory should verify the manufacturer's claims and any adjustments before initiating patient testing.

Method validation should provide evidence of the following:

1. *Accuracy—Perform either (a) or (b)*
   a. Run known value substance and compare results to expected value
   b. Perform split sample patient comparison between existing method of known accuracy and new method.

2. *Precision—Perform either (a) or (b)*
   a. Run 10 replicates of 2 levels of quality control (QC) samples
   b. Gather 21 results; 7 results in each of 3 separate runs (better estimate of day-to-day precision, as well as without-run precision).

With results from (a) or (b) determine mean, standard deviation (SD) and coefficient of variation (CV). Determine whether within-run SD is acceptable.

3. *Sensitivity—Perform (a), (b) or (c)*
   a. Assess manufacturer's claims
   b. Use concentration of low calibrator or another sample or fluid with low levels of analyte
   c. Run a series of dilutions and assess acceptability of performance.

4. *Specificity—Perform (a) or (b)*
   a. Use published list of interfering substances, check with manufacturer
   b. Assess known or suspected interfering substances by spiking specimens or use patient material with known conditions.

5. *Linear reportable range*
   a. Establish upper and lower limits for reporting patient values based on calibration materials
   b. For the lower limit, there should be confirmation of the discriminatory ability of the test
   c. The highest calibration point is the maximum upper limit and the lowest calibration point or zero should be the minimum lower limit for reporting patient results.
6. *Linearity—Perform either (a) or (b)*
   a. Determine by analyzing multiple dilutions of either a high calibrator, control or patient samples with increased levels of analyte
   b. Analyze calibrators of variable, known concentrations
   c. Linearity should be established at the time of validation and whenever new or altered reagents are used.
7. *Reference intervals*
   a. The laboratory should establish or validate existing reference intervals for each method and species before reporting results
   b. Parallel tests should be run to confirm reference intervals for controls when changing reagents or QC lot number.

C. Instrumentation

1. *Instrument performance*
   The equipment and instrument used must be capable of providing test results within the laboratory's stated performance characteristics. These include:
   a. Detection limits
   b. Precision
   c. Accuracy
   d. Specificity
   e. Sensitivity
   f. Freedom from interferences and related test variables (refer to previous section on method validation)
   g. Additional points to consider:
   Instruments with adjustable setting for different substances and/or species should be carefully checked for compliance
   Compare and make adjustments for performance characteristics as defined by the laboratory and the manufacturer.
   Make certain species differences are accommodated.
2. *Function checks*
   a. Appropriate function checks should be made on all instruments. These are critical operating characteristics of an instrument, i.e. stray light, zeroing, electrical levels, optical alignment, background checks, etc.
   b. Laboratory personnel should recheck and/or calibrate each instrument daily or once per shift, prior to patient testing, to ensure that it is functioning correctly and is properly calibrated. This includes daily QC.
3. *Calibration*
   a. Instruments should be calibrated every 6 months or more frequently if indicated by:
   Manufacturer's recommendation
   After major service
   QC outside limits or troubleshooting indicates need
   Laboratory determination that volume, equipment performance or reagent stability indicate a need for more frequent calibration
   b. After calibration, controls should be run.
4. *Laboratory personnel knowledge of equipment and its use, including, but not limited to:*
   a. Linearity differences from possible manufacturer's range (human) to animal
   b. Effects to hemolysis, lipemia, icterus, carotenoid pigments (especially large animals), and different anticoagulants on each assay
   c. Reportable ranges
   d. Species-specific ranges and reference intervals
   e. Expected abnormal ranges
   f. Common problems encountered with veterinary samples
   g. Regular instrument maintenance schedule
   h. Replacement of inadequate or faulty equipment
   i. Problem solving procedures, troubleshooting.

D. Quality Control

1. For each run, at least 2 controls should be assayed. Use of "high" and "low" abnormal controls is recommended.
2. Maximum length of a run is 24 hours. If the instrument manufacturer requires more frequent controls, observe the recommended frequency (i.e. some blood gas instruments).
3. Verify that the instrument is stable over the "run time". During a validation check, controls are assayed more frequently to establish run time.
4. Establish QC frequency; consider the following:
   a. Test volume (number performed each run on day) and frequency
   b. Technique dependence of the method
   c. Analyte or reagent stability
   d. Frequency of QC failures

e. Training and experience of personnel
f. Cost of QC (increasing frequency adds to cost-per-test).

5. *Quality control parameters*
   a. Mean, SD and CV should be calculated (minimum n = 20)
   b. Controls should be assayed in the same manner as patient specimens
   c. A mechanism should be in place to determine whether testing personnel follow policies and procedures correctly
   d. Use of Westgard multirule procedures or other rules based on QC validation is recommended
   e. Policies and procedures should be written and available in a laboratory Standard Operating Procedures (SOP) manual to ensure accurate and reliable test results
   f. An SOP manual should have clearly marked and dated entries of current procedures (manufacturer package inserts are sufficient as long as verified) and when any changes are made and implemented
   g. QC records should be reviewed frequently to ensure that when QC values fail to meet the criteria for acceptability, suitable action is taken
   h. Control products should be purchased commercially, if possible. If using calibrators as controls, use a different lot as QC material. If patient pooled samples are used, establish the mean value of all analytes (minimum n = 10 to establish a mean)
   i. Monitor results of clinical specimens for various sources of error by use of parameters such as anion gap, comparison of test results with previous submissions from same patient (delta checks), and investigation of markedly abnormal results (limit checks).

E. Procedures Manual

1. All procedures currently in use should be included. Protocols may be organized in manuals and/or stored in computers, and be written form. They should contain such information as:
   a. Patient preparation
   b. Specimen collection, processing and handling
   c. Criteria for rejection of specimens
   d. Limitations of and things that interfere with the method in use
   e. Step-by-step procedures
   f. Reagent preparation or manufacturer
   g. Reference interval
   h. Reportable range
   i. Literature references
   j Reagent labeling: content, storage requirements, expiration
   k. Laboratory-specific information, such as:
   Identification of instrument used
   Result reporting method
   Actions to take when system is down
   Criteria for specimen referrals to outside laboratories ("send outs")
   Quality control procedures
   Documentation of critical values
   Clearly stated and dated entries of procedure implementation or change.

F. Comparison of Test Results

If the laboratory performs the same test by more than 1 method or at more than 1 test site, or the test is sometimes also sent to a referral laboratory, comparisons should be run at least twice annually to define the relationships between methods and sites. Comparison of different test methods for the same analyte within the laboratory or between laboratories (if samples are tested in-house and at a referral laboratory) is recommended. This should be done every 6 months or at a frequency determined by the laboratory manager. The following steps should be included:

1. Perform a 20 sample or greater comparison using specimens covering the analytical range.
   a. Group data in an X-Y comparison plot
   b. Calculate slope and intercept by a least squares method.
2. Laboratory director or qualified personnel should define acceptable performance limits.
3. If individual test results performed on the same patient or material do not correlate with each other (i.e. BUN/creatinine, electrolyte balance), the cause should be investigated and corrective action taken.

Postanalytical Factors Important in Clinical Chemistry

A. Computer Entry of Data

Reports should be accurate whether created manually or electronically, and in a standard format as established by the laboratory. Established laboratory standards for uniform reporting should be met.

B. Report Generation

Reports should be in a format that is readable and easily understood, with appropriate references or explanations as needed. They should be generated in a timely manner relative to preanalytical and analytical components.

C. Report Delivery

Report delivery should be timely, to the correct client, and in a manner agreed upon by the client and the laboratory.

D. Specimen Storage

Specimens should be stored under appropriate conditions for a predetermined time period, as determined by specimen stability, laboratory policy and/or certification/accreditation requirements.

E. Specimen Disposal

Laboratories should appropriately dispose of biohazardous and non-biohazardous materials and specimens, including timely emptying of all containers and trash bins.

F. Personnel Safety

Conditions should be appropriate for computer entry, transcription, handling of specimens, specimen disposal and all other postanalytical tasks.

G. Laboratory Environment

Laboratory environment should meet standard requirements necessary for safe, rapid, efficient and effective performance.

H. Personnel Requirements

Personnel should meet training requirements as indicated for specific areas of the laboratory.

CHAPTER 20

Enzymology

For most enzymic estimations, special care has to be taken regarding storage specifications of reagents/kits. During testing one has to be extremely cautious about temperature (incubation), pH, time settings, etc. otherwise reproducible results may not be obtained. All precautions listed in this chapter or those mentioned by manufacturers should be cautiously followed to every minutest detail.

ALPHA-AMYLASE

Serum and Urine—α Amylase (Direct Substrate Method)

(*Courtesy*: Tulip Group of Companies)

For the determination of α amylase activity in serum, plasma or urine (for in vitro diagnostic use only).

Summary

α amylase is secreted by the pancreas into the duodenum where it aids the catabolism of carbohydrates to simple sugars. Damage to the pancreas or obstruction to the pancreatic duct causes the enzyme to enter the blood-stream. Elevated levels are found in acute pancreatitis, perforated/penetrating peptic ulcers, parotitis (mumps). Patients with chronic pancreatic disorders having pancreatic cell destruction do not have high levels as less amylase is produced by the pancreas.

Principle

α Amylase catalyzes the hydrolysis of a 2-chloro-4 nitrophenol salt to chloronitrophenol (CNP). The rate of hydrolysis is measured as an increase in absorbance due to the formation of chloro nitrophenol which is proportional to the α Amylase activity in the sample.

$$\text{CNP - Gal - G2} + H_2O \xrightarrow{\alpha\ \text{Amylase}} \text{CNP} + \text{Gal - G2}$$

Normal Reference Values

Serum : Up to 90 U/L at 37°C
Urine : Up to 490 U/L at 37°C

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*2 × 10 mL*	*2 × 30 mL*
L1: Amylase reagent	2 × 10 mL	2 × 30 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the label.

Reagent Preparation3

Reagents are ready to use. Do not pipette with mouth.

Sample Material

Serum, heparinized plasma, urine.

α Amylase is reported to be stable in the sample for 5 days at 2–8°C. Separate serum from clot as soon as possible.

Procedure

Wavelength/filter : 405/(Hg 405)/violet
Temperature : 37°C
Light path : 1 cm

For serum as sample:

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) 37°C*
Amylase reagent (L1)	1.0 mL
Sample	0.02 mL

Mix well and read the initial absorbance A_0 after 1 minute and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

For Urine as Sample

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) 37°C*
Amylase reagent (L1)	1.0 mL
Sample	0.01 mL

Mix well and read the initial absorbance A_0 after 1 minute and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

α Amylase activity in U/L (Serum) = ΔA/min × 3954
α Amylase activity in U/L (Urine) = ΔA/min × 7830

Linearity

The procedure is linear up to 1000 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.300, use only the value of the first 2 minutes to calculate the result, or dilute the sample 1 + 9 with normal saline (NaCL 0.9%) and repeat the assay (Results × 10).

Note
Anticoagulants like oxalate and EDTA bind Calcium which is needed for α Amylase activity and should not be used. Heparin may be used. Saliva and sweat contain α Amylase. Avoid contamination of reagent and sample during use.

For users to convert the values obtained by this method to the EPS substrate methods, multiply the results obtained by 2.45.

System Parameters

Reaction	:	Kinetic	**Interval**	:	60 sec.
Wavelength	:	405 nm	**Sample vol**	:	0.02 mL (s)/ 0.01 mL (u)
Zero setting	:	Distilled water	**Reagent vol**	:	1.00 mL
Incubation temperature	:	37°C	**Standard**	:	
Incubation time	:	—	**Factor**	:	3954/7830
Delay time	:	60 sec	**Factor**	:	Increasing
Read time	:	180 sec	**Linearity**	:	1000 U/L
No. of read	:	4	**Units**	:	U/L

Normal Values

Normal values urine	
Mayoclonic method	10–80 Amylase U/h
Somogyi method	26–950 U/24 h
Beckman method	1–17 U/h

Normal values serum		*SI units*
Somogyi method	50–180 U/dL	92–330 U/L
Beckman method		20–125 U/L
Over age 70		20–160 U/L
Panic level	Three times the upper limit of normal	

Clinical Relevance

Increased levels are found in pronounced elevation (5 or more times normal)

- Acute pancreatitis
- Pancreatic pseudocyst
- Morphine administration.

Moderate elevation (3 to 5 times normal)

- Pancreatic carcinoma affecting head of pancreas (late manifestation)
- Mumps
- Salivary gland inflammation
- Perforated peptic ulcer (sometimes)
- Acute exacerbation of chronic pancreatitis
- Partial gastrectomy
- Obstruction of pancreatic duct
- Alcohol poisoning
- Acute cholecystitis
- Intestinal obstruction with strangulation
- Ruptured tubal ectopic
- Ruptured aortic aneurysm.

Decreased Levels are found in

- Acute pancreatitis subsidence
- Hepatitis
- Cirrhosis of liver
- Toxemia of pregnancy
- Severe burns
- Severe thyrotoxicosis.

LIPASE

Lipase Serum (Turbidimetric Method)

(Courstesy: Randox)

Intended Use

For the quantitative in vitro determination of lipase in serum and plasma. This product is suitable for manual use.

Clinical Significance

A lipase test system is a device intended to measure the activity of the enzyme lipase in serum and plasma. Lipase measurements are used in the diagnosis and treatment of diseases of the pancreas such as acute pancreatitis and obstruction of the pancreatic duct.

Turbidimetric Method

Principle

$$\text{Triolein} + 2H_2O \xrightarrow{\text{Lipase}} \text{Monoglyceride} + 2\ \text{oleic acid}$$

The decrease in turbidity is measured at 340 nm.

Sample

Serum or heparinized plasma.

Lipase is stable in the sample for 5 days at +2 to +8°C or 24 hours at +20° to +25°C.

Reagent Composition

Contents	*Concentrations in the test*
1. Buffer	
Tris buffer	26 mmol/L, pH 8.9
2. Substrate	
Sodium deoxycholate	16.7 mmol/L
Calcium chloride	0.04 mmol/L
Triolein	0.3 mmol/L
Colipase	4 mg/L
3. Standard	See assigned value on lot specific insert

Safety Precautions and Warnings

For in vitro diagnostic use only. Do not pipette by mouth. Exercise the normal precautions required for handling laboratory reagents.

Solution 1 contains sodium azide. Avoid ingestion or contact with skin or mucous membranes. In case of skin contact, flush affected area with copious amounts of water. In case of contact with eyes or if ingested, seek immediate medical attention.

Sodium azide reacts with lead and copper plumbing, to form potentially explosive azides. When disposing of such reagents flush with large volumes of water to prevent azide build up. Exposed metal surfaces should be cleaned with 10% sodium hydroxide.

The reagents must be used only for the purpose intended by suitably qualified laboratory personnel, under appropriate laboratory conditions.

Stability and Preparation of Reagents

Buffer

Contents ready for use. Stable until expiry date when stored at +2 to +8°C.

Substrate

Reconstitute the contents of one vial of substrate 2 with the appropriate volume of buffer 1:

2.5 mL for the 20 × 2.5 mL kit
30 mL for the 4 × 30 mL kit

Stable for 2 weeks at +2 to +8°C for 5 days at +15 to +25°C.

Standard

Dissolve the contents of one vial of standard 3 in 3.0 mL of redistilled water, swirling gently for 30 mins before use. Stable for 5 days at +2 to +8°C.

Materials Provided

- Buffer
- Substrate
- Standard.

Materials Required but not Provided

Randox assayed multisera level 2 and level 3.

Procedure Notes

In rare cases, a patient's serum may give an increase in absorbance rather than a decrease. The lipase activity of these samples usually falls within the normal range. Extremely high lipase activities can lead to considerable substrate consumption, with A_1 being less than 0.500. In such cases, dilute the sample 1+9 with 0.9% NaCl solution and repeat the assay. Multiply the result by 10. It is preferable to use disposable cuvettes. Glass cuvettes should be cleaned thoroughly especially after being used for triglyceride or cholesterol assays.

Manual—Lipase

Procedure

Wavelength:	340 nm (Hg 365 nm or Hg 334 nm)			
Cuvette:	1 cm light path			
Temperature:	37°C			
Measurement:	Against air or distilled/deionized water			
Pipette into cuvette:				
	Standard		Sample	
	Macro	Semi	Macro	Semi
	Micro		Micro	

Contd...

Contd...

Reagent	2.5 mL	1.0 mL	2.5 mL	1.0 mL
Sample	—	—	0.1 mL	0.04 mL
Standard	0.1 mL	0.04 mL	—	—
Mix. Avoid the formation of foam. Read absorbance A_1 of standard and sample after 4 minutes. After a further 5 minutes read absorbance A_2 of standard and sample.				
ΔA of sample of standard = $A_1 - A_2$				

Calculation

Calculate the assay factor as follows:

$$\text{Factor} = \frac{\text{Activity standard}}{\Delta A \text{ standard}}$$

$$\text{Sample lipase activity} = \text{Factor} \times \Delta A_{\text{sample}}$$

Quality Control

Randox Assayed Multi-sera, level 2 and level 3 are recommended for daily quality control. Two levels of controls should be assayed at least once a day. Values obtained should fall within a specified range. If these values fall outside the range and repetition exclude errors, the following steps should be taken:

1. Check instrument settings and light source.
2. Check cleanliness of all equipment in use.
3. Check water, contaminants, i.e. bacterial growth may contribute to inaccurate results.
4. Check reaction temperature.
5. Check expiry date of kit and contents.

Interference

Hemolysis interferes with the assay.

Normal Values

Serum: Up to 190 U/l (37°C).

It is recommended that each laboratory establish its own reference range to reflect the age, sex, diet and geographical location of the population.

Linearity

If the lipase activity exceeds 500 U/l, dilute the sample 1 + 1 with 0.9% NaCl solution and reassay. Multiply result by 2.

Normal Value

< 200 U/L with triolein; < 160 U/L with olive oil.

		SI Units
Adults	13–141 U/L	0.22–2.40 µKat/L
Age 20–60	31–186 U/L	0.53–3.16 µKat/L

Contd...

Contd...

Over age 60	< 302 U/L	< 5.13 µKat/L
Over age 90	26–267 U/L	0.44–4.54 µKat/L
Children	20–136 IU/L	
Infants	9–105 IU/L	

Clinical Relevance

Increased

Cholecystitis, cirrhosis, duodenal ulcers, fat embolism, gallstone colic, pain (abdominal), pancreatic carcinoma, pancreatic cholera, pancreatic trauma, pancreatitis, peritonitis trauma, pancreatitis, peritonitis, renal disease with impaired output, and strangulated bowel. Drugs include bethanechol, heparin, and narcotic analgesics.

Description

Lipase is a pancreatic enzyme that changes fats and triglycerides into fatty acids and glycerol. The pancreas is the only body organ that demonstrates significant lipase activity. In acute pancreatitis, serum lipase begins to increase in 2–6 hours, peaks at 12–30 hours, and remains elevated, but slowly decreases for 2–4 days. Lipase rises and falls in tandem with amylase in acute pancreatitis, but is a more specific marker for this condition.

PHOSPHATASES

Phosphatases belong to the class of enzymes called hydrolases, and they are characterized, by their ability to hydrolyze a large variety of organic phosphate esters with the formation of an alcohol and a phosphate ion.

Phosphatases of diagnostic significance are of two kinds**:** alkaline phosphatase and acid phosphatase. These are differentiated by their reaction in alkaline and acidic medium. The pH for measuring the alkaline phosphatase activity is 10, and for acid phosphatase, it is 5.0. Various substrates have been used from time to time, but the current use of p-nitrophenyl phosphate (PNPP) appears to be universal. The PNNP is not a natural substrate for the phosphatases. It is used because it gives a reasonably rapid rate of reaction and because it is analytically convenient to measure the product formed (p-nitrophenol). The liberated phenol is yellow in color in alkaline medium and is colorless in acid medium. Continuous assay can be done for alkaline phosphatase by measuring the rate of formation of p-nitrophenol at pH 10. For this one will need a recording spectrophotometer. The manual two-point method is described here.

Units for Reporting Phosphatase Activity

Several units are used in expressing phosphatase activity—Bodansky unit, King-Armstrong unit, Bessey-Lowry-Brock unit and U/L (International unit). As the current trend is to express all enzyme activity by the International unit, one U/L signifies 1.0 mmole of chromogen from the substrate used per minute (and thus releases 1.0 mmole of chromogen from the substrate per minute) under the conditions of the assay.

Specimen

Serum is the preferred specimen but plasma (heparinized) can also be used. Other anticoagulants inhibit the activity of ALP. A blood specimen after overnight fasting is recommended, but a specimen collected at any other time can also be used. Separate the serum promptly and store in a refrigerator if immediate analysis is not possible. Red blood cells are high in acid phosphatase concentration and hence, a hemolyzed serum specimen is not acceptable for acid phosphatase determination of serum. In case of increased acid phosphatase activity due to hemolysis, check for "tartrate-inhibition." Acid phosphatase of red blood cells is not "tartrate-labile" and hence is not inhibited. Alkaline phosphatase (ALP) activity increases with storage, hence, as a general rule, it is best to analyze ALP specimens the same day they are drawn. Acid phosphatase (ACP) is best stored in acid medium, hence, maintain an acid pH with citric acid ("acid stabilizer"). Control sera must be treated in the same way after reconstitution. ACP is measured in vaginal washings in suspected rape cases and this must also be acidified.

Alkaline Phosphatase (ALP)

Clinical Significance

Increased alkaline phosphatase activity may be related to hepatobiliary and bone diseases. Very high alkaline phosphatase activity in serum is seen in patients with bone cancer, and marked increase also occur in obstructive jaundice and biliary cirrhosis. Moderate elevations have been noted in case of Hodgkin's disease, congestive heart failure, infective hepatitis and abdominal problems.

Alkaline Phosphatase, Serum

Normal Values

Total alkaline phosphatase		*SI Units*
King-Armstrong method		
Adult age 20–60	4.5–13 U/dL	32–92 U/L
Eldery	Slightly higher	
Newborn	5–15 U/dL	36–107 U/L
Premature newborn	1.5–2 times adult value	
Children: Values remain high until epiphyses close		
1 month	10–30 U/dL	71–213 U/L
3 years	10–20 U/dL	71–142 U/L
10 years	15–30 U/dL	107–213 U/L
Bodansky Method		
Adults age 20–60	2–4 U/dL	10.7–21.5 U/L
Eldery	Slightly higher	
Children	5–14 U/dL	27–75 U/L
Bessey-Lowrey-Brock Method		
Adults age 20–60	0.8–2.3 U/dL	13.3–38.3 U/L
Eldery	Slightly higher	
Bowers and McComb Method		
Females		
Age 1–12	< 350 U/L	< 5.95 µKat/L
Puberty: Values may triple		
Age >15	25–100 U/L	0.43–17.0 µKat/L
Males		
Age 1–12	< 350 U/L	< 5.95 µKat/L
Age 12–14	< 500 U/L	< 8.50 µKat/L
Puberty: Values may triple		
Age > 20	25–100 U/L	0.43–1.70 µKat/L
Isoenzyme		Normal Values
	Percentage of Isoenzyme Inactivated	Fraction of Isoenzyme Inactivated
Heat inactivation	After 16 minutes	After 16 minutes
Method	at 55°C	at 55°C
Liver isoenzyme	50–70	0.50–0.70
Bone isoenzyme	90–100	0.90–1.00
Intestinal isoenzyme	50–60	0.50–0.60
Placental isoenzyme		
Trimester 1 to 1 month postpartum 50% of total		

Alkaline Phosphatase (Mod. Kind and King's Method)

(*Courtesy*: Tulip Group of Companies)

For the determination of alkaline phosphatase activity in serum (for in vitro diagnostic use only).

Summary

Alkaline phosphatase (ALP) is an enzyme of the hydrolase class of enzymes and acts in an alkaline medium. It is found in high concentrations in the liver, biliary tract epithelium and in the bones. Normal levels are age dependent and increase during bone development. Increased levels are associated mainly with liver and bone disease. Moderate increases are seen in Hodgkin's disease and congestive heart failure.

Principle

ALP at an alkaline pH hydrolyzes disodium phenyl phosphate to form phenol. The phenol formed reacts with 4-aminoantipyrine in the presence of potassium ferricyanide, as an oxidizing agent, to form a red colored complex. The intensity of the color formed is directly proportional to the activity of ALP present in the sample.

Disodium phenyl phosphate + H_2O —(ALP, pH 10.0)→ Phenol + Disodium hydrogen phosphate

Phenol + 4-Aminoantipyrine —(Alkaline medium, $K_3Fe(CN)_6$)→ Red colored complex

Normal Reference Values

Total ALP activity : 3.0–13.0 KA units

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*15 Tests*	*30 Tests*
L1: Buffer reagent	60 mL	120 mL
L2: Substrate reagent	6 mL	12 mL
L3: Color reagent	60 mL	120 mL
S: Phenol standard (10 mg/dL)	5 mL	5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

All reagents are ready to use.

Sample Material

Serum free from hemolysis. ALP is reported to be stable in serum for 3 days at 2–8°C.

Procedure

Wavelength/filter : 510 nm (Hg 546 nm)/green
Temperature : 37°C
Light path : 1 cm
Assay:

Pipette into four clean dry test tubes labeled as Blank (B), Standard (S), (C), Test (T).

Addition Sequence	*B mL*	*S mL*	*C mL*	*T mL*
Distilled water	1.05	1.00	1.0	1.0
Buffer reagent (L1)	1.0	1.0	1.0	1.0
Substrate reagent (L2)	0.10	0.10	0.10	0.10
Mix well and allow to stand at 37°C for 3 minutes and add.				
Sample	-	-	-	0.05
Phenol standard (S)	-	0.05	-	-
Mix well and allow to stand at 37°C for 15 minutes and add.				
Color reagent (L3)	1.0	1.0	1.0	1.0
Sample	-	-	0.05	-

Mix well after each addition. Measure the absorbances of the blank (Abs. B), standard (Abs. S), control (Abs. C), and test (Abs. T) against distilled water.

Calculations

$$\text{Total ALP activity in KA units} = \frac{\text{Abs. T–Abs. C}}{\text{Abs. S–Abs. B}} \times 10$$

Linearity

If enzyme activity 60 KA. Units dilute the sample with distilled water and repeat the assay. Multiply the value with the proper dilution factor.

Note

In case or multiple samples to be assayed simultaneously, only one blank and standard can be run for the entire series, however for each sample a control and test assay has to be run additionally.

System Parameters

Reaction	: End point abs	Interval	: —
Wavelength	: 510 nm	Sample volume	: 0.05 mL
Zero setting	: Distilled water	Reagent volume	: Calculate
Incubation temperature	: 37°C	Standard	: Calculate
Incubation time	: 15 min	Factor	: —
Delay time	: —	Reaction slope	Increasing
Read time	: —	Linearity	: 60 KA units
No. of read	: —	Units	: KA units

Alkaline Phosphatase (DEA) (PNPP Kinetic Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of alklaline phosphatase activity in serum (For in vitro diagnostic use only).

Summary

Alkaline phosphatase (ALP) is an enzyme of the hydrolase class of enzymes and acts in an alkaline medium. lt is found in high concentrations in the liver, biliary tract epithelium and in the bones. Normal levels are age dependent and increase during bone development. Increased levels are associated mainly with liver and bone disease. Moderate increases are seen in Hodgkin's disease and congestive heart failure.

Principle

ALP at an alkaline pH hydrolyzes p-Nitrophenylphosphate to form p-Nitrophenol and Phosphate. The rate of 'formation of p-Nitrophenol is measured as an increase in absorbance which is proportional to the ALP activity in the sample.

p-Nitrophenylphosphate $\xrightarrow{\text{ALP}}$ p-Nitrophenol + Phosphate

Normal Reference Values

Serum (Adults) : 80–290 U/L at 37°C.
(Children) : 245–770 U/L at 37°C.

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*10 × 3 mL*	*5 × 15 mL*
L1 : Buffer reagent	35 mL	80 mL
T1 : Substrate reagent	10 Nos	5 Nos

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Working reagent : Dissolve 1 substrate tablet in 3.2 mL (10 × 3 mL pack) or 15 mL (5 × 15 mL pack) of buffer reagent.

This working reagent is stable for at least 15 days when stored at 2–8°C.

The substrate is light and temperature sensitive. Take adequate care, especially after reconstitution.

Sample Material

Serum. Free from hemolysis. ALP is reported to be stable in serum for 3 days at 2–8°C.

Procedure

Wavelength/filter : 405 nm
Temperature : 37°C/30°C/25°C
Light path : 1 cm
Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) (mL)*
Working Reagent	1.0
Incubate at the assay temperature for 1 minute and add	
Sample	0.02

Mix well and read the initial absorbance A_0 after minute and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

ALP activity in U/L = ΔA/min. × 2754.

Temperature Conversion Factors

Assay Temperature	*Desired 25°C*	*Reporting 30°C*	*Temperature 37°C*
25°C	1.00	1.22	1.64
30°C	0.82	1.00	1.33
37°C	0.61	0.75	1.00

Linearity

The procedure is linear up to 700 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.250, use only the value of the first 2 minutes to calculate the result, or dilute the sample 1 + 9 with normal saline (NaCI 0.9%) and repeat the assay (Results × 10).

Note
Samples having a very high activity show a very high initial absorbance. If this is suspected then dilute the sample and repeat the assay.

System Parameters

Reaction	:	Kinetic	Interval	:	30
Wavelength	:	405 nm	Sample volume	:	0.02 mL
Zero setting	:	Distilled water	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C	Standard	:	—
Incubation time	:	—	Factor	:	2754
Delay time	:	30 sec	Factor	:	Increasing
Read time	:	120 sec	Linearity	:	700 U/L
No. of read	:	4	Units	:	U/L

Clinical Relevance

Elevated Levels

1. Liver disease (correlates with abnormal liver function tests). An elevation of serum alkaline phosphatase is often associated with elevated SGOT/SGPT and raised bilirubin.
   a. Marked increases
      i. Obstructive jaundice (gallstones obstructing major biliary ducts, accompanies elevated bilirubin).
      ii. Space occupying lesions of the liver such as cancer and abscesses.
      iii. Hepatocellular cirrhosis.
      iv. Biliary cirrhosis.
   b. Moderate increases
      1. Hepatitis
      2. Cirrhosis of liver.
2. Bone disease
   a. Marked increases
      i. Paget's disease
      ii. Metastatic bone disease
      iii. Osteitis deformans.
   b. Moderate increases
      i. Osteomalacia (in osteoporosis, no increase in ALP)
      ii. Rickets.
3. Other diseases
   a. Hyperparathyroidism (accompanied by hypercalcemia)
   b. Infectious mononucleosis.

Reduced Levels

1. Hypophosphatasia (markedly reduced)
2. Malnutrition
3. Hypothyroidism
4. Pernicious anemia
5. Scurvy
6. Milk-alkali syndrome
7. Placental insufficiency.

Interfering Factors

1. Many drugs produce mild to moderate elevations of ALP, e.g.
   a. Phenothiazine tranquillizers
   b. Methyltestosterone
   c. Oral contraceptives
   d. Allopurinol
   e. Methyldopa
   f. Procainamide
   g. Tolbutamide
   h. Isoniazid
   i. PAS
   j. Erythromycin
   k. Oxacilin
   l. Ergoesterol.
2. Young children, pregnant women in the third trimester, and all women have physiologically high levels of alkaline phosphatase.
3. The level is slightly increased in older people.
4. After IV administration of albumin, there is sometimes a marked increase lasting for several days.
5. Drugs that may cause decreased levels
   a. Fluoride
   b. Oxalates
   c. Phosphates
   d. Propranolol
   e. Vitamin D.

Alkaline Phosphatase Isoenzymes

Normal Values

AP-1, Alpha 2:	Values are reported as weak, moderate, or strong
AP-2, Beta 1:	Values are reported as weak, moderate, or strong
AP-3, Beta 2:	Values are reported as weak, moderate, or strong

The isoenzymes of alkaline phosphatase (ALP) are produced by various tissues. AP-1, Alpha 2 is heat labile and is produced in the liver and by proliferating blood vessels. AP-2, Beta 1 is heat stable and is produced by bone and placenta. The intestinal isoenzymes AP-3, Beta 2 is present in small quantities in Group O and B individuals AP-1 and 2 can be distinguished partially in the laboratory by heating and urea testing. Placental alkaline phosphatase is still more stable to heat than urea.

The test is conducted (when the total alkaline phosphatase is raised) to distinguish between bone and liver origin of alkaline phosphatase.

Clinical Relevance

1. Osteoblastic bone tumors, increase the bone alkaline phosphatase in the blood serum; less than 25% is thermostable in bone disease.
2. Liver diseases such as cancer and biliary obstruction increase the liver isoenzyme, more than 25% in thermostable in hepatic disease.
3. The intestinal isoenzyme may be increased in patients with cirrhosis.
4. The placental isoenzyme is increased in some patients with cancer (Carcino placental antigen) and normally in pregnancy.

Acid Phosphatase

Clinical Significance

Request for the analysis of serum acid phosphatase is often done in male patients with suspected prostatic cancer. The increase of serum acid phosphatase activity in such patients is found to be inhibited by tartrate. Acid phosphatase is also present in very high concentrations in semen, a fact utilized in forensic medicine in the investigations of rape offences.

Normal Values

Methods		*SI units*
Bodansky	0.5–2 U/L	2.7–10.7 IU/L
King-Armstrong	0.1–5 U/L	0.2–8.8 IU/L
Bessey-Lowery-Brock	0.1–0.8 U/L	1.7–13.4 IU/L
Gutman	0.1–2 U/L	1.7–13.4 IU/L

Specimen

Serum is the most commonly used specimen; hemolyzed serum specimens are contaminated with red cell acid phosphatase and should be rejected.

Mod. King's Method

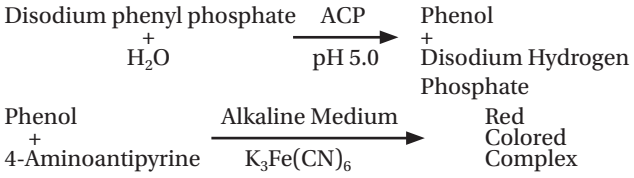
(*Courtesy*: Tulip Group of Companies)
For the determination of Acid Phosphatase activity in serum (For in vitro diagnostic use only).

Summary

Acid phosphatase (ACP) is an enzyme of the hydrolase class of enzymes and acts in an acidic medium. It is widely distributed and found in high concentrations in the liver, RBCs and the prostate. Increased levels of the prostatic fraction are associated with prostatic carcinomas. Increased levels of the non-prostatic fraction are associated with liver diseases, hyperparathyroidism, and Paget's disease.

Principle

ACP at an acidic pH hydrolyzes di-sodium phenyl phosphate to form phenol. The phenol formed reacts with 4-amino-antipyrine in the presence of potassium ferricyanide, as an oxidizing agent, to form a red colored complex. The intensity of the color formed is directly proportional to the activity of ACP present in the sample. Tartrate inhibits prostatic ACP and the testing in its presence is done to find the non prostatic ACP. The difference between the activities of the total and non-prostatic ACP gives the activity of the prostatic ACP.



Normal Reference Values

Total ACP activity : 1.0–4.0 KA units
Prostatic ACP activity : 0.0–0.8 KA units

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*10 Tests*	*25 Tests*
L1: Buffer reagent	50 mL	125 mL
L2: Substrate reagent	5 mL	12.5 mL
L3: Color reagent	50 mL	125 mL
L4: Tartrate reagent	2 mL	2 mL
S : Phenol Standard (10 mg/dL)	5 mL	5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

All reagents are ready to use.

Sample Material

Serum. Free from hemolysis.

The ACP, especially the prostatic fraction, is unstable in a collected sample hence, the serum should be separated from the clot, as soon as possible, and assayed. In case of a delay in testing the serum should be acidified to a pH of 5.0 with 0.02 mL Acetate Buffer (5M) for each mL of serum.

Procedure

Wavelength/filter : 510 nm (Hg 546 nm)/green
Temperature : 37°C
Light path : 1 cm

Assay

Pipette into 5 clean dry test tubes labeled as blank (B), standard (S), control (C), Test (T), and tartrate stable (TS).

Addition Sequence	*B (mL)*	*S (mL)*	*C (mL)*	*T (mL)*	*TS (mL)*
Distilled water	1.1	1.05	1.0	1.0	1.0
Buffer reagent (L1)	1.0	1.0	1.0	1.0	1.0
Substrate reagent (L2)	0.10	0.10	0.10	0.10	0.10
Mix well and allow to stand at 37°C for 3 minutes and add.					

Contd...

Contd...

Tartrate reagent (L4)	-	-	-	-	0.02
Sample	-	-	-	0.1	0.1
Phenol standard (S)	-	0.05			
Mix well and allow to stand at 37°C for 60 minutes and add.					
Color	1.0	1.0	1.0	1.0	1.0
reagent (L3)					
Sample	-	-	0.1	-	

Mix well after each addition. Measure the absorbances of the Blank (Abs.B), Standard (Abs.S), Control (Abs.C), Test (Abs.T), and Tartrate Stable (Abs.TS) against Distilled water.

Calculations

$$\text{Total ACP activity in KA Units} = \frac{\text{Abs. T-Abs. C}}{\text{Abs. S-Abs. B}} \times 5.0$$

$$\text{Prostatic ACP activity in KA units} = \frac{\text{Abs. T - Abs. TS}}{\text{Abs. S - Abs. B}} \times 5.0$$

Linearity

If enzyme activity exceeds 40 KA.Units dilute the sample with distilled water and repeat the assay. Multiply the value obtained with an appropriate dilution factor.

Notes

In case of multiple samples to be assayed simultaneously, only one Blank and Standard can be run for the entire series, however for each sample, a Control, Test and Tartrate Stable assay has to be run additionally. It has been seen that in a collected sample ACP, especially the prostatic form, may loose around 50% of its activity in an hour at RT.

System Parameters

Reaction	:	End point Abs	Interval	:	
Wavelength	:	510 nm	Sample volume	:	0.10 mL
Zero setting	:	Distilled water	Reagent volume	:	3.10 mL
Incubation temperature	:	37°C	Standard	:	Calculate
Incubation time.	:	60 min	Factor	:	
Delay time	:	—	Factor	:	Increasing
Read time	:	—	Linearity	:	40 KA Units
No. of read	:	—	Units	:	KA Units

Acid Phosphatase (a Naphthyl Phosphate Kinetic Method)

(*Courtesy*: Tulip Group of Companies)

For the determination of acid phosphatase activity in serum (For in vitro diagnostic use only).

Summary

Acid phosphatase (ACP) is an enzyme of the hydrolase class of enzymes and acts in an acidic medium. It is widely distributed and found in high concentrations in the liver, RBCs and the prostate. Increased levels of the prostatic fraction are associated with prostatic carcinomas. Increased levels of the non-prostatic fraction are associated with liver diseases, hyperparathyroidism, and Paget's disease.

Principle

ACP at an acidic pH hydrolyzes α naphthyl phosphate to form α naphthol and inorganic phosphate. The α naphthol formed is coupled with fast red TR salt to form a diazo dye complex. The rate of formation of this complex is measured as an increase in absorbance which is proportional to the ACP activity in the sample. Tartrate inhibits prostatic ACP and the testing in its presence is done to find the non-prostatic ACP. The difference between the activities of the total and non-prostatic ACP gives the activity of the prostatic ACP.

$$\alpha \text{ Naphthyl phosphate} + H_2O \xrightarrow{\text{ACP}} \alpha\text{Naphthol} + \text{Phosphate}$$

$$\alpha \text{ Naphthol} + \text{Fast Red TR Salt} \longrightarrow \text{Diazo dye complex}$$

Normal Reference Values

Serum (male) : Up to 4.2 U/L at 30°C/up to 4.7 U/L at 37°C
(female) : Up to 3.0 U/L at 30°C/up to 3.7 U/L at 37°C
Prostatic ACP : Up to 1.5 U/L at 30°C/up to 1.6 U/L at 37°C

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*10 × 2 mL*	*30 × 2 mL*
L1 : Buffer reagent	25 mL	75 mL
T1 : Substrate tablets	10 Nos	30 Nos
L2 : Tartrate reagent	2 mL	2 mL
L3 : Acetate buffer	2 mL	2 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents L2 and L3 are ready to use.

The Buffer (L1) when retrieved from 2–8°C may appear turbid. However, the turbidity clears up on attaining RT. In case, the turbidity persists a little warming of the Buffer to 30/37°C may be required.

Working reagent: Dissolve 1 Substrate tablet (T1) in 2.2 mL of Buffer reagent (L1). Allow the tablet to hydrate for around 5 minutes and then shake to dissolve. This working reagent is stable for at least 3 days when stored at 2–8°C. The working reagent may be used for the total ACP assay or the non-prostatic ACP assay as required.

Sample Material
Serum. Free from hemolysis.

ACP, especially the prostatic fraction, is unstable in a collected sample, hence the serum should be separated from the clot, as soon as possible, and assayed. In case of a delay in testing the serum should be acidified to a pH of 5.0 with 0.02 mL acetate buffer (5M) provided for each mL of serum.

Procedure

Wavelength/filter : 405 nm
Temperature : 30/37°C
Light path : 1 cm

Total ACP Assay
Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) (mL)*
Working reagent	1.0
Sample	0.1

Mix well and read the initial absorbance A_0 after 5 minutes and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Non-prostatic ACP Assay : (Tartrate Inhibited)

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) (mL)*
Working reagent	1.0
Tartrate reagent	0.02
Incubate at the assay temperature for 1 minute and add	
Sample	0.1

Mix well and read the initial absorbance A_0 after 5 minutes and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations
ACP activity in U/L = ΔA/min × 750
Prostatic ACP activity in U/L = Total ACP–non-prostatic ACP.

Linearity
The procedure is linear up to 75 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.100, dilute the sample 1 + 4 with normal saline (NaCI 0.9%) and repeat the assay (Results × 5).

Notes
Samples having a high activity show a very high initial absorbance. If this is suspected then dilute the sample and repeat the assay.

The working reagent should have an absorbance below 0.800 against distilled water at 405 nm. Discard the reagent if the absorbance is above 0.800. It has been seen that in a collected sample ACP, especially the prostatic form, may lose around 50% of its activity in an hour at RT.

System Parameters

Reaction : Kinetic		Interval	: 60
Wavelength : 405 nm		Sample volume	: 0.1 mL
Zero setting : Distilled water		Reagent volume	: 1.00 mL
Incubation temperature : 30/37°C		Standard	:
Incubation time : —		Factor	: 750
Delay time : 300 sec		Read scope	: Increasing
Read time : 180 sec		Linearity	: 75 U/L
No. of read :		Units	: U/L

Clinical Relevance

1. A significantly elevated value nearly always indicative of metastatic cancer of the prostate. If the tumor is successfully treated, this enzyme level will drop within 3 to 4 days after surgery or 3 to 4 weeks after estrogen administration.
2. Moderately increased values also occur in the absence of prostate disease in:
   a. Paget's disease
   b. Gaucher's disease
   c. Hyperparathyroidism
   d. Multiple myeloma
   e. Any cancer that has metastasized to the bone
   f. Hepatitis
   g. Obstructive jaundice
   h. Acute renal impairment
   i. Sickle cell crisis/hemolytic anemia
   j. Excessive destruction of platelets.
3. Levels are reported to be elevated in the bone marrow of patients with prostatic cancer metastatic to the bone.

Interfering Factors

1. Drugs that may cause increased levels include:
   a. Androgens in females
   b. Clofibrate.
2. Drugs that may cause decreased levels include:
   a. Fluorides
   b. Oxalates
   c. Phosphates.

Serum Alkaline Phosphatase and Acid Phosphatase

The following table gives the differences between the two:

Serum alkaline phosphatase	*Serum acid phosphatase*
1. Alkaline optimum (pH = 10.0)	Acid optimum (pH = 4.9)
2. Three isoenzymes known — from bone, liver and intestine	Two isoenzymes known— prostatic and non-prostatic
3. Inhibited by EDTA fluoride	Inhibited by oxalate and— Prostatic acid phosphatase is inhibited by tartrate
4. Tissue sources—Osteoblast of bone, liver cells, intestine, kidney and placenta	Bone, liver, spleen, kidney, prostate and red cells
5. Normal values	
3–13 KA units	1–4 KA units

TRANSAMINASES

Transamination is a process in which an amino group is transferred from an amino acid to an alpha-ketoacid. It is an important step in the metabolism of amino acids. The enzymes responsible for transamination are called transaminase (now called, amino-transferases). Two diagnostically useful transaminases are glutamate oxaloacetate transaminase or GOT or aspartate, aminotransferase and glutamate pyruvate transaminase or GPT alanine amino transferase. These enzymes catalyse the following reactions**:**

L-aspartate + Oxoglutarate $\xrightarrow{\text{GOT/AST}}$ Oxaloacetate+
(or ketoglutarate) Glutamate

L-alanine + Oxoglutarate $\xrightarrow{\text{GPT/ALT}}$ Pyruvate +
(or ketoglutarate) Glutamate

Clinical Significance

Increased serum transaminase activity is seen in liver dysfunction. Greater activity of GOT (AST) over GPT (ALT) is typical of myocardial infarction.

Evaluation of Methods

The two methods applied in the analysis of transaminase activity are colorimetry and ultraviolet spectrophotometry. The latter procedure requires NADH, coenzyme. The colorimetric method is discussed below.

Specimen

The serum specimen submitted for the enzyme assay of SGOT and SGPT should be free from hemolysis. Collect the serum by the usual procedure described earlier. Prompt analysis is recommended and if this is not possible, refrigerate the specimen.

Serum Glutamic oxaloacetic transaminase (SGOT) (AST) (Reitman and Frankel's Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of SGOT (AST) activity in serum (For in vitro diagnostic use only).

Summary

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in bloodstream. Elevated levels are found in myocardial infarction, cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases, primary muscle diseases. Decreased levels may be found in pregnancy, beri beri and diabetic ketoacidosis.

Principle

SGOT converts L-aspartate and α-ketoglutarate to oxaloacetate and glutamate. The oxaloacetate formed reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown colored complex whose intensity is measured. The reaction does not obey Beer's law and hence, a calibration curve is plotted using a pyruvate standard. The activity of SGOT (ASAT) is read off this calibration curve.

L-Aspartate + αKetoglutarate $\xrightarrow[\text{pH 7.4}]{\text{SGOT}}$ Oxaloacetate + L-Glutamate

Oxaloacetate + 2,4,DNPH $\xrightarrow[\text{Medium}]{\text{Alkaline}}$ 2,4,Dinitrophenyl Hydrazone (Brown colored complex)

Normal Reference Values

Serum : 8–40 Units/mL

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*40 assays*
L1 : Substrate reagent	25 mL
L2 : DNPH reagent	2 × 12.5 mL
L3 : NaOH reagent (4N)	25 mL
S : Pyruvate standard (2 mM)	5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels. Sodium hydroxide can be stored at RT till the expiry mentioned.

Reagent Preparation

All reagents are ready to use except NaOH reagent (4N) which has to be diluted 1**:**10 with distilled/deionized water.

Working NaOH reagent: Dilute the sodium hydroxide to 250 mL or for every 1.0 mL of NaOH reagent (4N) add 9.0 mL of distilled water. The working sodium hydroxide reagent is stable at RT till the expiry mentioned, in a plastic bottle.

Sample Material

Serum. Free from hemolysis SGOT (ASAT) is reported to be stable in serum for 3 days at 2–8°C.

Procedure

Wavelength/filter : 505 nm (Hg 546 nm)/green
Temperature : 37°C and RT
Light path : 1 cm

Plotting of the calibration curve.

Pipette into five clean dry test tubes labeled as 1, 2, 3, 4, and 5.

Addition sequence Enzyme activity (U/mL)	*1 0 mL*	*2 24 mL*	*3 61 mL*	*4 114 mL*	*5 190 mL*
Substrate reagent (L1)	0.50	0.45	0.40	0.35	0.30
Pyruvate standard (S)	-	0.05	0.10	0.15	0.20
Distilled water	0.10	0.10	0.10	0.10	0.10
DNPH reagent (L2)	0.50	0.50	0.50	0.50	0.50
Mix well and allow to stand at RT for 20 minutes					
Working NaOH reagent (L3)	5.00	5.00	5.00	5.00	5.00

Mix well and allow to stand at RT for 10 minutes. Measure the absorbances of the tubes 2–5 against tube 1 (blank). Plot a graph of the absorbances of tubes 2–5 on the 'Y' axis versus the corresponding enzyme activity on the 'X' axis.

Assay

Pipette into clean dry test tubes labeled as blank (B) and Test (T):

Addition Sequence	*B mL*	*T mL*
Substrate reagent (L1)	0.50	0.50
Incubate at 37°C for 3 minutes		
Sample	-	0.10
Mix well and incubate at 37°C for 60 minutes		
DNPH reagent (L2)	0.50	0.50
Mix well and allow to stand at RT for 20 minutes		
Distilled water	0.10	-
Working NaOH reagent (L3)	5.00	5.00

Mix well and allow to stand at RT for 10 minutes. Measure the absorbance of the test (T) against blank (Blank) and read the activity of the test from the calibration curve plotted earlier.

Note

One sample blank is sufficient for each assay series.

If enzyme activity exceeds 190 U/mL dilute the sample with distilled water and repeat the assay. Multiply the value with the proper dilution factor.

High concentration of aldehydes and ketones in the sample or icteric or lipemic, samples may cause slightly elevated results. It is recommended to run a sample blank for these samples using serum instead of distilled water in the blank. High levels of serum pyruvate may interfere with results.

System Parameters

Reaction	:	End point	Interval	:	—
Wavelength	:	505 nm	Sample volume	:	0.10 mL
Zero setting	:	Reagent blank	Reagent volume	:	6.00 mL
Incubation temperature	:	37°C	Standard	:	Calib curve
Incubation time	:	80 min	Factor	:	—
Delay time	:	—	React slope	:	Increasing
Read time	:	—	Linerity	:	190 U/mL
No. of read	:	—	Units	:	U/mL

SGOT (AST) (Mod. IFCC Method)

(Courtesy: Tulip Group of Companies)

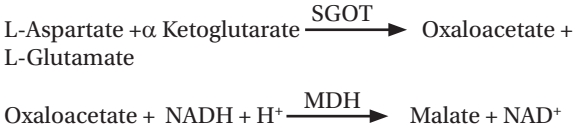
For the determination of SGOT (AST) activity in serum (For in vitro diagnostic use only).

Summary

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood. Elevated levels are found in myocardial infarction, Cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases, primary muscle diseases. Decreased levels may be found in pregnancy, beri beri and diabetic ketoacidosis.

Principle

SGOT (AST) catalyzes the transfer of amino group between L-aspartate and α ketoglutarate to form oxaloacetate and Glutamate. The oxaloacetate formed reacts with NADH in the presence of Malate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGOT (AST) activity in the sample.



Normal Reference Values

Serum (males) : Up to 37 U/L at 37°C
(females) : Up to 31 U/L at 37°C.

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*25 mL*	*75 mL*
L1 : Enzyme reagent	20 mL	60 mL
L2 : Starter reagent	5 mL	15 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent : For sample, start assays a single reagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottle of L1 (Enzyme Reagent). This working reagent is stable for at least 3 weeks when stored at 2–8°C.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent) and 1 part of L2 (Starter Reagent). Alternatively, 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum. Free from hemolysis. SGOT (AST) is reported to be stable in serum for 3 days at 2–8°C.

Procedure

Wavelength/filter : 340 nm
Temperature : 37°C/30°C/25°C
Light path : 1 cm

Substrate Start Assay

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) 25°C / 30°C*	*(T) 37°C*
Enzyme reagent (L1)	0.8 mL	0.8 mL
Sample	0.2 mL	0.1 mL
Incubate at the assay temperature for 1 minute and add		
Starter reagent (L2)	0.2 mL	0.2 mL

Mix well and read the initial absorbance A_0 and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change perminute (ΔA/min).

Sample Start Assay

Pipette into a clean dry test tube labeled as Test (T):

Addition	*(T)*	*(T)*
Sequence	*25°C / 30°C*	*37°C*
Working reagent	1.0 mL	1.0 mL
Incubate at the assay temperature for 1 minute and add		
Sample	0.2 mL	0.1 mL

Mix well and read the initial absorbance A_0 after 1 minute and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

Substrate/sample start
SGOT (AST) activity in U/L 25°C/30°C = ΔA/min × 952
SGOT (AST) activity in U/L 37°C = ΔA/min × 1746

Temperature Conversion Factors

Assay	*Desired Reporting Temperature*		
Temperature	25°C	30°C	37°C
25°C	1.00	1.37	2.08
30°C	0.73	1.00	1.54
37°C	0.48	0.65	1.00

Linearity

The procedure is linear up to 500 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.250, use only the value of the first 2 minutes to calculate the result, or dilute the sample 1+9 with normal saline (NaCL 0.9%) and repeat the assay (Results × 10).

Note

Samples having a very high activity show a very low initial absorbance as most of the NADH is consumed prior to the start of measurement. If this is suspected then dilute the sample and repeat the assay.

The working reagent or the combined reagent should have an absorbance above 1.000 against distilled water at 340 nm. Discard the reagent if the absorbance is below 1.000.

System Parameters

Reaction	:	UV kinetic	Interval	:	60
Wavelength	:	340 nm	Sample volume	:	0.10 mL
Zero setting	:	Distilled water	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C	Standard	:	—
Incubation time	:	—	Factor	:	1746
Delay time	:	60 sec	Reac. slope	:	Decreasing
Read time	:	180 sec	Linerity	:	500 U/L
No. of read	:	4	Units	:	U/L

SGPT (ALT) (Reitman and Frankel's Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of SGPT (ALT) activity in serum (For in vitro diagnostic use only).

Summary

SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction.

Principle

SGPT converts L-alanine and α ketoglutarate to pyruvate and glutamate. The pyruvate formed reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown colored complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a pyruvate standard. The activity of SGPT (ALT) is read off this calibration curve.

L - Alanine + αKetoglutarate —SGPT, pH 7.4→ Pyruvate + L-Glutamate

Pyruvate + 2,4,DNPH —Alkaline Medium→ 2,4,Dinitrophenyl hydrazone (Brown colored complex)

Normal Reference Values

Serum = 5–35 Units/mL

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*40 assays*
L1 : Substrate reagent	25 mL
L2 : DNPH reagent	2 × 12.5 mL
L3 : NaOH reagent (4 N)	25 mL
S : Pyruvate standard (2 mM)	5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels. Sodium hydroxide can be stored at RT till the expiry mentioned.

Reagent Preparation

All reagents are ready to use except NaOH Reagent (4N) which has to be diluted 1:10 with distilled/deionized water.

Working NaOH reagent: Dilute the sodium hydroxide to 250 mL or for every 1.0 mL of NaOH reagent (4N) add 9.0 mL of water. The working sodium hydroxide reagent is stable at RT till the expiry mentioned, in a plastic bottle.

Sample Material

Serum. Free from hemolysis. SGPT (ALT) is reported to be stable in serum for 3 days at 2–8°C.

Procedure

Wavelength/filter : 505 nm (Hg 546 nm)/Green
Temperature : 37°C and RT
Light path : 1 cm

Plotting of the Calibration Curve

Pipette into 5 clean dry test tubes labeled as 1, 2, 3, 4, and 5:

Addition Sequence Enzyme	*1 0 (mL)*	*2 28 (mL)*	*3 57 (mL)*	*4 97 (mL)*	*5 150 (mL)*
Activity (U/mL)					
Substrate reagent (L1)	0.50	0.45	0.40	0.35	0.30
Pyruvate standard (S)	-	0.05	0.10	0.15	0.20
Distilled water	0.10	0.10	0.10	0.10	0.10
DNPH reagent (L2)	0.50	0.50	0.50	0.50	0.50
Mix well and allow to stand at RT for 20 minutes					
Working NaOH Reagent (L3)	5.00	5.00	5.00	5.00	5.00

Mix well and allow to stand at RT for 10 minutes. Measure the absorbances of the tubes 2–5 against tube 1 (Blank). Plot a graph of the absorbances of tubes 2–5 on the Y-axis versus the corresponding enzyme activity on the 'X'-axis.

Assay

Pipette into clean dry test tubes labeled as Blank (B) and Test (T).

Addition Sequence	*(B) (mL)*	*(T) (mL)*
Substrate reagent (L1)	0.50	0.50
Incubate at 37°C for 3 minutes		

Contd...

Contd...

Addition Sequence	*(B) (mL)*	*(T) (mL)*
Sample	–	0.10
Mix well and incubate at 37°C for 30 minutes		
DNPH reagent (L2)	0.50	0.50
Mix well and allow to stand at RT for 20 minutes		
Distilled water	0.10	
Working NaOH reagent (L3)	5.00	5.00

Mix well and allow to stand at RT for 10 minutes. Measure the absorbances of the Test (T) against Blank (Blank) and read the activity of the test from the calibration curve plotted earlier.

Note

One sample blank is sufficient for each assay series.

If enzymes activity exceeds 150 U/mL dilute the sample with distilled water and repeat the assay. Multiply the value with proper dilution factor.

High concentration of aldehydes and ketones in the sample or icteric or lipemic, samples may cause slightly elevated results. It is recommended to run a sample blank for these samples using serum instead of distilled water in the blank. High levels of serum pyruvate may interfere with the results.

System Parameters

Reaction	:	End point	Interval	:	—
Wavelength	:	505 nm	Sample volume	:	0.10 mL
Zero setting	:	Reagent blank	Reagent volume	:	6.00 mL
Incubation temperature	:	37°C	Standard	:	Calib curve
Incubation time	:	50 min	Factor	:	—
Delay time	:	—	Reac. slope	:	Increasing
Read time	:	—	Linearity	:	150 U/mL
No. of read	:	—	Units	:	U/mL

SGPT (ALT) (Mod. IFCC Method)

(*Courtesy*: Tulip Group of Companies)

For the determination of SGPT (ALT) activity in serum (For in vitro diagnostic use only).

Summary

SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction.

Principle

SGPT (ALT) catalyzes the transfer of amino group between L-alanine and α ketoglutarate to form pyruvate and glutamate. The pyruvate formed reacts with NADH in the presence of Lactate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGPT (ALT) activity in the sample.

$$\text{L- Alanine} + \alpha\text{ Ketoglutarate} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^{+}$$

Normal Reference Values

Serum (males) : Up to 40 U/L at 37°C
(females) : Up to 31 U/L at 37°C.

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*25 mL*	*75 mL*
L1 : Enzyme reagent	20 mL	60 mL
L2 : Starter reagent	5 mL	15 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent : For sample start assays a single reagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottle of L1 (Enzyme Reagent). This working reagent is stable for at least 3 weeks when stored at 2–8°C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent) and 1 part of L2 (Starter Reagent). Alternatively, 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum. Free from hemolysis. SGPT (ALT) is reported to be stable in serum for 3 days at 2–8°C.

Procedure

Wavelength/filter : 340 nm
Temperature : 37°C/30°C/25°C
Light path : 1 cm

Substrate Start Assay

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) 25°C / 30°C*	*(T) 37°C*
Enzyme reagent (L1)	0.8 mL	0.8 mL
Sample	0.2 mL	0.1 mL
Incubate at the assay temperature for 1 minute and add		
Starter reagent (L2)	0.2 mL	0.2 mL

Mix well and read the initial absorbance A_0 and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Sample Start Assay

Pipette into a clean dry test tube labeled as Test (T):

Addition	*(T)*	*(T)*
Sequence	*25°C / 30°C*	*37°C*
Working reagent	1.0 mL	1.0 mL
Incubate at the assay temperature for 1 minute and add		
Sample	0.2 mL	0.1 mL

Mix well and read the initial absorbance A_0 after 1 minute and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

Substrate/sample start

SGPT (ALT) Activity in U/L 25°C/30°C = ΔA/min × 952

SGPT (ALT) Activity in U/L 37°C. = ΔA/min × 1746

Temperature Conversion Factors

Assay Temperature	*Desired 25°C*	*Reporting 30°C*	*Temperature 37°C*
25°C	1.00	1.32	1.82
30°C	0.76	1.00	1.38
37°C	0.55	0.72	1.00

Linearity

The procedure is linear up to 500 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.250, use only the value of the first 2 minutes to calculate the result, or dilute the sample 1 + 9 with normal saline (NaCI 0.9%) and repeat the assay (Results ×10).

Note

Samples having a very high activity show a very low initial absorbance as most of the NADH is consumed prior to the start of measurement. If this is suspected then dilute the sample and repeat the assay.

The working reagent or the combined reagent should have an absorbance above 1.000 against distilled water at 340 mn. Discard the reagent if the absorbance is below 1.000.

System Parameters

Reaction	:	UV kinetic	Interval	:	60
Wavelength	:	340 nm	Sample volume	:	0.10 mL
Zero setting	:	Distilled water	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C	Standard	:	
Incubation time	:	—	Factor	:	1746
Delay time	:	60 sec.	Reac slope	:	Decreasing
Read time	:	180 sec.	Linearity	:	500 U/L
No. of read	:	4	Units	:	U/L

Clinical Relevance of SGPT/ALT/ALAT

A. Increased levels are found in:

1. Hepatocellular disease (moderate to high increase)
2. Active cirrhosis (mild increase)
3. Metastatic liver tumor (mild increase)
4. Obstructive jaundice/biliary obstruction (mild to moderate increase)
5. Infection or toxic hepatitis (markedly increased)
6. Liver congestion (mild to moderate increase)
7. Pancreatitis (mild increase)
8. Hepatic injury in myocardial infarction complicated by shock
9. Infectious mononucleosis (moderate increase)
10. Chronic active hepatitis (moderate increase)
11. Reye's syndrome (moderate increase)
12. Laennec's cirrhosis (mild increase)
13. Alcoholic fatty liver (mild increase).

B. SGOT/SGPT comparison:

1. Although SGOT level is always increased in acute myocardial infarction, the SGPT level does not always increase proportionately.
2. SGPT is usually increased more than SGOT in acute extrahepatic biliary obstruction.

Clinical Relevance of SGOT/AST/ASAT

A. Increased levels occur in:

1. *Myocardial infarction (MI):*
   a. In MI, the SGOT level may be increased 4 to 10 times the normal values.
   b. The SGOT level reaches a peak in 24 hours and returns to normal by the 3rd or 4th day. Secondary rises in SGOT levels suggest extension or recurrence of MI.
   c. The SGOT curve in MI parallels that of CPK.
   d. Elevated levels do not always indicate MI in suspected patients. Severe arrhythmias and severe angina can also cause elevation.
2. *Liver disease:*
   a. Level is always enhanced in cirrhosis of the liver.
   b. In liver disease, the level may be 10 to 100 times the normal.
   c. Liver disorders associated with elevated SGOT levels
      1. Acute hepatitis
      2. Active cirrhosis
      3. Infectious mononucleosis with hepatitis
      4. Hepatic necrosis
      5. Metastatic or primary tumor of liver.
3. *Other diseases associated with elevated SGOT levels:*
   a. Acute pancreatitis
   b. Trauma and irradiation of skeletal muscle
   c. Acute hemolytic anemia
   d. Acute renal disease
   e. Severe tumors
   f. Cardiac catheterization and angiography
   g. Recent brain trauma with brain necrosis
   h. Crushing injuries
   i. Progressive muscular dystrophy
   j. Delirium tremens
   k. Pulmonary infarction
   l. Pericarditis
   m. Cerebrovascular accident.

B. Decreased levels occur in:

1. Beriberi.
2. Uncontrolled diabetes mellitus with acidosis.
3. Occasional liver disease may cause a decrease instead of the expected increase.

C. Interfering factors

1. Slight decreases occur during pregnancy when there is abnormal metabolism of pyridoxine.
2. Drugs that can cause elevated levels:
   a. Aspirin
   b. Codeine
   c. Cortisone
   d. Cholinergics
   e. Theophylline
   f. Vitamin A
   g. Large doses of nicotinic acid
   h. Hydralazine
   i. Meperidine
   j. Erythromycin
   k. Morphine
   l. Tolbutamide
   m. Guanethidine analogs
   n. Griseofulvin.
3. Salicylates may cause falsely decreased or increased SGOT levels.

For diagnosis of myocardial infarction, the SGOT levels should be done on three consecutive days because the peak is reached in 24 hours and levels are back to normal in 3 to 4 days.

GAMMA-GLUTAMYL TRANSPEPTIDASE (GGTP) BLOOD

Normal Values

Adult females	4–25 U 9–31 mU/mL 3.5–13 IU/L 3–33 U/L at 37°C
Adult males	7–40 U 12–38 mU/mL 4–23 IU/L 9–69 U/L at 37°C
Children	
Cord blood	190–270 U/L at 37°C
Premature infants	< 140 U/L at 37°C
1–3 days	56–233U/L at 37°C
4–21 days	0–130U/L at 37°C
3–12 weeks	4–120U/L at 37°C
3–6 months, female	5–35U/L at 37°C
3–6 months, male	5–65U/L at 37°C
> 6 months, female	15–85 IU/L
> 6 months, male	5–55 IU/L
1–15 years	0–23 U/L at 37°C

Glutamyl Transferase (Carboxy Substrate Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of γ-glutamyl transferase activity in serum (For in vitro diagnostic use only).

Summary

γ-glutamyl transferase (GGT) is an enzyme found mainly in serum from hepatic origin, though the highest levels are in the kidneys. Elevated levels are found in hepatobiliary

and pancreatic diseases, chronic alcoholism, myocardial infarction with secondary liver damage, and diabetics.

Principle

GGT catalyzes the transfer of amino group between L-γ-glutamyl-3-carboxy-4 nitroanilide and glycylglycine to form L-γ-Glutamyl-glycylglycine and 5-amino-2-nitrobenzoate. The rate of formation of 5-amino-2-nitrobenzoate is measured as an increase in absorbance, which is proportional to the GGT activity in the sample.

L-γ-Glutamyl-3-carboxy-4-nitroanilide + Glycylglycine $\xrightarrow{GGT}$ L-γ Glutamylglycyl-glycine + 5-amino-2-nltrobenzoate

Normal Reference Values

Serum (Males) : 10–50 U/L at 37°C
(Females) : 7–35 U/L at 37°C.

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*10 × 2 mL*	*35 × 2 mL*
L1 : Buffer reagent	25 mL	80 mL
T1 : Substrate tablets	10 Nos	35 Nos

Storage/stability

Contents are table at 2–8ºC till the expiry mentioned on the labels.

Reagent Preparation

Working reagent: Dissolve 1 substrate tablet in 2.2 mL of buffer reagent. This working reagent is stable for at least 15 days when stored at 2–8°C.

Sample material

Serum. Free from hemolysis. GGT is reported to be stable in serum for 3 days at 2–8°C.

Procedure

Wavelength/filter : 405 nm
Temperature : 37°C / 30°C / 25°C
Light path : 1 cm
Pipette into a clean dry test tube labeled as test (T).

Addition Sequence	*(T) (mL)*
Working reagent	1.0
Incubate at the assay temperature for 1 minute and add	
Sample	0.1

Mix well and read the initial absorbance A_0 after one minute and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

GGT activity in U/L = ΔA/min × 1158.

Temperature Conversion Factors

Assay Temperature	*Desired 25°C*	*Reporting 30°C*	*Temperature 37°C*
25°C	1.00	1.37	1.79
30°C	0.73	1.00	1.30
37°C	0.56	0.77	1.00

Linearity

The procedure is linear up to 700 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.250, use only the value of the first 2 minutes to calculate the result, or dilute the sample 1 + 9 with normal saline (NaCI 0.9%) and repeat the assay (Results × 10).

Note
Samples having a very high activity show a very high initial absorbance. If this is suspected then dilute the sample and repeat the assay.

System Parameters

Reaction	:	Kinetic	Interval	:	30
Wavelength	:	405 nm	Sample volume	:	0.10 mL
Zero setting	:	Distilled water	Reagent volume	:	1.00 mL
Incubation Temperature	:	37°C	Standard	:	
Incubation time	:	—	Factor	:	1158
Delay time	:	30 sec	React slope	:	Increasing
Read time	:	120 sec	Linearity	:	700 U/L
No. of read	:	4	Units	:	U/L

Clinical Relevance

1. Increased GGTP levels are associated with:
   a. Cholecystitis
   b. Cholelithiasis
   c. Cancer metastasis to the liver
   d. Cirrhosis of the liver
   e. Acute pancreatitis
   f. Cancer of the bile duct
   g. Alcoholism
   h. Barbiturate use

i. Lipoid nephrosis
j. Obstruction of biliary tract
k. Hepatotoxic drugs for treatment of cancer increase levels more than the cancer itself.

2. In myocardial infarction, GGTP is usually normal. However, if there is an increase, it occurs about the fourth day after myocardial infarction and probably implies liver damage secondary to cardiac insufficiency.
3. Values are not enhanced in:
   a. Bone disorders
   b. Pregnancy
   c. Skeletal muscle disease
   d. Neonatal hepatitis
   e. Renal failure.

LACTIC DEHYDROGENASE

Lactic dehydrogenase (LDH) is a hydrogen transfer enzyme that catalyzes the following reaction:

$$\text{Lactic acid} + \text{NAD} \overset{\text{LD}}{\rightleftharpoons} \text{Pyruvic acid} + \text{NADH}$$

The reaction is reversible but the conditions for the reverse reaction are different than those for the forward (e.g. the pH for the forward reaction is 8.8 to 9.8 and for the reverse reaction is 7.4 to 7.8).

LD activity can be determined colorimetrically using 2, 4-dinitrophenyl-hydrazine (2, 4-DNPH) as the chromogen in alkaline medium. It is a discrete or two-point method. The alternative method of kinetic measurement or continuous monitoring enzyme assay is definitely superior to the colorimetric method.

LD activity is present in almost all the tissues of the body yet its increased activity in serum reflects several pathologic states. The five isoenzymes of LD (1 to 5) can be separated by electrophoresis. Increased activity of LD-1 is related to myocardial infarction while that of LD-5 is interpreted to be due to liver disorder. LD-1 and LD-5 can also be separated by thermal treatment. If serum is heated to 65ºC for 30 minutes, the thermolabile LD-5 is destroyed. Thus, the difference between the total LD activity of a non-heated serum specimen and the activity of the thermostable isoenzyme (LD-1) gives the measure of LD-5 activity.

Clinical Significance

Serum LD activity is related to myocardial infarction, liver diseases, pernicious anemia, megaloblastic anemia, renal diseases, malignant diseases, and progressive muscular dystrophy.

Normal Values

		SI Units
Wroblewski method 30°C	150–450 U/L	72–217 U/L
Adult		
< Age 60	45–90 U/L	45–90U/L
> Age 60	55–102 U/L	55–102 U/L
Newborn	160–500 U/L	160–500 U/L
Neonate	300–1500 U/L	300–1500 U/L
Infant	100–250 U/L	100–250 U/L
Child	60–170 U/L	60–170 U/L

LDH (P-L) (Mod. IFCC Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of LDH activity in serum (For in vitro diagnostic use only).

Summary

LDH is found in many body tissues particularly heart, liver, skeletal muscle, kidney and RBCs. LDH is found in the form of isoenzymes based on their electrophoretic mobility with each isoenzyme being primarily from different organs. Increased levels are found in myocardial infarction, pulmonary diseases, hepatic diseases, hemolytic anemias, renal diseases and muscular dystrophy.

Principle

LDH catalyzes the reduction of pyruvate with NADH to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the LDH activity in the sample.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

Normal Reference Values

Serum : 230–460 U/L at 37°C

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*25 mL*	*2 × 75 mL*
L1 : Buffer Reagent	20 mL	2 × 60 mL
L2 : Starter Reagent	5 mL	2 × 15 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned.

Reagent Preparation

Reagents are ready to use.

Working reagent: For sample start assays, a single reagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottle of L1 (Buffer Reagent). This working reagent is stable for at least 1 week when stored at 2–8°C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Buffer Reagent) and 1 part of L2 (Starter Reagent). Alternatively, 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum. Free from hemolysis. Total LDH is reported to be stable in serum for 1–3 days at 2–8°C. Freezing inactivates the liver isoenzyme.

Procedure

Wavelength/filter : 340 nm
Temperature : 37°C/30°C/25°C
Light path : 1 cm

Substrate Start Assay

Pipette into a clean dry test tube labeled as test (T):

Addition Sequence	*(T) 25°C / 30°C*	*(T) 37°C*
Buffer reagent	0.8 mL	0.8
Sample	0.05 mL	0.02
Incubate at the assay temperature for 1 minute and add		
Starter reagent	0.2 mL	0.2 mL

Mix well and read the initial absorbance A_0 and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Sample Start Assay

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) 25°C/30°C*	*(T) 37°C*
Working reagent	1.0 mL	1.0 mL
Incubate at the assay temperature for 1 minute and add		
Sample	0.05 mL	0.02 mL

Mix well and read the initial absorbance after 1 minute and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

Substrate/Sample Start

LDH activity in U/L = ΔA/min × 3333
25°C/30°C
37°C = ΔA/min × 8095

Temperature Conversion Factors

Assay	*Desired Reporting Temperature*		
Temperature	*25°C*	*30°C*	*37°C*
25°C	1.00	1.33	1.92
30°C	0.75	1.00	1.44
37°C	0.52	0.70	1.00

Linearity

The procedure is linear up to 2000 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.250, use only the value of the first 2 minutes to calculate the result, or dilute the sample 1 + 9 with normal saline (NaCl 0.9%) and repeat the assay (results × 10).

Note

Samples having a high activity, show a very low initial absorbance as most of the NADH is consumed prior to the start of measurement. If this is suspected then dilute the sample and repeat the assay.

The working reagent or the combined reagent should have an absorbance above 1.000 against distilled water at 340 nm. Discard the reagent if the absorbance is below 1.000.

RBCs have a very high LDH content and hence, hemolyzed samples should not be used.

System Parameters

Reaction	:	UV Kinetic	Interval	:	60
Wavelength	:	340 nm	Sample volume	:	0.02 mL
Zero setting	:	Distilled water	Reagent volume	:	1.00 mL
Incubation temperature	:	37°C	Standard	:	
Incubation time	:	—	Factor	:	8095
Delay time	:	60 sec	React. slope	:	Decreasing
Read time	:	180 sec	Linearity	:	2000 U/L
No. of read	:	4	Units	:	U/L

Clinical Relevance

Myocardial Infarction

The elevation of LDH that follows an MI is characterized by:

1. High levels within 12 to 24 hours of infarction (18 hours) and 2 to 10 times normal.
2. Elevation that may continue for 6 to 10 days (larger than SGOT or CK). For this reason, LDH determination may be useful in the late diagnosis of MI. Elevations return to normal in 8 to 14 days.

Pulmonary Infarction

In pulmonary infarction, there is usually an increased LDH within 24 hours of the onset of pain. The pattern of normal SGOT and elevated LDH that levels 1 to 2 days after an episode of chest pain provides evidence for pulmonary infarction.

Conditions in general and according to degree of increase in levels.

1. Elevated levels of LDH are observed in a variety of conditions:
   a. Acute MI
   b. Acute leukemia
   c. Hemolytic anemias
   d. Hepatic disease
   e. Skeletal muscle necrosis
   f. Sprue
   g. Acute pulmonary infarction
   h. Malignant neoplasms, extensive cancer
   i. Acute renal infarction and chronic renal disease
   j. Shock with necrosis of minor organs
   k. Myxedema.
2. The greatest increase (2–40 times normal) is seen in:
   a. Megaloblastic anemia
   b. Extensive cancer (especially hepatic metastases)
   c. Shock and anoxia.
3. Moderate increase (2–4 times normal) is seen in:
   a. MI
   b. Pulmonary infarction
   c. Hemolytic anemia
   d. Granulocytic or acute leukemia
   e. Infectious mononucleosis
   f. Progressive muscular dystrophy.
4. Slight increase occurs in:
   a. Delirium tremens
   b. Hepatitis
   c. Obstructive jaundice/cholangitis
   d. Cirrhosis
   e. Nephrotic syndrome
   f. Hypothyroidism.

Decreased LDH levels are associated with a good response to cancer therapy

Elevated urine LDH levels occur in:

1. Cancer of kidney or bladder
2. Glomerulonephritis
3. Malignant hypertension
4. Lupus nephritis
5. Acute tubular necrosis
6. Renal transplantation and hemograft rejection
7. Pyelonephritis (sometimes).

Interfering Factors

1. Strenuous exercise and the muscular exertion involved in childbirth will cause increased levels.
2. Skin diseases can cause falsely increased levels.
3. Hemolysis of RBCs due to freezing, heating, or shaking the blood sample will cause falsely increased levels.
4. Drugs that may elevate levels comprise:
   a. Codeine
   b. Clofibrate
   c. Meperidine
   d. Mithramycin
   e. Morphine
   f. Procainamide.
5. Oxalate is known to cause decreased levels.

Electrophoresis of LDH Isoenzymes

Normal Values

Isoenzyme	Organ related	Percentage of total LDH
Isoenzyme 1	Cardiac	(25–40%)
Isoenzyme 2	Cardiac	(35–46%)
Isoenzyme 3	Pulmonary	(17–32%)
Isoenzyme 4	Hepatic	(9–18%)
Isoenzyme 5	Hepatic	(6–17%)

Variation of 2% to 4% are considered physiologically normal (isoenzymes 1 to 5 are also present in human skeletal muscle).

Test Significance

Electrophoresis or separation of LDH identifies the 5 isoenzymes or fractions of LDH, each with its own characteristics physical and chemical properties. Fractionating the LDH activity multiplies its diagnostic relevance since LDH is found in many organs. The LDH isoenzymes are released into the bloodstream when tissue necrosis occurs. However, a complete knowledge of the clinical history is necessary to properly interpret the resulting patterns. The isoenzymes are evaluated in terms

of patterns established, not on the basis of the value of a single isoenzyme. The 5 isoenzyme fractions of LDH show different patterns in various disorders. Abnormalities in the pattern suggest, which tissue has been damaged and help to diagnose myocardial infarction, pulmonary infarction, and liver disease. This test is sensitive enough to detect hepatic fraction in infectious hepatitis before clinical jaundice appears. It is in confirming the diagnosis of suspected MI that the separation of LDH isoenzymes finds its most frequent application, especially when a second infarct occurs shortly after the first. In these cases, the ECG is already abnormal, but the isoenzyme pattern will show increased LDH_1, indicating the release of more of the cardiac enzyme.

Clinical Relevance

Abnormal patterns reflect damaged tissue

1. LDH_1 and LDH_2 are increased in MI and in some hemolytic anemias.
2. LDH_3 is increased in pulmonary infarction and extensive pneumonia.
3. LDH_5 is increased in liver disease.
4. An increase in LDH_2, LDH_3, LDH_4 is common in malignant disease.
5. The LDH pattern will be essentially the same in MI, pernicious anemia, and renal infarction. This is because RBC's and the kidney have an isoenzyme pattern similar to that of heart muscle.
6. In most cancers, one to three of the bands (LDH_2, LDH_3, and LDH_4) are frequently increased. A notable exception is in seminomas and dysgerminomas when LDH_1, and LDH_2 are increased. Frequently, an increase in LDH_3 may be the first indication of the presence of cancer.

Creatine Kinase K (NAC Act) (Mod. IFCC Method)

(*Courtesy*: Tulip Group of Companies)
For the determination of CK activity in serum (For in vitro diagnostic use only).

Summary

Creatine kinase (CK) is mainly found in all muscle and brain tissue. It plays an important role in the energy storing mechanism of the tissues. Increased levels are found in myocardial infarction, cerebrovascular diseases, muscular dystrophy, pulmonary infarction and, electrical shocks. Increased levels can also be caused by intramuscular injections, strenuous exercise and recent surgery. Early pregnancy may produce decreased levels.

Principle

Creatine kinase catalyzes the reaction between creatinine phosphate and ADP to form creatine and ATP. The ATP formed along with glucose is catalyzed by hexokinase to form glucose 6 phosphate. The glucose 6 phosphate reduces NADP to NADPH in the presence of glucose 6 phosphate dehydrogenase. The rate of reduction of NADP to NADPH is measured as an increase in absorbance, which is proportional to the CK activity in the sample.

$$\text{Creatine Phosphate} + \text{ADP} \xrightarrow{\text{Creatine Kinase}} \text{Creatine} + \text{ATP}$$

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{G-6-PDH}]{\text{Hexokinase}} \text{Glucose 6 phosphate} + \text{ADP}$$

$$\text{G - 6 - P} + \text{NADP} \longrightarrow \text{Gluconate - 6 - P} + \text{NADPH} + \text{H}$$

Normal Reference Values

Serum (male) : 24–195 U/L at 37°C
(female) : 24–170 U/L at 37°C.

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*2 × 10 mL*	*2 × 25 mL*
L1 : Enzyme reagent	2 × 8 mL	2 × 20 mL
L2 : Starter reagent	2 × 2 mL	2 × 5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: For sample start assays, a single reagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottle of L1 (Enzyme Reagent). This working reagent is stable for at least 10 days when stored at 2 to 8°C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent) and 1 part of L2 (Starter Reagent). Alternatively, 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum. Free from hemolysis. CK is reported to be stable in serum for 3 days at 2 to 8°C.

Procedure

Wavelength/filter : 340 nm

Temperature : 37°C / 30°C / 25°C
Light path : 1 cm

Substrate Start Assay

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	*(T) 25°C / 30°C*	*(T) 37°C*
Enzyme reagent (L1)	0.8 mL	0.8 mL
Sample	0.05 mL	0.02 mL
Incubate at the assay temperature for 5 minutes and add		
Starter reagent (L2)	0.2 mL	0.2 mL

Mix well and read the initial absorbance A_0 and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Sample Start Assay

Pipette into a clean dry test tube labeled as test (T):

Addition Sequence	*(T) 25°C / 30°C/*	*(T) 37°C*
Working reagent	1.0 mL	1.0 mL
Incubate at the assay temperature for 1 minute and add		
Sample	0.05 mL	0.02 mL

Mix well and read the initial absorbance A_0 after 10 minutes and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

Substrate/Sample start

CK Activity in U/L 25°C/30°C = ΔA / min × 3333
37°C = ΔA / min × 8095

Temperature Conversion Factors

Assay Temperature	*Desired 25°C*	*Reporting 30°C*	*Temperature 37°C*
25°C	1.00	1.56	2.44
30°C	0.64	1.00	1.56
37°C	0.41	0.63	1.00

Linearity

The procedure is linear up to 2000 U/L at 37°C. If the absorbance change (ΔA/min) exceeds 0.250, use only the value of the first 2 minutes to calculate the result, or dilute the sample 1+ 9 with normal saline (NaCl 0.9%) and repeat the assay (Results × 10).

Note

Samples having a high activity show a very high initial absorbance as most of the NADP is converted prior to the start of measurement. If this is suspected then dilute the sample and repeat the assay.

The working reagent or the combined reagent should have an absorbance below 0.800 against distilled water at 340 nm. Discard the reagent if the absorbance is above 0.800.

System Parameters

Reaction	: UV Kinetic	Interval	: 60
Wavelength	: 340 nm	Sample volume	: 0.02 mL
Zero setting	: Distilled water	Reagent volume	: 1.00 mL
Incubation temperature	: 37°C	Standard	:
Incubation time	: -	Factor	: 8095
Delay time	: 60 sec	React. slope	: Increasing
Read time	: 180 sec	Linearity	: 2000 U/L
No. of read	: 4	Units	: U/L

CK MB (NAC Act) (Immunoinhibition/Mod. IFCC method)

(*Courtesy*: Tulip Group of Companies)

For the determination of CK-MB activity in serum (For in vitro diagnostic use only).

Summary

CK is dimeric molecule composed of M and B subunits, which are immunologically distinct. It exists as three main isoenzymes CK - MM, CK - MB, and CK - BB. The CK - MM is found in the muscle while CK - MB is found mainly in the myocardial cells. The CK - BB is found mainly in the brain and lungs, and enters the bloodstream only on injury to these organs like cerebrovascular accident or pulmonary infarction. Normally CK - MM is found in the blood. CK - MB levels increase significantly 4–6 hours following a myocardial infarction and peak at around 12 to 24 hours after the infarct. The levels return to normal, in case of no further myocardial damage, after 24 to 48 hours. Hence, the increased levels of CK - MB along with elevated levels of total CK is a good indicator of myocardial infarction. CK - MB levels usually do not rise in chest pain caused by angina, pulmonary embolism or congestive heart failure.

Principle

CK - M fractions of the CK - MM and the CK - MB in the sample are completely inhibited by an anti CK - M antibody present in the reagent. Then the activity of the CK - B fraction is measured by the CK (NAC act) method, the CK - MB activity is obtained by multiplying the CK - B activity by two.

Normal Reference Values

Serum up to 24 U/L at 37°C.

Indication of myocardial infarction is based on the following factors:

Total CK (male) : < 195 U/L at 37°C
(female) : < 170 U/L at 37°C
CK - MB : < 24 U/L at 37°C
CK - MB to Total CK : < 6%

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	*2 × 10 mL*	*2 × 25 mL*
L1 : Enzyme reagent	2 × 8 mL	2 × 20 mL
L2 : Starter reagent	2 × 2 mL	2 × 5 mL

Storage/stability

Contents are stable at 2–8°C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent: For sample start assays, a single reagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottle of L1 (Enzyme Reagent). This working reagent is stable for at least 10 days when stored at 2–8°C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent) and 1 part of L2 (Starter Reagent). Alternatively, 0.8 mL of L1 and 0.2 mL of L2 may also be used instead of 1 mL of the working reagent directly during the assay.

Sample Material

Serum. Free from hemolysis.

Procedure

Wavelength/filter : 340 nm
Temperature : 37°C / 30°C / 25°C
Light path : 1 cm

Substrate Start Assay

Pipette into a clean dry test tube labeled as test (T):

Addition Sequence	*(T) 25°C / 30°C/37°C*
Enzyme reagent (L1)	0.8 mL
Sample	0.05 mL
Incubate at the assay temperature for 1 minute and add	
Starter reagent (L2)	0.2 mL

Mix well and read the initial absorbance A_0 after 5 minutes and repeat the absorbance reading after every 1, 2, and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Substrate Start Assay

Pipette into a clean dry tube labeled as test (T).

Addition Sequance	*(T) 25°C/30°C/37°C*
Working reagent	1.0 mL
Incubate at the assay temperature for 1 minute and add	
Sample	0.05 mL

Mix well and read the initial absorbance A_0 after 10 minutes and repeat the absorbance reading after every 1, 2 and 3 minutes. Calculate the mean absorbance change per minute (ΔA/min).

Calculations

Substrate/Sample start

CK - B activity in U/L 25°C / 30°C /37°C = ΔA/min × 3333
CK-MB activity in U/L 25°C / 30°C /37°C = ΔA/min × 6666

Temperature Conversion Factors

Assay Temperature	*Desired 25°C*	*Reporting 30°C*	*Temperature 37°C*
25°C	1.00	1.56	2.44
30°C	0.64	1.00	1.56
37°C	0.41	0.63	1.00

Linearity

This procedure is linear up to 1000 U/mL. Inhibition of CK - MM is up to 1500 U/L at 37°C. If the total CK activity exceeds this limit dilute the sample 1 + 9 with normal saline (NaCl 0.9%) before estimating CK - MB (Results × 10).

Note

This method will also measure any CK - BB isoenzyme present in the sample. The amount of CK - BB is usually negligible in serum from normal individuals or in patients with myocardial infarction.

A macro form of BB has been observed and this will be measured as CK - B activity, if the CK - B activity exceeds 20% of the total CK activity the presence of macro BB should be suspected.

The working reagent or the combined reagent should have an absorbance below 0.800 against distilled water at 340 nm. Discard the reagent if the absorbance is above 0.800.

System Parameters

Reaction	:	UV Kinetic	Interval	:	60 sec
Wavelength	:	340 nm	Sample volume	:	0.05 mL
Zero setting	:	Distilled water	Reagent volume	:	1.00 mL
Incubation temprature	:	37°C	Standard	:	
Incubation time	:	—	Factor	:	6666
Delay time	:	300 sec	React. slope	:	Increasing
Read time	:	180 sec	Linearity	:	1000 U/L
No. of read	:	4	Units	:	U/L

Clinical Relevance

The two enzymes CK and adenylate kinase (AK) play a decisive role in the synthesis of ATP, the immediate energy source of the muscle, the CNS and many proliferating tissues. Human creatinine kinase is synthesized by a number of different genes. The respective gene products are called CK-M (muscle), CK-B (brain) and CK-Mi (mitochondria). The total CK activity measurable in serum is composed of the activities of the cytoplasmic, dimeric isoenzymes (CK-MM, CK-MB, CK-BB) and their postsynthetically modified forms, and the activities of the macrocreatinine kinase (macro-CK).

CK Isoenzymes

Isoenzyme of total CK:

CK-BB (brain) 0–3 (found mainly in brain, also in smooth muscle, thyroid, lungs and prostate)

CK-MB (heart) 0–6 (found mainly in myocardium, also in tongue, diaphragm and skeletal muscle)

CK-MM (muscle) 90–97 (found mainly in the skeletal muscle).

Approximate distribution of the CK isoenzymes in human organs

Tissue	U/g	CK-MM	CK-MB	CK-BB	CK-mito
Skeletal	800–4000	++++	(+)	(+)	+ muscle
Myocardium	240–800	+ + +	++	*(+)*	+ +
Brain	≤ 550	-	-	+ + +	+ +
Bladder	≤ 135	-		++++	+
Blood	≤ 0.2	++++	(+)		
Colon	≤ 200	(+)	(+)	++++	+
Umbilical cord blood	≤ 1.0	+ + + +	(+)	+	?
Prostate	≤ 135	-	-	++++	?
Uterus	≤ 400	-	-	++++	+
Vein wall	≤ 60	-	-	++++	?

(+ + + + :> 75%, + + + :50-75%, + + :25-50%/ +:5-25%, (+):< 5% at 37°C)

Normal Values (at 37°C)

Adult males 24–195 U/L, Adult females 24–170 U/L

Children**:** Umbilical cord 175–402 U/L, Newborns; ≤ 5 days 195–700 U/L, < 6 months 41–330 U/L, > 6 months 24–229 U/L. (Conversion of U/L into μ Kat/L: 1 μKat/L = 60 U/L)

CK-MB: Normal value ≤ 24 U/L CK-BB:, For adults < 2 U/L.

CK-MM: Reference values for total CK activity for adults can be used.

CK-mito: Normal value is < 2U/L.

Clinical data, ECG findings and the results of CK determination complement each other with regard to clinical sensitivity and specificity. In spite of determination of CK-MB the differential diagnosis of myocardial infarction /skeletal damage presents problems in the following circumstances**:** extensive skeletal muscle damage and concomitant small infarction, chronic skeletal muscle disease and myocardial involvement or MI after coronary artery bypass grafting. In these cases, determination of one of the cardiospecific troponins is necessary.

Diagnostic Alert

As adenylate kinase (AK) interferes with CK estimation and AK is found to a greater extent in the Indian population. It becomes imperative to use reagents that are capable of inhibiting AK so as not to overestimate CK. A report generated by employing inappropriate kits can initiate unnecessary therapy.

Total CK and CK-MB trends in acute myocardial infarction:

	Total CK	*CK-MB*
Initial rise:	2–6 hours after onset of damage	4–8 hours after onset of damage
Peak levels:	18–36 hours after onset of damage	18–24 hours after the onset of damage
Return to basal levels:	3–6 days after onset of damage	3 days after onset of damage

6% Rule

The decision criterion is an increase in the total CK activity to > 240 U/L (37°C) within the diagnostic time window and a simultaneous increase in CK-MB activity. A CK-MB fraction more than 6% of the total CK activity is regarded as diagnostic for MI. A fraction < 6% indicates skeletal muscle damage. The clinical specificity of the 6% rule is high as the number of false positive results caused by presence of extracardiac CK-MB is small. However, following this rule, smaller MIs may be missed. False positive values can be caused by Adenylate Kinase, which occurs in large quantities in the liver and in blood cells.

Increased Total CK

Amyotrophic lateral sclerosis, anoxia, atresia (biliary), bowel injury, brain tumor, burns (thermal, electrical), cancer (breast, lung, oat cell, gastrointestinal, prostatic), carbon monoxide poisoning, cardiomyopathy (cobalt-beer), carrier state (for Duchenne's muscular dystrophy), cerebrovascular accident, CNS trauma, coma (hepatic), convulsions, coughing (severe), delirium tremens, dermatomyositis, eosinophilia-myalgia syndrome, exercise, head injury, hemodialysis, hypokalemia (severe), hypothermia, hypothyroidism, infarction (bowel, cerebral, myocardial, prostate), intoxication (alcohol, salicylate), intramuscular injection (recent), labor, leptospirosis, malignant hyperthermia, meningoencephalitis, muscle spasms, muscular dystrophy (Duchenne's, limb-girdle, fascioscapulohumeral), myocarditis, myoglobinuria, myopathy (from alcoholism), myotonic dystrophy, myxedema, necrosis of striated muscle, organ rejection (heart transplant), parturition, polymyositis, pregnancy, prostatic injury, psychosis (acute with agitation), pulmonary edema, pulmonary embolism, renal failure, renal insufficiency (chronic), Reye's syndrome, rhabdomyolysis, Rocky Mountain spotted fever, shock, skeletal muscle disorders, status epiepticus, striated muscle atrophy (acute), subarachnoid hemorrhage, surgery (bowel, cardiac, CNS, prostate), tachycardia, thyrotoxicosis, toxic shock syndrome (day 7), trauma (muscular), typhoid fever, and very muscular people.

Increased CK-BB

Anoxia, atresia (biliary), cancer (breast, gastrointestinal, oat cell, prostatic, widespread malignancies), cerebrovascular accident (hemorrhage, infarction), hemodialysis, hypothermia, intestinal necrosis, labor, malignant hyperthermia, renal failure, shock, surgery (CNS) and uremia.

Increased CK-MB

Anoxia, burns (electrical, thermal), cancer (lung), carbon monoxide poisoning, cardiomyopathy (cobalt-beer), collagen vascular diseases, congestive heart failure (rare), coronary angiography (rare), coronary insufficiency (rare), hypothermia, hypothyroidism, malignant hyperthermias, muscular dystrophy (Duchenne's), myocardial infarction, myocarditis, myoglobinuria (severe), polymyositis, pulmonary embolism, renal insufficiency (chronic), Reye's syndrome, rhabdomyolysis, Rocky Mountain spotted fever, surgery (cardiac, valve replacement), SLE, and trauma (cardiac).

Increased CK-MM

Cardiac catheterization (with myocardial damage), cardioversion, coronary arteriography (with myocardial damage), hypothyroidism, intramuscular injection, muscle trauma, myocardial infarction, psychosis (acute with agitation), Reye's syndrome, shock, surgery, and trauma (skeletal muscle).

Decreased Total CK

Addison's disease, anterior pituitary hyposecretion, connective tissue disease, hepatic disease (alcoholic), low muscle mass, metastatic neoplasia, and pregnancy (first half). Drugs include steroids.

Decreased CK-BB, CK-MB, CK-MM

Clinically insignificant/not applicable.

Interfering Factors

1. Strenuous exercise (up to 3 times normal) and surgical procedures that damage skeletal muscle may cause increased levels.
2. High doses of salicylates may cause increased levels.
3. Athletes have a higher value because of greater muscle mass.
4. Multiple intramuscular injections may cause increased levels.
5. Drugs that may cause increased levels include
   a. Amphotericin B
   b. Ampicillin IM

c. Carbenicillin IM
d. Chlorpromazine IM
e. Clofibrate.

Liver Disease (Serum Enzyme Patterns) Values are x Times the Upper Normal Limits

Condition	*GOT*	*GPT*	*LDH*	*SAP*
Acute viral hepatitis	15–20	15–20	6–8	1–2
Obstructive jaundice				
(intra/extrahepatic)	3–4	3–4	1–2	3–6
Cirrhosis (portal)	2–3	2–3	1–2	1–2
Secondary deposits in liver without jaundice	1–2	1–2	1–3	1–3

GOT—glutamic-oxaloacetic transaminase
GPT—glutamic pyruvic transaminase (tends to be higher than GOT in acute liver disorders)
LDH—lactate dehydrogenase
SAP—(Serum) alkaline phosphatase
(In obstructive jaundice in addition to others, 5-nucleotidase rises 4–6 times the upper normal limit (UNL) and in tumor deposits in liver GGTP-Gamma-Glutamyl Transpeptidase rises 4–20 times the UNL.

AUTOMATION IN CLINICAL CHEMISTRY: RANDOM ACCESS AUTOANALYZER

These kinds of completely automatic analyzers are best suited for laboratories with moderate to heavy workload.

For a laboratory considering an automated clinical chemistry system, there are a number of criteria, which are very important to the Indian/tropical environment:

1. Design
2. Support
3. Cost.

Design

System design is an important factor and one should answer questions as**:**

a. Does it have miles of tubing which can leak and which will need replacing?
b. Is the dispensing mediated by banks of syringes which can (and will) leak, and will need replacing?
c. Are the moving parts easily accessible?
d. Does the system need external drains?
e. Does the system need external water supplies?
f. Is the software open (can I change volumes, times, etc.)?
g. Is the system open (can I use any reagent I like)?
h. Is the system flexible (can I do drugs, drug abuse in urine—DAU, special proteins, and general developmental work, etc.)?
i. Is the system truly walkaway?
j. Can the system be interlinked with other equipment?
k. Can the system work with a data management system?

Support

Is the system supported in India by an organization with true accountability, professionalism, and infrastructure such that you can depend on getting help when you need it?

Cost

1. Capital cost of the equipment
2. Recurrent cost of reagents and consumables.

When looking at the cost of an instrument it is vital to compare "like" with "like". This means that it is important to develop an understanding of the design feature differences which can translate into very real benefits to the user. This means in turn that one should not just compare quoted prices on the assumption that one system is much like another, they are not.

To summarize, when choosing an analyzer one should think very seriously about the suitability of the equipment to India. Issues of throughput, and unit price should not distract the buyer from fundamentals of good design because in the long run good design will save money.

Roche Hitachi 911 Chemistry Analyzer

(Courtesy: Recho Hitachi)
The Hitachi 911 is a fully automated, discrete, computerized chemistry analyzer (Fig. 20.1) that uses serum, urine, plasma and CSF sample types to perform in vitro quantitative and qualitative tests on a wide range of alalytes. In addition, it is capable of performing potentiometric and photometric assays. The Hitachi 911 analyzer is composed of two units; the analytical unit and the control unit: The analytical unit consists of an ISE system, a photometric measuring system, and a CPU. The control unit consists of a monitor (CRT), a keyboard, and a printer. Features of the Hitachi 911 include; STAT results available quickly, ready to use 24 hours per day, 360 tests/hr throughput (photometric), 720 tests/hr throughput with ISE (Ka^+, Na^+, Cl^-), 46 programmable tests, automatic calibration, and refrigerated storage for 64 reagent containers.

Assay Types

- Monochromatic
- Bichromatic

- Endpoint
- Kinetic
- Simultaneous endpoint and kinetic
- Endpoint with sample blanking
- Kinetic with sample blanking
- Simultaneous double endpoint and double kinetic.

Sampling System

- Photometric 360 tests/h
- Photometric and ISE 760 tests/h
- Sample volume per test: 3–50 mL
- Sampling rate: Once every 10 seconds for photometric chemistries, once every 20 seconds for ISE
- Bar code reader formats: Coda bar, interleaf 2 of 5, code 39, code 128.

ISE System

- Sample volume:15 mL
- Photometric and ISE 760 tests/h.

Reagent System

Reagent dispense volume: 25–300 mL per reagent (in 1 mL increments).

Reagent storage capacity: 2 compartments (12 C or less) 32 position each for reagent. Each reagent compartment has an additional position #33 for hitergent total reaction volume/test: 250–500 mL.

Quality Control Criteria given with Clinical Chemistry Chapter apply to Enzymology section also.

FIG. 20.1: Roche Hitachi 911 chemistry analyzer

CHAPTER 21

Blood Gases and Electrolytes

BLOOD GASES

Introduction

Reasons for obtaining blood gases:

1. Assessment of adequacy of oxygenation
2. Assessment of adequacy of ventilation
3. Assessment of acid-base status by measuring the respiratory and non-respiratory components.

Reasons for using arterial blood rather than venous blood to measure blood gases:

1. *Arterial blood is a good way to sample a mixture of blood that has come from various parts of the body.*
   a. Venous blood in an extremity gives information mostly about that extremity. The metabolism in the extremity can differ from the metabolism in the body as a whole. This difference is accentuated.
      i. In shock, when the extremity is cold or under perfused
      ii. With local exercise of extremity, as opening and closing the fist
      iii. In local infection of the extremity.
   b. Blood from a central venous catheter usually is an incomplete mix of venous blood from various parts of the body. For a sample of completely mixed blood, a sample would have to be obtained from the right ventricle or pulmonary artery, and even then information is not obtained about how well the lungs are oxygenating the blood.
2. *Arterial blood gives the added information of how well the lungs are oxygenating the blood.*
   a. If it is known that arterial O_2 concentration is normal (indicating that the lungs are functioning normally), but the mixed venous O_2 concentration is low, it can be inferred that the heart and circulation are failing.
   b. Oxygen measurements of central venous catheter blood can tell if the tissues are getting oxygenated, but they do not separate the contribution of the heart from the lungs. If central venous catheter blood has a low O_2 concentration, it means either that:
      i. The lungs have not oxygenated the arterial blood well, so that venous blood has a low concentration, or
      ii. The heart is not circulating the blood well. In this case, the tissues of the body must take more than the usual amount of O_2 from each cardiac cycle because the blood is flowing slowly. This produces a low venous O_2 concentration.

Note: The site of arterial puncture must satisfy three requirements:

1. Available collateral blood flow
2. Superficial or easily accessible
3. Periarterial tissues (should be nonsensitive).

The radial artery satisfies the criteria tested above, although the brachial and femoral are also arteries of choice.

Procedure for Obtaining Arterial Blood Sample

1. Place the patient either in a sitting or supine position.
2. Elevate the wrist with a small pillow and ask the patient to extend fingers downward (this will flex the wrist and move the radial artery closer to the surface).
3. Palpate the artery and rotate the patient's hand back and forth until a good strong pulse is felt.

4. Swab the area liberally with an antiseptic agent such as betadine.
5. Optional: Anesthetize the area with a small amount of 1% xylocaine (approximately ¼ mL or less). This allows a second attempt without undue pain if the first attempt is a failure.
6. Using a 20- or 21-gauge needle, make the puncture and then attach the preheparinized 12 mL syringe once the artery has been entered.
7. Pull the plunger on the syringe (being careful not to accidentally pull the needle out of the artery) and collect a 3 to 5 mL sample.
8. Withdraw needle and place a 4" × 4" absorbent bandage over the puncture site and maintain pressure with two fingers for a minimum of 2 minutes.
9. Meanwhile, any air-bubbles in the blood sample should be expelled as quickly as possible; the syringe should be capped and gently rotated to mix heparin with blood.
10. If the sample is not going to be analyzed for 15–20 minutes, place it in an icewater container until it can be analyzed.

Clinical Alert

1. Arterial gases will not indicate to what degree the patient is suffering from an abnormality. For this reason, the vital signs and mental function of the patient must be used as guides to determine adequacy of tissue oxygenation.
2. Arterial puncture site must have pressure applied and be watched carefully for bleeding.
3. Blood for gases (and electrolytes) must be drawn without trauma and be protected from room air at all times. Be aware that air bubbles in the syringe will also change gas values.

Blood Gas Symbols

Large capital letters are used as primary symbols for blood.

C = Concentration of gas in blood
S = Percent saturation of hemoglobin with CO_2 or O_2
Q = Volume of blood
QT = Volume of blood per unit time
P = Gas pressure or partial pressure of a gas in a gas mixture or in blood.

To indicate whether blood is capillary, venous, arterial, lower case letters are used as subscripts:

v = Venous blood
a = Arterial blood
c = Capillary blood
s = Shunted blood.

Combination of Symbols

PO_2 = Oxygen tension or partial pressure of oxygen.
PvO_2 = Venous oxygen tension or partial pressure of oxygen in venous blood.
PaO_2 = Arterial oxygen tension or partial pressure of oxygen in arterial blood.
PCO_2 = Partial pressure of carbon dioxide.
$PaCO_2$ = Partial pressure of carbon dioxide in arterial blood.
$PvCO_2$ = Partial pressure of carbon dioxide in venous blood
SO_2 = Oxygen saturation
SaO_2 = Percent saturation of oxygen in arterial blood
SvO_2 = Percent saturation of oxygen in venous blood
TCO_2 = Total carbon dioxide content

Blood Gases, Arterial (ABG) Blood

Normal Values

Must be corrected for body temperature.

		SI units
pH		
Adults	7.35–7.45	7.35–7.45
Panic values	< 7.2 and > 7.6	< 7.2 and > 7.6
Children		
Birth–2 months	7.32–7.49	7.32–7.49
2 months–2 years	7.34–7.46	7.34–7.46
Over 2 years	7.35–7.45	7.35–7.45
$PaCO_2$	35–45 mm Hg	4.7–6.0 kPa
Panic values	< 20 mm Hg	< 2.7 kPa
PaO_2	75–100 mm Hg	10.0–13.3 kPa
Panic values	< 40 mm Hg	< 5.3 kPa
HCO_3	22–26 mEq/L	22–26 mmol/L
Panic values	< 10 mEq/L	< 10 mmol/L
O_2 saturation	96–100%	0.96–1.00
Panic values	< 60%	< 0.60
Oxyhemoglobin dissociation		
Curve	No shift	

Blood Gases, Capillary Blood

Normal Values

Must be corrected for body temperature.

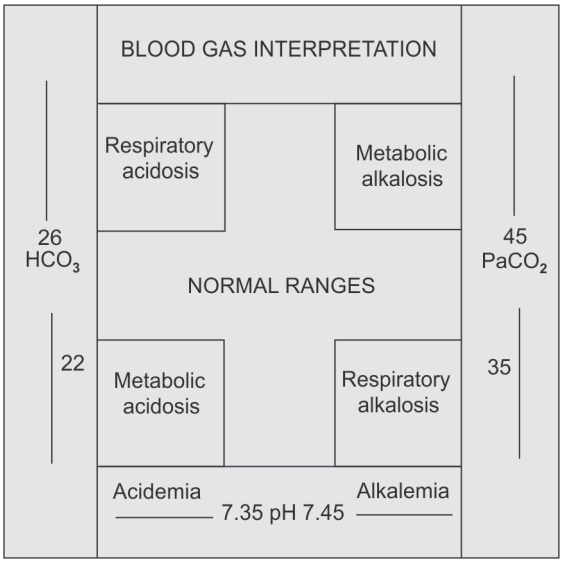
		SI units
pH		
Adults	7.35–7.45	7.35–7.45
Panic values	< 7.2 or > 7.6	< 7.2 or > 7.6
Children (arterialized capillary sample)		
Birth–2 months	7.32–7.49	7.32–7.49
2 months–2 years	7.34–7.46	7.34–7.46
Over 2 years	7.35–7.45	7.35–7.45
PCO_2	26.4–41.2 mm Hg	3.5–5.4 kPa
Panic values	< 20 mm Hg	< 2.7 kPa
	> 70 mm Hg	
PO_2	75–100 mm Hg	10.0–13.3 kPa
Panic values	< 40 mm Hg	< 5.3 kPa
HCO_3	22–26 mEq/L	22–26 mmol/L
Panic values	< 10 mEq/L	< 10 mmol/L
O_2 saturation	96–100%	0.96–1.00
Panic value	< 60%	< 0.60
Oxyhemoglobin Dissociation Curve	No shift	

Blood Gases, Capillary Blood

Normal Values

Must be corrected for body temperature.

		SI Units
pH		
Adults	7.35-7.45	7.35-7.45
Panic Values	<7.2 or >7.6	<7.2 or >7.6
Children (arterialized capillary sample)		
Birth-2 months	7.32-7.49	7.32-7.49
2 months-2 years	7.34-7.46	7.34-7.46
Over 2 years	7.35-7.45	7.35-7.45
PCO_2	26.4-41.2 mm Hg	3.5-5.4 kPa
Panic values	<20 mm Hg	<2.7 kPa
	>70 mm Hg	
PO_2	75-100 mm Hg	10.0-13.3 kPa
Panic values	<40 mm Hg	<5.3 kPa
HCO_3	22-26 mEq/L	22-26 mmol/L
Panic values	<10 mEq/L	<10 mmol/L
O_2 Saturation	96-100%	0.96-1.00
Panic value	<60%	<0.60



Blood gases interpretation

Blood Gases, Venous Blood

Normal Values

Must be corrected for body temperature.

		SI units
pH	7.32–7.43	7.32–7.43
Panic value	< 7.2 or > 7.6	< 7.2 or > 7.6
PCO_2	38–50 mm Hg	5.0–6.7 kPa
PO_2	20–49 mm Hg	2.6–6.5 kPa
HCO_3	22–26 mEq/L	22–26 mmol/L
Panic value	< 10 mEq/L	< 10 mmol/L
	> 40 mEq/L	> 40 mEq/L
O_2 saturation	60–80%	0.60–0.80

Partial Pressure of Carbon Dioxide (PCO_2)

Normal Values

$PaCO_2$ (arterial blood) 35–45 torr
$PvCO_2$ (venous blood) 38–50 torr.

Carried in blood in two ways: 10% carried in plasma, 90% carried in RBCs.

Explanation of Test

This test is a measurement of the pressure or tension exerted by dissolved CO_2 in the blood and is proportional to the partial pressure of CO_2 in the alveolar air. The test is commonly used to detect a respiratory abnormality

and to determine the alkalinity or acidity of the blood. In order to maintain CO_2 within normal limits, the rate and depth of respiration vary automatically with changes in metabolism. It is an index of alveolar ventilation and is the most physiologically reflective blood gas measurement. When taken as an arterial sample, it directly reflects how well air is exchanging with blood in the lungs.

CO_2 tension in the blood and cerebrospinal fluid (CSF) is the major chemical factor regulating alveolar ventilation. When the CO_2 of arterial blood rises from 40 to 45 torr, it causes a three-fold increase in alveolar ventilation. A CO_2 of 63 torr in arterial blood increases alveolar ventilation tenfold. When the CO_2 concentration of breathed air exceeds 5%, the lungs can no longer be ventilated fast enough to present a dangerous rise of CO_2 concentration in tissue fluids. Any further increase in CO_2 begins to depress the respiratory center, causing a progressive decline in respiratory activity rather than an increase.

Procedure

1. Obtain an arterial blood sample.
2. Do not expose sample to air.
3. A small amount of blood is then introduced into a blood gas analyzer and the CO_2 tension is measured with a silver-silver chloride electrode (Severinghaus electrode).

Clinical Implications

1. A rise in PCO_2 is usually associated with hypoventilation, a decrease, with hyperventilation. Reduction in PCO_2 through its effect on plasma bicarbonate concentration decreases renal bicarbonate reabsorption. For each mEq/L fall in HCO_3, the PCO_2 falls by 1 to 1.3 mm of Hg. Because HCO_3 and PCO_2 bear this close mathematical relationship, and this ratio in turn defends the hydrogen ion concentration, the outcome is that the steady state PCO_2 in simple metabolic acidosis is equal to the last two digits of the pH. Also, addition of 15 to the bicarbonate level also equals the last two digits of the pH. Failure of the PCO_2 to achieve predicted levels defines the presence of superimposed respiratory acidosis on alkalosis.
2. The causes of decreased *PCO_2* include:
   - Hypoxia
   - Nervousness
   - Anxiety
   - Pulmonary emboli
   - Pregnancy
   - Other cause of hyperventilation.
3. The causes of increased *PCO_2* are:
   a. Obstructive lung disease
      - Chronic bronchitis
      - Emphysema.
   b. Reduced function of respiratory center
      - Over-reaction
      - Head trauma
      - Anesthesia.
   c. Other more rare causes of hypoventilation, such as Pickwickian syndrome.

Clinical Alert

Increased PCO_2 may occur even with normal lungs if the respiratory center is depressed. Always check laboratory reports for abnormal values. In interpreting laboratory reports remember that PCO_2 is a gas and is regulated by the lungs, not the kidneys.

Oxygen Saturation (SO_2)

Normal Values

Arterial blood saturation SaO_2 = 95% or higher mixed venous blood saturation SvO_2 = 75%.

Explanation of Test

This measurement is a ratio of the actual oxygen (O_2) content of the hemoglobin compared to the potential maximum O_2 carrying capacity of the hemoglobin. The percentage of SO_2 saturation is a measure of the relationship between O_2 and hemoglobin. The percentage of saturation does not indicate the O_2 content of arterial blood. The maximum amount of O_2 that can be combined with hemoglobin is called the O_2 capacity. The combined measurements of O_2 saturation, partial pressure of O_2, and of hemoglobin will indicate the amount of O_2 available to the tissue (tissue oxygenation).

Procedure

Obtain an arterial blood sample. Two methods for determining oxygen saturation are used.

1. The blood sample is introduced into the oximeter, which is a photoelectric device for determining the oxygen saturation of the blood. The value is measured directly with an oximeter (i.e. spectrometry).
2. Oxygen saturation is calculated from the oxygen content and oxygen capacity determinations.

$$\text{Percentage saturation} = \frac{100 \times O_2 \text{ content volume\%}}{O_2 \text{ capacity volume\%}}$$

i.e., Percentage saturation =

$$\frac{100 \times \text{volume of } O_2 \text{ actually combined with Hb}}{\text{volume of } O_2 \text{ with which Hb is capable of combination}}$$

The O_2 content of blood sample is measured before and after exposure to atmosphere.

Oxygen (O_2) Content

Normal Values

Aterial blood : 15–22 volume%
Venous blood : 11–16 volume%
(Volume% = volume percentage = mL/100 mL of blood).

Explanation of Test

The actual amount of oxygen (O_2) in the blood is termed the oxygen content. Blood can contain less O_2 than it is capable of carrying. About 98% of all O_2 delivered to the tissues is transported in chemical combination with hemoglobin. One gram of hemoglobin can carry or is capable of combining with 1.34 mL of O_2, whereas 100 mL of blood plasma can carry upto 0.3 mL of O_2. This measurement is determined mathematically by multiplying the number of grams of hemoglobin in 100 mL of blood by 1.34 times the partial pressure of oxygen in the blood.

Procedure

1. An arterial or venous blood sample is obtained
2. Mathematical formula
   O_2 content = $SaO_2 \times Hb \times 1.34 + PaO_2 \times 0.003$.

Clinical Implications

Decreased arterial blood O_2 associated with increased arterial blood CO_2 can be due to:

1. Chronic obstructive lung disease
2. Patients with respiratory complications postoperatively
3. Flail chest
4. Kyphoscoliosis
5. Neuromuscular impairment
6. Obesity hypoventilation.

Partial Pressure of Oxygen (PO_2)

Normal Values

PaO_2 80 torr or greater: arterial sample
PvO_2 30–40 torr: venous or peripheral blood sample.

Background

Oxygen (O_2) is carried in the blood in two forms dissolved and in combination with hemoglobin. Most of the O_2 in the blood is carried by hemoglobin. It is the partial pressure of a gas that determines the force it exerts in attempting to diffuse through the pulmonary membrane. The partial pressure reflects the amount of O_2 passing from the pulmonary alveoli into the blood and is directly influenced by the amount of O_2 being inhaled.

Explanation of Test

This is a measure of the pressure exerted by the amount of O_2 dissolved in the plasma. It is a test that measures the effectiveness of the lungs to oxygenate the blood. The severity of impairment of the ability of the lungs to diffuse O_2 across the alveolar membrane into the circulating blood is indicated by the level of partial pressure of oxygen (PO_2).

Procedure

1. An arterial blood sample is obtained.
2. A small amount of blood is then introduced into a blood gas analyzing machine and the O_2 tension is measured with a polarographic electrode (Clark electrode).

Clinical Implications

1. Increased levels are associated with:
   a. Polycythemia
   b. Hyperventilation in an arterial blood sample.
2. Decreased levels are associated with:
   a. Anemias
   b. Cardiac decompensation
   c. Insufficient atmospheric O_2
   d. Intracardiac shunts
   e. Chronic obstructive disease
   f. Restrictive pulmonary disease
   g. Hypoventilation due to neuromuscular disease.
3. Decreased arterial PO_2 with normal or decreased arterial blood PCO_2 tension is associated with:
   a. Diffuse interstitial pulmonary infiltration
   b. Pulmonary edema
   c. Pulmonary embolism
   d. Postoperative extracorporeal circulation.

Carbon Dioxide (CO_2) Content or Total Carbon Dioxide (TCO_2)

Normal Values

24–30 mEq/L.

Background

In normal blood plasma, more than 95% of the total CO_2 content is contributed by bicarbonate (HCO_3), which is regulated by the kidneys. The other 5% of the CO_2 is contributed by the dissolved CO_2 gas and carbonic acid (H_2CO_3). Dissolved CO_2 gas, which is regulated by the

lungs, therefore, contributes little to the total CO_2 content. Total CO_2 content gives little information about the lungs.

HCO_3^- in the extracellular spaces exists first as CO_2 then as H_2CO_3, and thereafter, much of it is changed to sodium bicarbonate ($NaHCO_2$) by the buffers of the plasma and red cells.

Explanation of Test

This test is a general measure of the alkalinity or acidity of the venous, arterial or capillary blood. This test measures CO_2 from:

1. Dissolved CO_2
2. Total H_2CO_3
3. HCO_3^-
4. Carbamino carbon dioxide

Total $CO_2 = HCO_3^- + 0.03 \times PCO_2$

Procedure

1. A venous or arterial blood sample of 6 mL is collected in a heparinized syringe.
2. If the collected blood sample cannot be studied immediately, the syringe should be placed in an iced container.

Clinical Implications

1. Elevated CO_2 content levels occur in:
   a. Severe vomiting
   b. Emphysema
   c. Aldosteronism
   d. Use of mercurial diuretics.
2. Decreased CO_2 content levels occur in:
   a. Severe diarrhea
   b. Starvation
   c. Acute renal failure
   d. Salicylate toxicity
   e. Diabetic acidosis
   f. Use of chlorothiazide diuretics.

(**Note:** In diabetic acidosis the supply of ketoacids exceeds the demands of the cell. Blood plasma acids rise. Blood plasma HCO_3 decreases because, it is used in neutralizing these acids).

Clinical Alert

A double use of the CO_2 is one of the main reasons why understanding acid-base problems may be difficult. Use the terms CO_2 content and CO_2 gas to avoid confusion. Remember the following:

1. CO_2 content is mainly bicarbonate and a base. It is a solution and is regulated by kidneys.
2. CO_2 gas is mainly acid. It is regulated by the lungs.

Interfering Factors

1. Drugs that may cause increased or decreased levels include:
   a. Nitrofurantoin
   b. Salicylates.
2. Drugs that may cause decreased levels include:
   a. Dimercaprol (BAL)
   b. Lipomul oil injection
   c. Methicillin.

Blood pH

Normal Values

Arterial blood : 7.35–7.45
Venous blood : 7.32–7.43.

Background

The pH is the negative logarithm of the hydrogen ion concentration in the blood. The sources of hydrogen ions are: (i) volatile acids, which can vary between a liquid and a gaseous state, and (ii) non-volatile acids that cannot be volatilized and are fixed (e.g. dietary acids, lactic acids and ketoacids).

Explanation of Test

This is a measurement of the chemical balance in the body and is a ratio of acids to bases. A determination of the blood pH is one of the best ways to tell if the body is too acid or too alkaline. Low pH numbers (< 7.35) indicate an acid stage, and higher pH numbers (> 7.45) indicate an alkaline state. This balance is extremely intricate and must be kept within the very slight margin of 7.35 to 7.45 pH (alkaline) in the extracellular fluid. pH limits compatible with life are 6.9 to 7.8.

The respiratory response to changes in blood pH is almost instantaneous. Acidosis (CO_2 retained; pH falls) stimulates ventilation; alkalosis (CO_2 blown off; pH rises) depresses ventilation. The respiratory center in the medulla appears to respond to a pH intermediate between those of the blood and CSF (7.35–7.40).

Procedure

1. An arterial blood sample is obtained.
2. Two methods of determining the pH are used, the direct method and the indirect method:
   a. *Direct method:* A small amount of blood is introduced into a blood gas machine and the pH is measured.
   b. *Indirect method:* The Henderson-Hasselbalch equation is solved.

TABLE 21.1: Four basic forms of acid-base imbalance and their compensatory mechanism

Form	*Cause*	*Occurrence*	*Compensatory mechanism*
1. Respiratory acidemia	Primary increase in PCO_2 and decreased pH	*Depression of respiratory centers* (a) Drug overdose (b) Barbiturate toxicity (c) Use of anesthetics *Interference with mechanical function of thoracic cage* (a) Deformity of thoracic cage (b) Kyphoscoliosis *Airway obstruction* (a) Extrathoracic tumors (b) Asthma (c) Bronchitis (d) Emphysema *Circulatory disorders* (a) Congestive heart failure (b) Shock	Renal reabsorption of the bicarbonate ion. Examples: (a) Uncompensated respiratory acidemia (acute ventilatory failure) *Values* pH = 7.26 ↓ PCO_2 = 56 ↑ Bicarbonate = 4 normal (b) Compensated respiratory acidemia (chronic respiratory failure) *Values*: pH = 7.36 PCO_2 = 63 Bicarbonate = 34
2. Respiratory alkalemia	Primary decrease in PCO_2 and in increased pH	*Hyperventilation* —Hysteria *Lack of oxygen.* *Toxic stimulation of the respiratory centers* (a) High fever (b) Cerebral hemorrhage (c) Excessive artificial respiration (d) Salicylates	Glomerular filtration of the bicarbonate on. Examples (a) Uncompensated respiratory alkalemia (acute alveolar hyperventilation) *Values*: pH = 7.52 ↑ PCO_2=28 ↓ Bicarbonate = 22 normal (b) Compensated respiratory alkalemia (Chronic alveolar hyperventilation) pH = 7.43 PCO_2 = 24 Bicarbonate = 15
3. Nonrespiratory acidemia metabolic acidemia	Increase in hydrogen ions with a secondary decrease in bicarbonate	*Acid addition* (a) Renal failure (b) Diabetic ketoacidosis (c) Lactic acidosis (d) Anaerobic metabolism *Hypoxia* *Base subtraction* (a) Diarrhea (b) Renal tubular acidosis	Hyperventilation through stimulation of central chemoreceptors Examples (a) Uncompensated nonrespiratory acidemia (acute) *Values*: pH = 7.20 ↓ PCO_2 = 38 ↓ Bicarbonate = 15 ↓ (b) Compensated respiratory acidemia (chronic) *Values*: pH = 7.35 PCO_2 = 25 Bicarbonate = 15
4. Nonrespiratory alkalemia or metabolic alkalemia	Increase in bicarbonate secondary to a decrease in hydrogen ions	*Acid subtraction* (a) Loss of gastric juice (b) Vomiting *Potassium or chloride depletaion base addition* (a) Excessive bicarbonate or lactate administration	Hypoventilation. Examples (a) Uncompensated nonrespiratory alkalemia (acute) *Values*: pH = 7.56 PCO_2 = 44 ↑ Bicarbonate = 38 ↑ (b) Compensated respiratory alkalemia (chronic) *Values*: pH = 7.44 PCO_2 = 55 Bicarbonate = 38

Note

1. Although these four basic imbalances occur individually, more frequently a combination of two or more is observed. These disturbances may have an antagonistic or a synergistic effect upon each ones.
2. Compensation is most efficient is respiratory and nonrespiratory acidemia.
3. The degree of hypoventilation is precisely related to the degree of hypobicarbonatemia. For each mEq/L fall in bicarbonate, PCO_2 falls by 1–1.3 torr. A close mathematical relationship prevails between bicarbonate and PCO_2. Their ratio (HCO_3 and PCO_2) defines the prevailing hydrogen ion concentration. For this reason, the steady state PCO_2 in simple metabolic acidosis is equal to the last two digits of the pH. Failure of the PCO_2 to reach predicted levels defines the presence of superimposed respiratory acidosis or alkalosis.

$$pH = pk + \log \frac{\text{major blood base}}{\text{major blood acid}}$$

Clinical Implications

1. Generally speaking, the pH is decreased in acidemia because of increased formation of acids. pH is increased in alkalemia because of a loss of acids.
2. When attempting to interpret an acid-base abnormality, one must:
   a. Check the pH to see if there is an alkalemia or an acidemia.
   b. Check PCO_2 to see if there is a respiratory abnormality.
   c. Check HCO_3^- or base excess to see if there is a metabolic abnormality.
3. Refer to table given later for a more complete explanation of the changes occurring in respiratory and metabolic acidemia and respiratory and metabolic alkalemia (Table 21.1).
4. Metabolic acidemia:
   a. Renal failure
   b. Ketoacidosis in starvation and diabetes
   c. Lactic acidosis
   d. Strenuous exercise.
5. Metabolic alkalemia:
   a. Deficient potassium
   b. Hypochloremia
   c. Gastric suction or vomiting
   d. Massive administration of steroids
   e. Sodium bicarbonate administration
   f. Aspirin intoxication.
6. Respiratory alkalemia:
   a. Acute pulmonary disease
   b. Myocardial infarction
   c. Chronic and acute heart failure
   d. Adult cystic fibrosis
   e. Third trimester of pregnancy
   f. Anxiety, neurosis, psychosis
   g. CNS disease
   h. Pain
   i. Anemia
   j. Carbon monoxide poisoning
   k. Acute pulmonary embolus
   l. Shock.
7. Respiratory acidemia:
   a. Acute respiratory distress syndrome
   b. Ventilatory failure.

Clinical Alert

Ventilation failure is a medical emergency. Aggressive and supportive measures must be taken immediately.

Observing the rate and depth of respiration may give a clue to blood pH:

1. Acidosis usually increases respirations
2. Alkalosis usually decreases respirations.

Interfering Factors

1. Drugs that may cause increased levels include:
   - Potassium oxalate
   - Sodium bicarbonate
   - Sodium oxalate.
2. Drugs that may cause decreased levels include:
   - Acetazolamide
   - Ammonium chloride
   - Ammonium oxalate
   - Calcium chloride
   - EDTA
   - Methyl alcohol
   - Paraldehyde
   - Salicylates
   - Sodium citrate.

Base Excess/Deficit

Normal Values

(± 3 mEq/liter)

- Positive value indicates a base excess (i.e. a non-volatile acid deficit).
- Negative value indicates a deficit (i.e. a non-volatile acid excess).

Explanation of Test

This determination is an attempt to quantify the patient's total base excess or deficit so that clinical treatment of acid-base disturbances (specifically those that are nonrespiratory in nature) can be initiated. It is also referred to as the whole blood buffer base and is the sum of the concentration of buffer anions (in mEq/L) contained in whole blood. These buffer anions are the bicarbonate (HCO_3^-) ion in plasma and RBCs, and the hemoglobin, plasma proteins, and phosphates in plasma and RBCs.

Total quantity of buffer anions is 45 to 50 mEq per liter or about twice that of HCO_3^-, which is 24 to 28 mEq/L. Thus, the quantity of HCO_3^- ions accounts for only about half of total buffering capacity of the blood. Therefore, the base excess/deficit measurement provides a more complete picture of the buffering taking place and is a critical index of nonrespiratory changes in acid-base balance versus respiratory changes.

Procedure

Calculation is made from the measurements of pH, $PaCO_2$, and hematocrit. These values are plated on a nomogram and the base excess/deficit is read.

Clinical Implications

1. Negative value (below 3 mEq/L) reflects a nonrespiratory or metabolic disturbance. It indicates acid accumulation due to:
   a. Dietary intake of organic and inorganic acids
   b. Lactic acid
   c. Ketoacidosis.
2. Positive value (above 3 mEq/L) reflects a non-volatile acid deficit.

AUTOMATION IN BLOOD GAS ANALYSIS

The Basis of Blood Gases

With most lab blood work there are two types of tests that are in some way time-dependent: stat tests, which must be done as quickly as possible, and routine tests. If there were such a thing as "super stat," blood gas tests would fall into that category.

The values obtained represent a mere moment in time for the patient, and although trends and stabilization of blood gas values can be obtained, more often than not the results are worthless later if changes in treatment are contemplated based on their values. Such therapeutic changes often involve critical, life-saving and time-dependent interventions such as adjustment upwards or downwards of oxygen, carbon dioxide and pH values. There is no time to waste in a critical situation.

Most blood samples can be collected routinely, on rounds, and kept and transported at room temperature until they are analyzed. Temperature does not affect their results. This is not true for arterial blood gases. As a living tissue, blood collected for this panel degrades rapidly unless kept in an ice/water bath until analyzed if any delay at all is expected in performing the analysis. And at the moment of analysis, the sample must be rewarmed to body temperature for an accurate result as the partial pressure of oxygen and CO_2 decreases at lower temperatures and increases at higher ones. The most accurate reflection of these numbers lies in analyzing the correcting the values for the patient's actual body temperature if the patient is either hypothermic or febrile.

Sample Collection

Most blood labs are performed on tourniqueted venous blood drawn from a superficial vein that is easily palpated and often even visually apparent. Today, lab technologists use a special needle and a vacutainer containing an appropriate anticoagulant, other substance or nothing at all, depending on the test. Such tubes are identified by a color-coded cap that is never removed. This makes for unparalleled safety and protection from needlesticks and accidental exposure to bloodborne pathogens.

Arterial blood gases, as their name implies, must be drawn from an artery with a free-flowing, unimpeded flow of blood coursing through it. This procedure is known as an arterial stick and is usually performed on a palpable radial artery. If this site is unavailable, the brachial artery must be used. If no upper limb artery can be used, the next most favored site is one of the femoral arteries.

In critically ill patients requiring frequent samples, physicians often insert an arterial line that simplifies the procedure immeasurably. The blood must be drawn through a needle (or directly into a syringe if an a-line is available) into a heparinized (wet or dry lithium) syringe. A milliliter or less of blood is required to perform the procedure using most modern blood gas analyzers. Any air bubble left in the hub or top of the syringe must be carefully and gently expelled and the needle capped using the safety coverlet supplied with most arterial blood gas sampling kits. The syringe is then placed in a plastic bag containing crushed ice and immediately transported for analysis.

Blood Gas Analyzers

To save time in the transport and analysis of blood samples on critically ill patients, many blood gas operations are housed in or near intensive care units as well as in or near the operating or recovery room. Because of the immediate life-threatening nature of blood gas abnormalities and the need to correct them rapidly on an objective and rational basis, blood gas labs should be equipped with a minimum of two analyzers in case one goes down due to routine maintenance or through some unforeseen malfunction or equipment failure. There can be no excuse for not being able to provide blood gas analysis rapidly and accurately on site at all times. Failure to do so can result in a potentially avoidable patient death.

Modern blood gas analyzers are electronic marvels compared to the methods used for this purpose 20 years ago. On attaching the sample syringe to the cuvette, they automatically draw the sample into a heated sampling chamber with miniaturized electrodes that quickly and accurately (if properly calibrated) measure pH, PCO_2 and PO_2 values. Based on these three measured values, these

units automatically calculate HCO_3, total CO_2, percent oxygen saturation and O_2 content, which is based on entry of the patient's measured hemoglobin values.

A companion to such units, known as a co-oximeter, directly measures percent oxygen saturation and hemoglobin, then accurately calculates oxygen content and carboxyhemoglobin, a value that reflects the degree of carbon monoxide in the blood in smoke inhalation victims.

In addition to arterial sampling, critical care specialists often order blood gas panels in blood drawn through a central venous line since PO_2 and O_2 content values of this blood, when compared against arterial PO_2 and O_2 content, enable an estimate of cardiac output, another valuable service performed by blood gas testing. Such samples are often collected and run from patients undergoing cardiac catheterization and the results must be returned while the patient is still on the table.

What are blood gases?

These are two broad components to the blood gas panel: respiratory and metabolic. The values reported are as follows:

- pH—This is a logarithmic expression of hydrogen ion concentration—the acidity of alkalinity of the blood. The normal human arterial pH is 7.4. Any pH below this is acid, and any pH above it is alkaline. There is a narrow range of pH values (7.35 to 7.45) that the human body and its complicated system of enzyme-supported system operates within. pH values below 7.0 and above 7.6 are incompatible with life.
- HCO_3—This value is derived through the blood gas analyzer's manipulation of the Henderson-Hasselbalch Equation. An uncompensated decrease in the HCO_3 value causes a decline in pH. An increased HCO_3 results in alkalinization of the blood. Either condition can be life-threatening. Decreased HCO_3 is often the result of kidney or other major organ failure or uncontrolled diabetes. Increased HCO_3 is more rare and is usually the result of inappropriate administration of certain drugs such as some kinds of diuretics or an excess of $NaHCO_3$.
- PCO_2—This value is measured directly by the CO_2 electrode. An increased PCO_2 is often the result of acute, chronic or impending respiratory failure, whereas a decreased PCO_2 is the result of hyperventilation stimulated by a metabolic acidosis or hysteria and severe anxiety reactions. The normal arterial PCO_2 is 40 mm Hg.

FIG. 21.1: AVL compact 2 blood gas analyzer

- PO_2—The partial pressure of oxygen in the blood is measured directly by a polarographic O_2 electrode. The normal acceptable range is roughly between 85 and 100. An increased PO_2 is usually the result of excessive oxygen administration that needs to be adjusted downwards on such results. A decreased PO_2 is often the result of any number of respiratory or cardiopulmonary problems.

AVL COMPACT 2 BLOOD GAS ANALYZER (FIG. 21.1)

The AVL compact series is a marvel of design and function. As with all AVL blood gas analyzers, this little workhorse is designed to make operation as simple as possible allowing for faster, safer, more reliable results in hectic emergency situations. The AVL compact series focuses on the most important requirements of critical care analysis and is suitable for intensive care situations, neonatology and lung function testing. Patient data entry is a breeze via the built-in keyboard and LCD display. Furthermore, a logical menu guides the user through all functions making operation simple, reliable and safe. The simplicity and small footprint of the AVL compact series mask its advanced analytical performance and data processing capabilities. Measured parameters in the AVL compact series include PH, PCO_2, PO_2, and PBaro. Features of the AVL compact series include automatic calibration and cleaning cycles and 20-second analysis time, 32 samples/h. Other features of the AVL compact series are LCD display with integrated numeric keypad, automatic sample handling system, and on-board quality control features able to store up to 34 QC results for three levels.

AVL Compact 2 Blood Gas Analyzer

Specifications:
Dimensions: 14 × 13 × 14 (36 × 33 × 36 cm)
Weight: 29 lbs (13 kg).

ELECTROLYTE ANALYSIS BY FLAMEPHOTOMETER

In this instrument, the solution under test is passed under carefully controlled conditions as a very fine spray in the air supply to a burner. In the flame, the solution evaporates and the salt dissociates to give neutral atoms. Some of these though only a very small proportion, move into a higher energy state (their electrons move to the outer orbits). When the electrons of these atoms fall back to their original orbits, they release energy in the form of light. Which is used in flame photometry of this type and is called emission flame photometry. Light of characteristic wavelengths is emitted and passes through a suitable filter, grating or prism on to a photocell, and the amount of current thus produced is measured. This varies with the concentration of sodium; for example, in the solution being tested. Using solutions of known sodium concentrations, a calibration curve can be made and this is used for reading the sodium content of the fluids examined.

Many gases have been used for the flame. These include acetylene, propane, butane and coal gas. Both the gas pressure and air pressure have to be carefully regulated so as to maintain a constant steady flame, which should be blue in color and have no yellow streaks. The spray is formed by passing compressed air through an atomizer, into which the liquid which is being tested is drawn either by suction or gravity, and then enters the burner in its air supply. The pressure used is usually about 10 to 15 lb per square inch. The atomizer is a very important part of the apparatus. A steady fine spray of droplets of uniform size should be produced if there is to be a constant emission of light.

The light produced is first passed through a lens to focus it and then through suitable filters, a diffraction grating or prism before it falls on to the photocell. For determining sodium, an orange or yellow (589 nm) filter can be used. Potassium emits light at 404.4 and 766.5 nm. For the former, a violet; and for the latter a deep red filter should be used. Lithium (671 nm) needs a near red filter for its measurement.

The most satisfactory dilution to use should be established experimentally. For sodium, this may be 1 in 100, 1 in 200, even 1 in 500; for potassium, it is usually lower, often 1 in 50, but it is possible by varying the sensitivity to use the same dilution for both. Both sodium and potassium can interfere with each other, therefore, in standard solution both the elements are added.

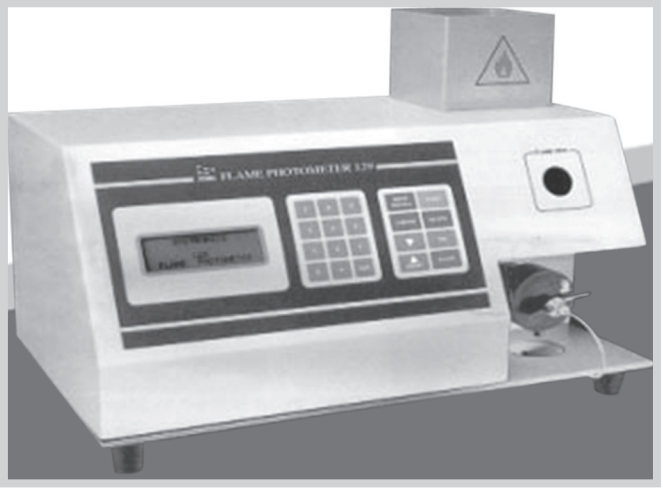


FIG. 21.2: Flame photometer

FIG. 21.3: Measurement results can be printed immediately or later on

Equipment: Systronics provides digital read-out flame photometer (Mediflame 127/128/129) that provides two parameters at a time.

Flame Photometer 129

(*Courtesy*: Systronics)

Microprocessor-based Automation

Systronics flame photometer 129 is a microprocessor-based unit designed for medical applications (Fig. 21.2). The microprocessor provides automation in operation, measurements, and end-result presentation. The unit can do the estimation of sodium (Na), potassium (K), calcium (Ca) and lithium (Li) in single aspiration of a sample.

For user's convenience, the unit offers three measuring modes: (i) serum, (ii) urine, and (iii) bio-fluid, general. The last mode is helpful for analyzing biological samples other than serum and urine.

Frequently used measurement set-ups can be stored once (in a battery-back-up memory) and recalled whenever required with a stroke of a button. This eliminates the typical chores of instructions required to be given to a microprocessor-based instrument before it starts the operation. Facility for restandardization with a single standard is available to minimize the effect of any unforeseen drift without going for the full recalibration.

A 4-line 20-character LCD readout provides easy user interface and presentation of results. A Centronics printer port is provided for Epson compatible dot matrix/inkjet and HP compatible laser printers. Printer is optional (Fig. 21.3).

Salient Features

- Specifically designed for medical applications
- Up to four elements measured with single aspiration
- Automatic filter selection (narrow band interference)
- Curve fittings for nonlinear range (up to 5 standards)
- Calibration stored in memory
- Restandardization cuts on full recalibration
- Record kept of date and time of analysis
- Saved set-ups cut operation steps
- Measurement results can be recalled later on for display and printout (330 max)
- Measurement results can be printed (individual, full Batch, of the day, all in memory)
- 4-line, 20-character alphanumeric LCD readout
- Centronics printer port for epson compatible dot matrix/inkjet and HP laser printers
- RS-232 interface (optional)
- Compressor with built-in air filter and air regulator.

Easy Menu Driven Operation

```
Operating mode
1. Serum
2. Urine
3. Biofluids (GEN)
```

Specifically designed for medical use.

```
Set flame
Ignite flame,
Aspirate DW,
Press enter
```

Step-by-step guidance for operation.

```
Set flame
Ignite Flame,
Aspirate DW,
Press enter
```

```
Adjust fuel for
best flame stability
< 0578.7)
```

Digital monitoring of flame stability.

```
Restandardization
Enter Std. Value
Na = 140
```

A restandardization facility cuts on full recalibration

```
Measurement recall
Enter date: 23/02
Enter batch no.: 1
Enter sample no.: 01
```

Measurement results can be recalled later on for display.

```
Set-up
1. Directory
2. Call set-up
3. Edit set-up
```

Saved set-ups drastically cut operation steps.

Technical Specifications

Range of operation

Element	*Serum*	*Urine*	*BIP-Fluids*
Na	100–200 mEq/L	0–250 mEq/L	All the four
	1:100 dil	1:100 dil	elements up
K	0–10 mEq/L	0–100 mEq/L	250 mEq/L
	1:100 dil	1:100 dil	with 1:100 dil
Li	0–2 mEq/L		
	1:10 dil		
Ca		0–10 mEq/L	
		1:2 dil	

i. Curve fit software is provided for urine and biofluids.
ii. Curve fit accuracy: ± 2% f.s.
iii. Suitable dilution for concentrations higher than given in the above table.

- Filters (10 nm typical): Na and K supplied; Li and Ca (optional)
- Reproducibility: ± 2% f.s.
- Minimum sample: Approximately 3 mL per element (at Avarage. time of 4 seconds)
- Averaging: 2 to 15 seconds, selectable

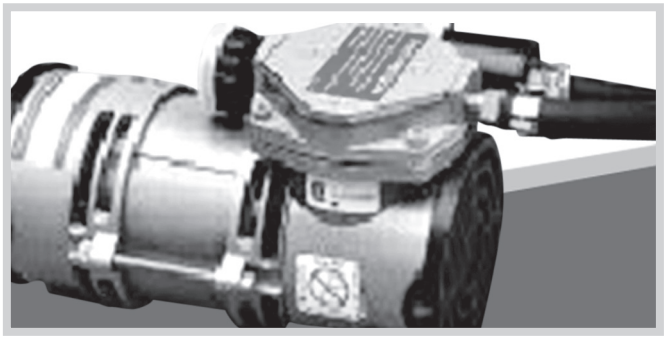


FIG. 21.4: Standard air compressor for flame photometers

- Aspiration time: (5 seconds + Average time) per element + 4 seconds.
- Operating air pressure: 0.45 kg/cm^2 (typical)
- Air compressor: With built-in air regulator and air filter to deliver stable and moisture/oil free air supply
- Fuel Gas: LPG
- Power Supply: 230 Vac +/– 10%, 50 Hz.

System: The system consists of main unit with one each of Na and K narrow band filters, a compressor with built-in air regulator and air filter. Li and Ca filters and printer are optional (Fig. 21.4).

Normal Values

		SI units
Potassium		
Adults	3.5–5.3 mEq/L	3.5–5.3 mmol/L
Premature infants	5.0–10.2 mEq/L	5.0–10.2 mmol/L
Cord blood	5.0–10.2 mEq/L	5.0–10.2 mmol/L
2 days	3.0–6.0 mEq/L	3.0–6.0 mmol/L
Full-term newborn		
Cord blood	5.6–12.0 mEq/L	5.6–12.0 mmol/L
Newborn	3.7–5.0 mEq/L	3.7–5.0 mmol/L
Infants	4.1–5.3 mEq/L	4.1–5.3 mmol/L
Children	3.4–4.7 mEq/L	3.4–4.7 mmol/L
Panic levels		
Adults	< 2.5 mEq/L	< 2.5 mmol/L
	or > 6.6 mEq/L	or > 6.6 mmol/L
Newborn	< 2.5 mEq/L	< 2.5 mmol/L
	or > 8.1 mEq/L	or > 8.1 mmol/L
Sodium		
Adults	136–145 mEq/L	136–145 mmol/L
Umbilical cord	116–166 mEq/L	116–166 mmol/L
Infants	139–146 mEq/L	139–146 mmol/L
Children	138–145 mEq/L	138–145 mmol/L

Alterations of Sodium and Extracellular Fluid (ECF)

Hyponatremia (Serum Sodium Concentration Lower than Normal)

Total-body sodium and ECF volume low:

- GIT fluid loss
- Burns
- "Third compartment" accumulation
- Salt losing renal disorders
- Diuretic overuse.

Total body sodium and ECF volume normal

- Acute water intoxication, usually iatrogenic
- Syndrome of inappropriate ADH secretion.
- Glucocorticoid deficiency.
- Severe whole body potassium depletion.

Total body sodium and ECF volume increased

- Acute renal failure with superimposed water load
- Congestive heart failure
- Cirrhosis
- Nephrotic syndrome.

Hypernatremia (serum sodium concentration higher than normal)

Total body sodium normal, ECF volume low

- Gastroenteritis
- Osmotic diuresis
- Pronounced sweating.

Total body sodium increased proportionately more than increased ECF volume

- Salt ingestion, deliberate or accidental.
- Inappropriate intravenous therapy.

Hyperosmolality, without sodium alterations

- High blood ethanol
- Hyperglycemia
- Radiographic contrast media.

Abnormalities of Serum and Whole Body Potassium

Hyperkalemia (serum potassium concentration more than normal)

Inappropriate cellular metabolism

- Insulin deficiency
- Acidemia
- Hypoaldosteronism
- Cell necrosis (burns, crush, hemolysis, anti-leukemia therapy).

Decreased renal excretion

- Acute renal failure
- Chronic interstitial nephritis
- Tubular unresponsiveness to aldosterone
- Hypoaldosteronism.

Increased potassium intake
- Inappropriate use of salt substitutes or K^+ replacement.
- Potassium salts of antibiotics.

Hypokalemia (serum potassium concentration lower than normal)
Inappropriate cellular metabolism
- Alkalemia
- Familial periodic paralysis
- Very rapid generation of cells (leukemia, treated megaloblastic anemia).

Increased excretion
- Vomiting and/or diarrhea
- Diuretic overuse
- Hyperaldosteronism
- Renal tubular acidosis.

Decreased potassium intake
- Anorexia nervosa
- Diet deficient in vegetables, meat
- Clay eating (binds potassium and prevents absorption).

RAPID DIAGNOSTICS IN ELECTROLYTE ANALYSIS

Within a remarkably short period of only a few years, a variety of analyzers have appeared in the market which use ion-selective electrodes (ISE) for quantitative measurements of biologically relevant cations and anions. The electrode (ISE) permits measurement of the activity of a specific ion under the presence of a given amount of other ions. The selective transport of a certain ion species from the solution into the membrane phase of the electrode allows a potential difference that can be calculated and the ion concentration can be deduced thereof.

Ion selective electrodes are available for H^+ (for pH measurement), Li^+ (Lithium), Na^+, K^+, Ca^{++} and Cl^-.

Bayer 614 Na^+ K^+ Electrolyte Analyzer (Fig. 21.5)

The Corning 614 is designed for whole blood, plasma—using lithium heparin as anti-coagulant. Fresh samples can be analyzed at temperatures up to 40°C.

Serum may be used (free from hemolysis) within a range of 5 to 40°C.

Urine may be used, diluted 1 part sample to 9 parts urine diluents as long as samples are not collected into strong acid preservatives.

Sample presentation may be syringe, blood collection tube, centrifuge tube, vial, vacutainer, etc. A sample measurement result will be displayed within 35 seconds of pressing analyze blood? or analyze urine? button. Sample to sample measurement result will be displayed within 35 seconds of pressing analyze blood? Or analyze urine? Button. Sample to sample measurement time is 60 seconds.

35 uL blood required
150 uL urine required.

Ranges

Whole blood, serum, plasma, and QC material)
80–200 mmol/L Na^+
0.5–9.99 mmol/L K^+.

Urine
10–350 mmol/L Na^+
5–250 mmol/L K^+.

FIG. 21.5: Bayer 614 electrolyte analyzer
- 20 character alphanumeric vacuum fluorescent display
- Caliberation and slope solutions are contained within instrument
- Extremely easy to use
- Stable: the measured calibration value will change by less than +/– 2 mmol/L na^+ and +/– 0.1 mmol/L K^+ in a 1 hour period at constant ambient temperature

Specifications	614	634	644	654	664
Test performed	Na^+/K^+	Ca^{++}/pH	$Na^+/K^+/Cl^-$	$Na^+/K^+/Li^+$	$Na^+/K^+/Cl^-/tCO_2$
Measurement method	ISE	ISE	ISE	ISE	ISE, thermal cond

Contd...

Contd...

Sample types	Whole blood, serum, plasma, diluted urine	Whole blood, serum, plasma, diluted urine	Whole blood, serum, plasma, diluted urine	Whole blood, serum, plasma, diluted urine	Whole blood, serum, plasma
Sample volume	35 uL, 150 uL Diluted urine	35 uL	65 uL, 250 uL Diluted urine	65 uL, 250 uL Diluted urine	170 uL, 85 uL in Micro mode, 100 uL diluted urine
Analysis time	35 seconds	Within 60 Sec	35 seconds	40 seconds	35 seconds, 100 samples/h
Measuring ranges:					
Na^+	80–200 mmol/L	N/A	80–200 mmol/L	80–200 mmol/L	50–200 mmol/L
Na^+ (Urine)	10–350 mmol/L	N/A	10–350 mmol/L	10–350 mmol/L	10–350 mmol/L
K^+	0.5–9.99 mmol/L	N/A	0.5–9.99 mmol/L	0.5–9.99 mmol/L	0.5–20 mmol/L
K^+ (Urine)	5–250 mmol/L	N/A	5–250 mmol/L	5–250 mmol/L	5–300 mmol/L
Cl^-	N/A	N/A	50–200 mmol/L	N/A	20–200 mmol/L
Cl^- (Urine)	N/A	N/A	10–350 mmol/L	N/A	15–400 mmol/L
tCO_2	N/A	N/A	N/A	N/A	3–60 mmol/L
Ca^{++}	N/A	0.2–5.0 mmol/L	N/A	N/A	N/A
pH	N/A	6.50–8.00 mmol/pH	N/A	N/A	N/A
Li^+	N/A	N/A	N/A	0.2–0.50 mmol/L	N/A
Dimensions (W×D×H)	11.0" × 0.16" × 12.6"	11.0" × 10.6" × 12.6"	11.0" × 10.6" × 12.6"	11.0" × 10.6" × 12.6"	25.5" × 19.0" × 16.0"
Weight	14 lbs	14 lbs	14 lbs	14 lbs	64 lbs
Additional features	604 autosampler interface	pH at 7.4 or manually adjustable between 7.2 and 7.6 pH	604 autosampler interface	604 interface autosampler. Dual mode of operation: $Na^+/K^+/Li^+$	Interface with 550 Express clinical chemistry analyzer

CHAPTER 22

Serology/Immunology

BASIC IMMUNOLOGY

The immune system offers protection against invading microorganisms, viruses and other foreign materials. Somehow, it must distinguish between Valuable what "belongs" and what doesn't "belong". Failure to detect and expel foreign materials can lead to problems due to immunodeficiency (i.e. AIDS) and misidentification of "self" (autoimmunity).

Antigen-Immunogen

Antigen is a molecule that binds with an antibody or T cell receptor (**antigenicity** is the ability to bind to the antibody).

Immunogen is a molecule that can elicit an immune response (**immunogenicity** is the ability to elicit an immune response).

Antigenicity

Several factors influence how "antigenic" a molecule is. Most important is how foreign it is, with molecules that are most unlike self-being the most antigenic. There are also numbers of physical and chemical determinants, which also matter molecular size — the larger the better, generally. 1000 Daltons are about the lower limit.

- Complexity: The more complex the better. For example, simple repeating polysaccharides like starch aren't very good, while proteins with a constantly changing sequence of 20 or so different amino acids are good
- Structural stability: A fixed shape is helpful. For example, gelatin (which wobbles) is a poor antigen unless it is stabilized
- Degradability
- Foreignness.

Epitopes (Fig. 22.1)

For a molecule such as a protein, a given antibody will "be directly against" only one of all the possible parts of the entire molecule. This part is known as an EPITOPE. A molecule may have several epitopes. Also, a complex antigen (such as a cell) will have many molecules, each of which will contain several epitopes.

An epitope is also known as an antigenic determinant. Some epitopes are better able to elicit antibodies than others. They are known as **Immunodominant Epitopes.**

How Big is an Epitope?

About 6 units of a polysaccharide chain, or about 6–8 amino acids. For a protein epitope, it is the shape of the epitope, rather than the specific amino acid sequence that is

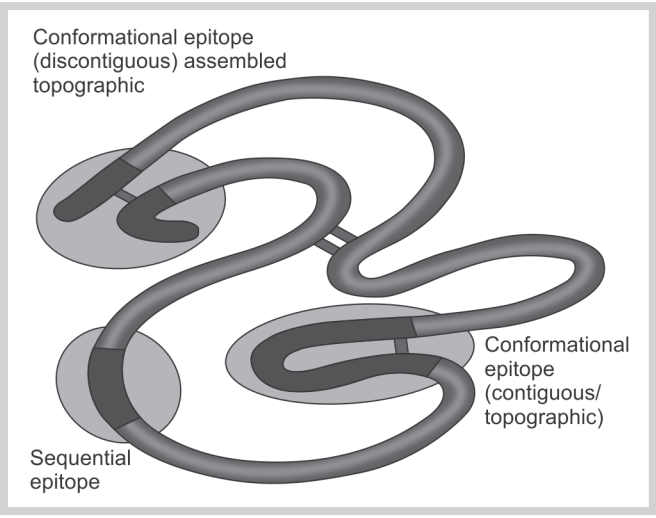


FIG. 22.1: Sites for obtaining blood by venipuncture from forearm

important. For example, a few amino acids, which come from different parts of the chain, can come together in one physical spot to create an epitope.

When an antibody directed against one epitope can bind to another epitope, this is known as "cross-reactivity". If this happens, it will be because the two epitopes 'look alike" in some way.

Some intestinal bacteria possess antigens that look like blood group A and B antigens which can be absorbed through the intestinal wall into the bloodstream; therefore, people of blood group A will have antibodies against the B antigens even if they never have been exposed to B-type blood cells.

Antibodies directed against human serum will cross-react with serum from chimpanzees, gorillas, orangutans and spider monkeys to an increasingly lesser extent.

What are the Different Kinds of Epitopes?

Conformational
Discontinuous.

Some Examples of Antigens

Proteins: Most antigens are proteins, such as the ones on the outer coverings of microorganisms.

Antibodies themselves: Human immunoglobulin G, which contains human antibodies, is immunogenic in experimental animals, because it is foreign to them.

Polysaccharides: Simple ones are not good. Longer ones, especially if they are complex and/or associated with proteins, can be good.

Blood Group Antigens: A, B, AB and O.

LPS or Lipopolysaccharides: From cell wall of gram-negative bacteria.

Lipids are generally poor antigens.

Nucleic acids are generally poor antigens.

Antibody
A class of proteins that migrate in the gamma fraction. They are classified on the basis of heavy chains.

- IgG — Eighty percent plasma immunoglobulin, present in all body fluids, transplacental,
- IgM — large molecule, pentameric in structure, present in vascular system, activates complement
- IgA — present in body secretion, respiratory and GI tract
- IgE — involved in hypersensitivity and allergic reactions
- IgD — present in B cell surfaces.

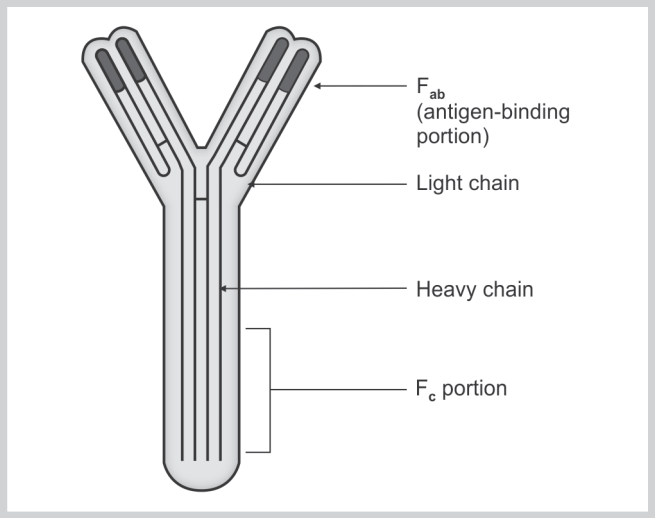


FIG. 22.2: Antibody structure

What is the Structure of Antibody?

Basic model consists of 4 polypeptide chains
2 small/light chains
2 large/heavy chains

Heavy chains are structurally different for different class of antibodies (Fig. 22.2).

What is the Kinetics of Antigen–Antibody Reaction?

The reaction complies with the law of mass action. The higher the K, the stronger the reaction. The forces governing the reaction are:

- Hydrogen bonds
- Hydrophobic bonds
- Electrostatic bonds
- van der Wall's bonds

$$K = \frac{(Ag.Ab)}{(Ag)(Ab)}$$

Immunological Reactions

What are the different ways of detection of antigen–antibody reaction?

- Immunodiffusion
- Electrophoresis
- Flocculation
- Complement assays
- Flow cytometry
- Immunohistochemical techniques

- Binder–ligand assays
- A clinical laboratory performs different kinds of tests for detection of antigen–antibody reactions;
- Agglutination blood grouping, Widal test
- Latex agglutination—CRP, RF test
- Flocculation—VDRL test for syphilis
- Electrophoresis—protein biochemistry
- Chromatography—pregnancy tests.

How is Binder-ligand Assays Classified?

- Isotopic assays—radioimmunoassays
- Non-isotopic assays—enzyme Immunoassays, fluorescence polarization immunoassays.

What is the Difference Between All these Reactions?

All are basically antigen-antibody reaction.

The indicator used will differentiate the technology (Fig. 22.3).

What form of Reaction Takes Place in HLA Typing?

It is also antibody reaction in which the end product is visualized by using a dye in a phase contrast microscope. The reaction can also be visualized using fluorescent dyes in a fluorescent microscope.

What is the Principle of HLA Typing?

It is called ad mixed lymphocytotoxicity test (MLT). In this the antibody (antisera) is coated in the microwell. The patient's B or T lymphocytes containing HLA antigens is added and incubated. Complement proteins are added which will destroy the complex, if they are formed. The dead and viable cells are differentiated and graded using an appropriate dye.

The principle is same for both cross-matching and tissue typing.

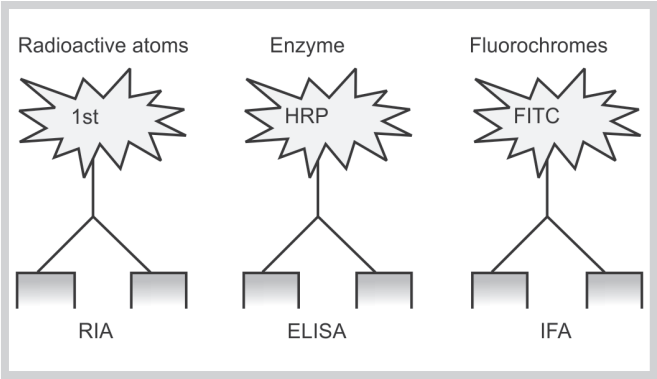


FIG. 22.3: Indicators used to differentiate immunological reactions

What are the Different Indicators Used in Immunoassay?

Indicator	*Example*	*Technology*
Enzyme	Horse radish peroxidase	EIA
Radio isotope	^{131}I	RIA
Fluorescence	Fluorescein iso thiocynate (FITC)	IFA
Chemiluminescent dyes	Acridinium ester	CLIA
Chromogen	Colloidal gold	Chromatography
Microparticles	Latex	Latex agglutination

Interferences in Immunoassays

Despite advances in the design of immunoassays, the problems of unwanted interference have yet to be completely overcome. An ideal immunoassay should have the following attributes:

- The immunochemical reaction behavior should be identical and uniform for both the reference preparation and the analyte in the sample
- The immunochemical reaction of the antibody reagent is uniform from batch to batch
- The immunochemical method is well standardized to ensure that the size of measurement signal is caused only by the antigen-antibody product
- For macromolecules the results declared in arbitrary units (IU – International Units), the conversion to (SI) units is not constant and depend on many factors.

Definition of Interference

Interference may be defined as " the effect of a substance present in an analytical system which causes a deviation of the measured value from the true value, usually expressed as concentration or activity."

IFCC (International Federation of Clinical Chemistry) offers the following d efinition – "Analytical interference is the systematic error of measurement caused by a sample component, which does not, by itself, produce a signal in the measuring system".

Assay interference can be "Analyte dependent or Analyte independent".

It can increase or decrease the measured result.

Increase (positive interference) is due to lack of specificity.

Decrease (negative interference) is due to lack of sensitivity.

Assay interference can be of different types:

Preanalytical Variables

All factors associated with the constituents of the sample are termed as preanalytical variables. They can be of two types:

Patient based: Such as incorrect sampling times and environmental factors such as smoking, etc. may change analyte concentration and consequently interpretation.

Specimen based: There are many factors that constitute this.

- Blood collection
- Nature of the sample: For all immunoassays, serum is the matrix of choice. Samples collected into tubes containing sodium fluoride may be unsuitable for some enzymatic immunoassay methods; preservation with sodium fluoride may affect results. Impurities in tracers interfere with direct dialysis methods for free hormones
- Hemolysis and hyperbilirubinemia
- Lipemia — may cause interference with assays for fat soluble compounds such as steroids
- Stability and storage.

Matrix Effects

A fundamental problem with the analysis of components in biological materials is the effect of the extremely complex and variable mixture of proteins, carbohydrates, lipids, and small molecules and salts constituting the sample. The effect of these compounds on analytical techniques is termed as matrix effect.

It can be defined as **" the sum of the effects of all the components, qualitative or quantitative, in a system with the exception of the analyte to be measured".**

The Effect of Reagents

Assay buffers: The ionic strength and pH of buffers are vitally important, particularly in the case of monoclonal antibodies with pI values of 5–9. The use of binding displacers (blockers) may change the binding characteristics of antibodies, particularly those of low affinity. Detergents used in the buffers may contain peroxides, which inhibit antigen-antibody reaction.

Immunoassay labels: Labels have a profound effect on assays. The structure of most molecules, especially haptens, may be dramatically changed by labeling, e.g. by attachment of a radioactive iodine atom to a steroid. Labeling antibodies with enzymes is less of a problem because of their large size.

Separation of the antibody-bound and free fractions: The proportion of free analyte in the bound fraction and vice versa is known as the "misclassification error". Antibody bound fraction may be efficiently separated from the free analyte using solid-phase systems in which the antibody is covalently linked to an inert support, e.g. the reaction tube, a polystyrene bead, a cellulose or nylon.

Effect of Proteins

Interfering proteins of general relevance include.

Albumin: May interfere as a result of its comparatively huge concentration and its ability to bind as well as to release large quantities of ligands.

Rheumatoid factors: These are autoantibodies usually IgM class, and directed against the Fc portion of IgG. They are not specific to rheumatoid arthritis and are found in other autoimmune diseases, including systemic lupus erythematosus, scleroderma and chronic active hepatitis.

Complement: These proteins bind to the Fc fragment of immunoglobulins, blocking the analyte specific binding sites.

Lysozyme: Strongly associates with proteins having low isoelectric points (pI). Immunoglobulins have a pI of around 5 and lysozyme may form a bridge between the solid–phase IgG and the signal antibody.

Endogenous hormone-binding proteins: These are present in varying concentrations in all serum and plasma samples and may markedly influence assay performance. For example:

SHBG (sex hormone binding globulin) interferes in immunoassay of testosterone and estradiol.

TBG, (thyroxine binding globulin) and **NEFA** (non esterified fatty acid) interfere with the estimation of free T4.

Abnormal forms of endogenous binding proteins: These are present in the plasma of some patients. They are present in familial dysalbuminemic hyperthyroxinemia (FDH) in which albumin molecules bind to thyroxine (T4). Individuals with FDH can be diagnosed as thyrotoxic, in spite of being normal.

Heterophilic antibodies: They may arise as a consequence of intimate contact, either intentional or unintentional, with animals. The most familiar effect of heterophilic antibodies is observed in two-site sandwich reagent-excess assays, in which a 'bridge' is formed between the two antibodies forming the sandwich. Assays that are affected by heterophilic antibodies include for CEA, CA 125, hCG, TSH, T3, T4, free T4, Prolactin, HBsAg and Digoxin.

Mechanical Interference

Fibrinogen from incompletely clotted samples interferes with sampling procedures on automated immunoassay instruments and may produce spurious results. Paraproteinemia causes interferences in many assays by increasing the viscosity of the sample. They may also non-specifically bind either analytes or reagents that may affect the result.

Nonspecific Interference

Non-specific interference may arise from excessive concentrations of other constituents of plasma. Free fatty acids affect some assays for free T4 by displacement of T4 from endogenous binding proteins.

Hook Effect

The "Hook Effect" is characterized by the production of artefactually low results from samples that have extraordinarily high concentrations of antigen (analyte), far exceeding the concentration of the upper standard in the assay concerned.

The Hook effect is most commonly found in single-step immunometric assays, a popular format, chosen for its specificity and speed, particularly with high-throughput immunoassay analyzers. The assays most affected are those that have analyte concentration that may range over several orders of magnitude. For example, α Feto protein (AFP), CA 125, hCG, PSA, TSH, prolactin and Ferritin are most affected by Hook effect.

Reduction of Hook Effect

The incidence of Hook effect can be reduced (but not eliminated) by careful assay design – incorporating a wash step prior to addition of the second antibody, thereby avoiding simultaneous saturation of both antibodies.

Despite attempts to eliminate or reduce the Hook effect by careful assay design the only reliable method of routinely eliminating the effect is to test the samples that are likely to be affected by Hook effect in undiluted and also at a suitable dilution. Such samples should be diluted using either the assay diluent or serum from a normal subject until a stable quantitative response is achieved.

Assay Specificity

It is one of the most important requirements of immunoassays. Interference occurs in all situations in which the antibody is not absolutely specific for the analyte. Consequently, assessment of specificity is a vital step in the optimization of every new immunoassay. Poor specificity results in interference from compounds of similar molecular structure or which carry similar immunoreactive epitopes. In determining the overall specificity of an assay, a major factor is the cross reactivity of the antibody.

Some the major specificity problem areas are related to measurement of steroids and structurally related compounds. All commonly used testosterone assays, cross react in varying degrees with 5α-dihydrotestosterone, and all cortisol assays cross react with prednisolone.

Assessment of the specificity of immunometric assays is complex and quite different from that used for single-site assays. In most assays, two different antibodies are employed, each having unique specificity for a different epitope on the antigen. It is usual practice to employ at least one monoclonal antibody, which can be selected by epitope mapping to react only with predetermined sites on the antigen molecule. Use of two monoclonal antibodies can introduce extreme specificity.

What is the difference between an antigen and immunogen?

The word "antigen" is conventionally used to describe as antibody generators, i.e. that can generate antibody against itself. Also, anything that is foreign to the body is also known as antigen. This definiton of foreignness has become irrelevant with autoantigens. Antigen can be defined as those that bind with the antibody. They need not be foreign in nature. Some antigens also require a carrier/helper to bind with the antibody.

Immunogens, as the name goes are those that can elicit an immune response. It may be either T-cell or B-cell response. All immunogens can be antigens. But all antigens need not be immunogens.

1. What are the different types of epitopes?

There are two different types — sequential and conformational. Sequential epitopes are made of linear region of peptides. Conformational epitopes are formed when the protein chain is folded. Disulfide bonds are important for maintaining the conformational integrity.

2. What is Hook effect?

Sometimes, the value of an analyte obtained by laboratory testing will be very low in spite of suspicion that it will be high. This false low values derived in spite of it being very high is known as Hook effect. This is due to very high concentration in the blood. The levels are so high that they actually mask the binding sites available in the immunoassay system, leading to very low values. (Imagine one hundred persons fighting to sit in 5 chairs. Even though there were hundred the actual number of people who sat were only 5). This is observed in parameters like PSA,hCG, CEA, etc. The solution for this is to dilute the sample and run the assay.

3. What is the difference between chemiluminescence and fluorescence? Which is better?
Fluorescence is a phenomenon where molecule absorsbs light in one wavelength and emits in another wavelength. In this, there is a source of excitation. Chemiluminescence is the production of light by a chemical reaction. The main difference is that there is no radiation is absorbed. The energy required to emit light comes from the energetics of chemical reaction. Definitely chemiluminescence is a better technology for use in immunoassays.

4. What is meant by apoptosis?
In an organism such as a human, the number of new cells created must be balanced by an equal number of cells dying. Sometimes cell death occurs as a result of injury; most often, however, it is a planned, natural process called apoptosis. Apoptosis is sometimes called cellular suicide because it is a cell's own gene products that carry out its death. While it kills a cell, apoptosis is beneficial to the host as a whole - it is important, for example, in development, in the immune response, etc.

5. How does secondary response differ from primary response?
It differs mainly in three ways:
- It involves an amplified population of memory cells
- The response is more rapid
- Higher levels of antibodies are formed than primary response.

6. What are primary and secondary lymphoid organs?
Primary lymphoid organs are the bone marrow and thymus. These organs function as sites for B-cell and T-cell maturation, respectively. Secondary lymphoid organs are spleen, lymph nodes and various mucous associated lymphoid tissues. All these trap antigens and provide sites lymphocytes can interact with antigen.

7. What is the difference between active and passive immunity?

Active immunity	Passive immunity
Produced actively by the host	Received passively by the host
Induced by infection	Conferred by introduction of readymade antibodies
Durable and effective protection	Protection transient and less effective
Immunity effective only	Immunity effective immediately after a lag time
Immunological memory present	No immunological memory
Negative phase may occur	No negative phase
Not applicable in immuno-deficient host	Applicable in immunodeficient hosts

8. What is the difference between analytical and functional sensitivity?
Analytical sensitivity refers to intra assay precision, whereas functional sensitivity refers to inter assay precision.

TECHNOLOGIES

Rapid Immunochromatographic Techniques

Perspective on Membrane-based Rapid Diagnostic Tests

The need for a rapid, reliable, simple, sensitive *in vitro* diagnostic assay for use at point-of-care, have lead to the commercialization of *in vitro* Rapid Diagnostic Tests based on the principle of immunochromatography.

Rapid Diagnostic Tests are membrane-based immunoassays that allow visual detection of an analyte in liquid specimens. In clinical assays, specimens such as urine, whole blood, serum or plasma, saliva and other body fluids may be employed.

What are the Principles of Membrane-based Rapid Diagnostic Tests?

Currently available Rapid Diagnostic Tests comprise of a base membrane such as nitrocellulose. A detector reagent (antigen/antibody-indicator complex) specific to the analyte, impregnated at one end of the membrane. A capture reagent is coated on the membrane at the test region.

When the specimen is added to the sample pad, it rapidly flows through the conjugate pad. Analyte if present in the specimen, binds to the detector reagent. As the specimen passes over the test band to which the capture reagent is coated, the analyte-detector reagent complex is immobilized. A colored band proportional to the amount of analyte present in the sample, develops. The excess unbound detector reagent moves further up the membrane and is immobilized at the control band.

What are the Components of Membrane-based Rapid Diagnostic Tests and how are they Constructed?

Rapid Diagnostic Test consists of (Fig. 22.4)

1. Sample pad
2. Detector reagent/conjugate: Antigen/antibody-indicator complex specific to the analyte, impregnated in the conjugate pad but remains unbound
3. Test band: Coated on nitrocellulose membrane; specific to the analyte
4. Control band: Usually antidetector antibodies coated on the membrane, served to validate the test results
5. Soak pad.

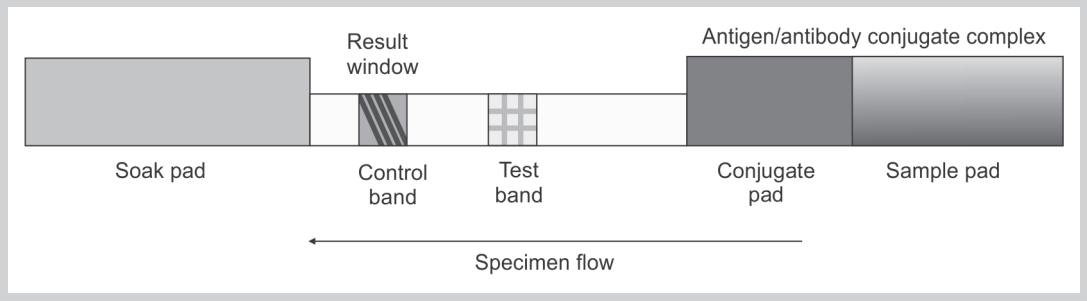


FIG. 22.4: Construction of rapid diagnostic tests

Currently, immunochromatography tests are available in two formats; "lateral flow" and "transverse flow or flow through". The lateral flow formats are available in device or dipstick format. The lateral flow formats are commonly employed where rapid detection of pregnancy, drug abuse, infectious disease or parasitology is required, and serve as qualitative screening assay at laboratories, physician's office or at homes due to their simplicity and ease of performance. The flow through format is less common as the assay requires greater operator involvement. However, some of these assays enable semi-quantitative estimation of the analyte by visual comparison with an in-built reference.

Regardless of the format used, the desired specificity, sensitivity and assay performance depends upon reliable formulation and proper assay assembly.

What are the Limitations and Effects of Various Components on the Performance of Membrane Rapid Diagnostic Tests?

This section highlights the role of various components of Rapid Diagnostic Tests and their effect on attaining the desired performance characteristics.

How does the Nitrocellulose Membrane Affect the Sensitivity of Rapid Diagnostic Tests?

Rapid Diagnostic Tests are fabricated on a solid support membrane, usually made of nitrocellulose. Membranes employed in Rapid Diagnostic Tests are porous. Depending upon the porosity, some membranes are better suited for applications with certain specimens than others. This is because, the pore size of the membrane has significant effect on the capture reagent binding properties and the lateral flow rate. The combined effects of these two phenomena in turn determine the sensitivity and performance of the test assay.

Pore Size and Capture Reagent Binding Properties

It has been observed that as the pore size decreased the effective surface area available for binding of capture reagent increases. Greater effective surface area available for binding, results in optimal coating of the capture reagent, which is essential for attaining the desired sensitivity of the assay.

Pore Size and Lateral Flow Rate

It has been observed that as the pore size increases, the lateral flow rate increases. However, slower flow rate increases the effective concentration (concentration required for interaction) of the analyte, since a slower flow rate allows the analyte and the capture reagent to be in close proximity for a longer times. As it is well known, immunological reactions are time-dependent and prolonged exposure of the analyte with the capture reagent allows better interaction and thus, results in increased sensitivity. The flow rate is important when the analyte is present in low concentrations, such as borderline samples. The relationship between lateral flow rate and effective analyte concentration is:

$$\text{Effective analyte concentration} \quad \alpha \frac{1}{(\text{Flow rate})^2}$$

Thus, it is important to optimize the membranes such that Rapid Diagnostic Tests can achieve rapid results which are also reliable and accurate.

Why are Colloidal Gold Sol Particles Commonly Employed in the Detector Reagent in Membrane-based Rapid Diagnostic Tests?

Interpretation of results in Rapid Diagnostic Tests depends upon the development of a signal at the stipulated time. A signal is generated when capture reagent—analyte-detector reagent complex is formed. The detector reagent/

conjugate consists of an antibody or antigen bound to the indicator. The indicator imparts color to the signal, enabling visual interpretation of results.

Colored latex particles, colloidal gold sol particles, dyes, enzymes and carbon particles are some of the indicator used in immunochromatographic assays. However, stability, protein-binding properties, and particles' size are critical factors that determine their use in immunochromatographic assays. The most popular indicators used in immunochromatographic assays is the colloidal gold sol particle.

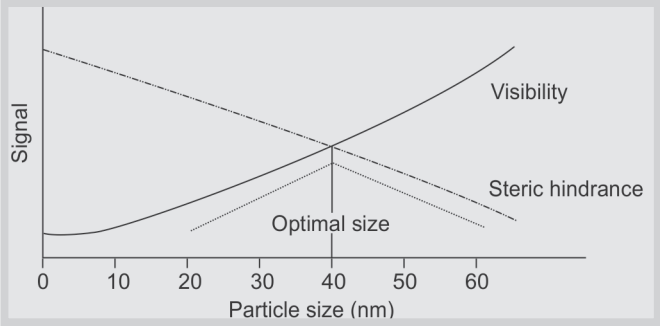


FIG. 22.5: Graph of particle's size v/s signal color of colloidal gold sol

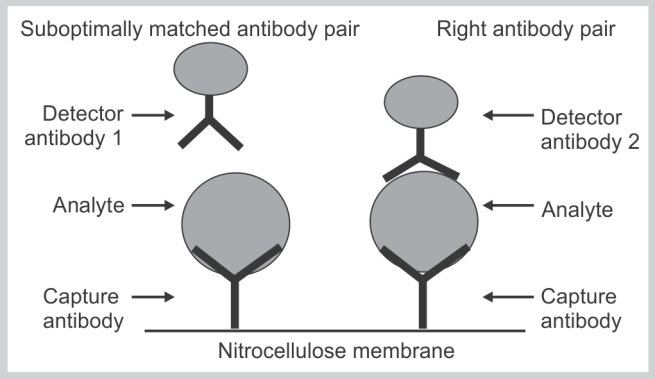


FIG. 22.6: Two-site sandwich immunoassay

Colloidal Gold Sol Particles as Indicator

Homogeneous colloidal gold sol particles are inert and can couple with antibody/antigen, which is stable in dry as well as in liquid forms. All the above-mentioned parameters are determined by the particles' shape and size of colloidal gold.

Effect of Shape of Colloidal Gold Sol Particles on Stability

Colloidal gold sol particles have a net negative charge called "zeta potential". This zeta potential maintains the minimal distance between two particles resulting in long-term stability. Ideally, colloidal gold sol particles should be spherical in shape, since, this shape allows uniform distribution of zeta potential at the surface. In case of nonhomogeneous particles, the zeta potential is not uniformly distributed, thus the particles may come together to form aggregates. These aggregates may permanently get impregnated into the conjugate pad, or during the test assay may deposit on the nitrocellulose membrane leading to discrepant results. Such nonhomogeneous colloidal gold is usually blue/black in color.

Effect of Shape of Colloidal Gold Sol Particles on Sensitivity

Spherical, homogeneous colloidal gold sol particles also allow uniform coating of the detector reagent at their surface. Whereas non-homogeneous colloidal gold sol particles do not allow uniform coating of detector reagent, resulting in decreased assay sensitivity and specificity.

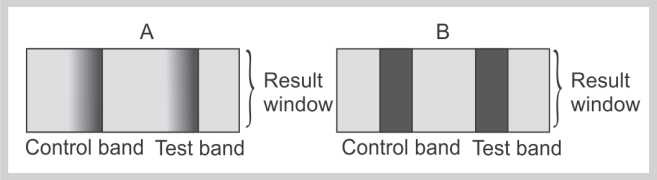
Effect of Size on Color of Colloidal Gold Sol Particles

It has been observed that as the colloidal gold sol particles increase in size, the color turns from light pink to cherry red to red-purple to blue-black to gray-black. Darker colored particles are preferred in Rapid Diagnostic Tests since darker colors allow easy interpretations of results. However, as the colloidal gold sol particles increase in size, these particles are less stable and aggregate together. Secondly, due to the steric hindrance, the larger colloidal gold sol particles tend to dwarf the coated antigen/antibody making interaction with the analyte difficult (Fig. 22.5).

Ideally, the colloidal gold sol used in immunochromatographic assay is ~40 nm in size and imparts a cherry red color, which enables optimal visualization of results against a clear white background and is stable in dry and liquid forms. However, purple colored colloidal gold sol particles if properly stabilized, can also be used in Rapid Diagnostic Tests.

Why are Variations in Band Appearance Commonly Observed in Membrane-based Rapid Diagnostic Tests Employed for Antigen Detection?

The sensitivity/specificity of Rapid Diagnostic Tests primarily depends upon the detector and capture reagent pair. Ideally, the detector reagent should be specific to one epitope of the analyte and the capture reagent specific to another epitope of the same analyte, thereby enabling two-site sandwich immunoassay. To illustrate the same, please refer to Figure 22.6.



FIGS 22.7A and B: A. Band appearance due to avid antibodies. B. Band appearance due to less avid antibodies

For higher analyte sensitivity, manufacturers of commercial Rapid Diagnostic Tests for antigen detection depend on the use of various combinations of capture reagents at the test and control band. Avid capture reagents have a high affinity for the analyte. When the sample containing the analyte reaches the avid capture reagent at the best band, due to high affinity, the avid reagent at the edge of the band captures most of the analyte. Thus, resulting in a distinct thin colored line at the edge of the test band (Figs 22.7A and B).

On the other hand, use of less avid capture reagent (lesser affinity for the analyte) results in capture of the analyte uniformly across the test or control band. Thus, broader bands are generated by less avid antibodies.

Variations in band appearance in different assays is due to use of varying avidity of the antibodies at the test/control band.

What is the Role of Sample Pad in Membrane-based Rapid Diagnostic Tests?

Rapid Diagnostic Tests enable detection of the analyte in several specimens such as urine, whole blood, serum or plasma. However, the pH, viscosity, ionic concentraction, turbidity, and total protein content may vary from specimen to specimen. Variations in these factors can cause alterations in the colloidal gold particles or the capture reagent leading to non-specific results. For example, highly turbid specimens can cause invalid results since the particles from the specimen may block the membrane preventing sample flow. Urine specimen becomes acidic on storage due to bacterial growth. Due to a shift in the pH, the colloidal gold particles come together to form aggregates which may interfere in the performance of the test.

Rapid Diagnostic Tests incorporating serum as specimen may give false-positive results due to the presence of heterophillic antibodies. These antibodies have multispecificity and bind the capture reagent to the detector reagent leading to false positive results. Use for Rapid Diagnostic Tests incorporating heterophillic blocking reagents (HBR) is recommended to avoid this intereference.

A sample pad with a bed volume of minimum retention capacity facilitates transfer of the entire specimen dispensed. This not only ensures minimal wastage of specimen but also the excess specimen can be used to wash away unbound conjugate from the test region for better visualization of results.

Thus, use of sample pad that allows incorporation of buffer salts, stabilizers and HBR, to a large extent eliminates variation in pH, ionic concentration and interference of heterophillic antibodies.

What is the Role of Soak Pad in Membrane-based Rapid Diagnostic Test?

Use of a soak pad with high bed volume is preferred in Rapid Diagnostic Tests because the total volume of specimen that enters the test assay can be increased. This increased volume can be used to dislodge the conjugate as well as wash away the unbound/unreacted conjugate from the test region contributing to clearer background and better visualization of results.

Why do "Faint Ghost Bands" Appear at the Test Region if the Device is Left Out on the Worktable?

A common phenomenon observed in the device format is appearance of faint ghost bands at the test region after some time. After completion of the test, if the device is exposed to warm ambient temperatures, evaporation occurs from the result window. Due to evaportion, the excess sample along with unreacted/unbound conjugate from the soak pad flows back to reaction area. This unreacted or unbound conjugate may then get deposited on the test band resulting in appearance of a "Faint Ghost Band" after sometime (Fig. 22.8).

Results must be recorded at the end of the recommended reaction time for correct interpretation.

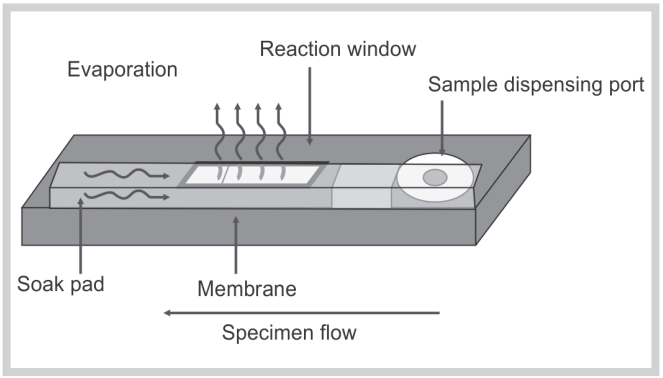


FIG. 22.8: Appearance of "Faint Ghost Band"

How do We Interpret "Broken Bands" at the Test/Control Region?

To prevent evaporation of the specimen from the test window, the membrane of the device is laminated with the help of a thin transparent tape. Sometimes, during the process of lamination, air pockets may be formed between the membrane and the tape. These air pockets prevent uniform sample flow, which may result in appearance of broken bands at the test/control region.

However, appearance of even a broken band at the test region indicates positive results.

In the following section, we shall discuss the role of hCG as a marker for diagnosing pregnancy and certain conditions that may give discrepant results.

Excess Sample Volume Dispensed

Adding excess sample in no way improves the performance of the test. The excess sample added, cannot be absorbed by the sample pad and thus flows out through the sides of the device. Sometimes, the excess sample may flow out along with the conjugate. The amount of the conjugate left in the device is insufficient to perform the assay, leading to invalid results. Secondly, once the specimen flows through the device, the soak pad cannot retain the excess volume of the sample, which then may flow out through the sides of the device or may also flow back to the membrane along with unreacted/unbound conjugate. This unreacted/unbound conjugate may then deposit onto the membrane resulting in apparently discrepant results.

ENZYME IMMUNOASSAY

Introduction

An immunoassay can be defined as a qualitative or quantitative assay, which relies on the reaction between an antigen and its specific antibody. The antigen being bound is called "ligand" and the antibody is the "binder" of the ligand. Enzyme labeled conjugates were introduced first in 1966 for localization of antigens in tissues, as an alternative for fluorescent conjugates. In 1971, enzyme-labeled antigens and antibodies were developed as serological reagents for assay of antibodies and antigens. Their versatility, sensitivity, simplicity, economy and absence of radiation hazard have made EIAs the most widely used procedure in clinical serology. The availability of test kits and facility of automation have added to their popularity.

The enzyme-linked immunosorbent assay (ELISA), [Enzyme immunoassay (EIA) or solid-phase immunosorbent assay (SPIA)] is a sensitive laboratory method used to detect the presence of antigens (Ag) or antibodies (Ab) of interest in a wide variety biological sample.

Many variations in the methodology of the ELISA have evolved since its development in the 1960s, but the basic concept is still the immunological detection and quantitation of single or multiple Ag or Ab in a patient sample (usually serum).

Classification of ELISA

ELISA can be classified in different ways (Fig. 22.16):

Direct ELISA

Direct ELISA is the most basic of ELISA configurations. It is used to detect an Ag after it has been attached to the solid phase (e.g. a membrane or dipstick). An Ab conjugated with a label (e.g. HRPO, AP, FITC) is then incubated with the captured antigen. After washing off excess conjugate and incubating with a substrate and chromogen, the presence of an expected color indicates a specific Ab-Ag interaction. The conjugate could be a commercial preparation specific for the Ag of interest, or an in-house conjugated monoclonal or polyclonal Ab, or even patient serum (Fig. 22.9).

Indirect ELISA

This is extensively used for the detection and/or titration of specific antibodies from serum samples. The specificity of the assay is directed by the antigen on the solid phase, which may be highly purified and characterized. The first, or **primary** Ab is incubated with the Ag, and then the excess is washed off. A second or **secondary** Ab conjugate is then incubated with the samples. The excess is again removed by washing. For color to develop, a primary Ab that is specific for the Ag must have been present in the sample (e.g. human serum, CSF or saliva). This indicates a positive reaction. It is important, during assay

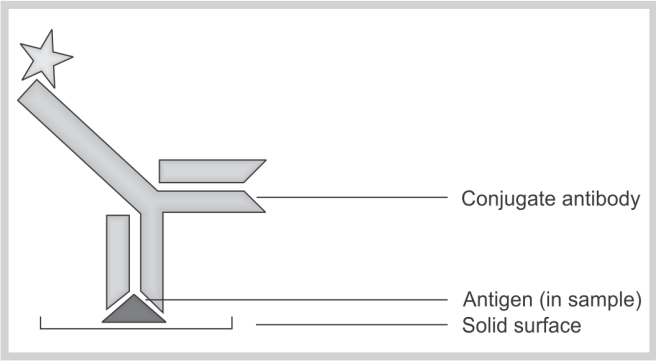


FIG. 22.9: Direct ELISA

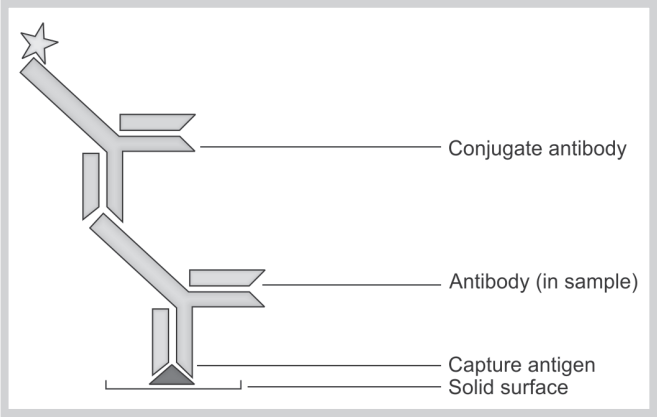


FIG. 22.10: Indirect ELISA

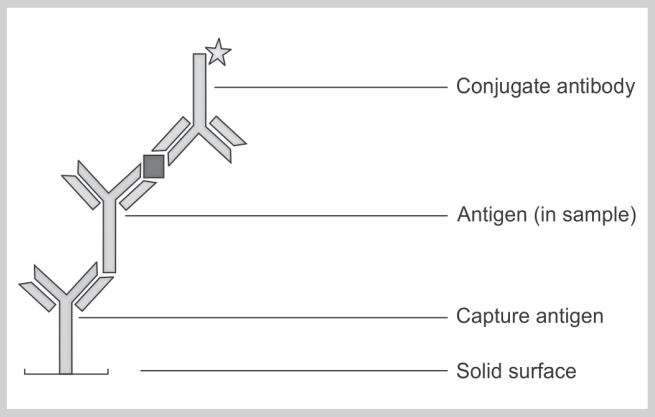


FIG. 22.12: Antibody capture

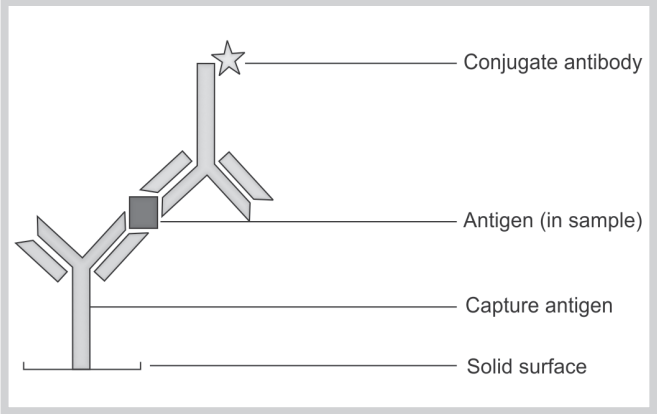


FIG. 22.11: Antigen capture

optimization, to ensure that the secondary Ab does not bind nonspecifically to the Ag preparation or impurities within it, nor to the solid phase (Fig. 22.10).

Capture ELISA

Antigen Capture

In this, more specific approach, a capturing Ab is adsorbed onto the solid phase. The capture antibody may be the reagent to be tested (e.g. the titer of a patients immune response to a known Ag). However, the Ab may be a standard reagent and the antigen the unknown (as when a patient's serum is being investigated). The same stringent optimization is required as for indirect ELISA. This will ensure that the Ab does not cross-react in the absence of Ag, or nonspecifically binds to the solid phase. It is also important, when detecting the Ag, to use Ab from different animal species to prevent same-species Ab binding (e.g. a polyclonal rabbit capture Ab will capture a monoclonal conjugate if it was raised in rabbits. This will produce a positive result in the absence of Ag) (Fig. 22.11).

Antibody Capture

In this approach, a capturing Ab is adsorbed onto the solid phase. The Ab is designed to capture a class of human Ab (e.g. IgG, IgA or IgM). Next, the sample is applied, containing the Ab under investigation. After washing, an Ag specific for the Ab is added and finally an anti-Ag conjugate provides the signal (Fig. 22.12).

Another approach is to coat antigen on the solid surface. The antibody (from the sample) binds with it. After washing an anti-antibody (antibody against antibody) conjugated with enzyme is added.

Competitive ELISA

This implies that two reactants are trying to bind to a third. Proper competition assays involve the simultaneous addition of two competitors. It can be of various types.

Direct Antibody Competition

In this, the solid phase is coated with antigen. The labeled and unlabeled antibodies both compete for the limited binding sites for the antigen (Fig. 22.13).

Direct Antigen Competition

This is same as above except that the solid phase is coated with antibody, while labeled and unlabeled antigens (from the patient sample) compete for the antibody.

In a competitive ELISA, the amount of color developed is inversely proportional to the amount of Ag-specific patient Ig present. Careful standardization is required to interpret the results.

Analytes tested by competitive ELISA

- Thyroid hormones T3, T4, FT3, FT4
- Steroid hormones:

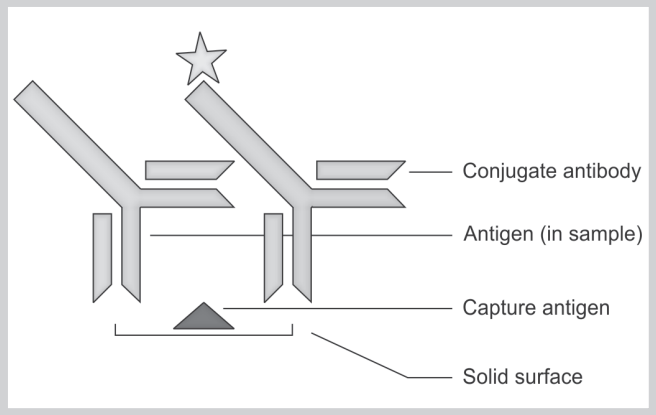


FIG. 22.13: Competitive ELISA-direct antibody competition

- Androgens, testosterone, androstenedione, DHEA-S
- Estrogens: Estradiol (E2), estriol (E3)
- Progesterones: Progesterone, 17-OH progesterone.
- Cortisol
- Hepatitis Markers: HAV, HBc Antibody.

Streptavidin-Biotin ELISA

This is also a type of sandwich ELISA. In this, the solid phase is coated with streptavidin instead of antigen or antibody.

Avidin, found in hen egg white, is a fascinating protein because of its high binding affinity for the vitamin Biotin (vitamin H). In fact, avidin and a related protein, streptavidin (found in the bacteria *Streptomyces avidinii*), exhibit the highest known affinity in nature between a protein and a ligand (Ka.1015 M/L). The rate constant for the avidin/biotin association reaction is also fast ($K=7 \times 10^7$ M-1s-1).

Because of its extraordinary binding capacity, biotin is used to develop modern ultrasensitive quantitative enzyme immunoassays. The tetrameric avidin/streptavidin system (cross-linking with biotinylated molecules) has been used for developing third generation ultrasensitive quantitative endocrine and other related immunoassays.

The active form of avidin is a tetramer composed of four glycosylated subunit (mol. wt = 62,400). The avidin tetramer has the capacity to bind up to four biotin molecules through noncovalent linkages. Each avidin monomer consists of 128 residues arranged in an orthogonal eight-stranded "B barrel". Biotin binds with the barrel towards one end only.

Avidin-biotin relation of Ag-Ab binding

Every antibody has two antigen binding sites (F_{ab}), the structure and shape of the particular antigen-binding site of an antibody (also termed as paratope) and its corresponding antigen (more specifically epitope) is such that they tightly fit (high attraction force and minimum repulsive force between participating molecules) to each other. Similarly, biotin gets tightly fit into the avidin molecule because of the following structural characteristics of avidin:

1. Strong hydrogen bonding between the monomers of avidin makes streptavidin an extremely stable molecule.
2. Properly placed hydrophobic and hydrophilic residues that create a tight fit for the biotin molecule.
3. Limited access to other parts of the protein molecule for non-specific binding.

The avidin-biotin system is well suited for use as a bridging or sandwich system in association with antigen-antibody reactions. The biotin molecule can be easily coupled to either antigens or antibodies, and avidin can be conjugated to enzymes and other immunological markers.

Advantages of Streptavidin

Streptavidin is used in preference to avidin because of the following reasons:

- It has a neutral isoelectric point and it does not contain carbohydrates
- Streptavidin is more inert in assay systems
- It reportedly exhibits less non-specific binding than avidin; and hence, offer, greater specificity.

Streptavidin-biotin based IEMA systems are a better choice for Indian laboratories because of the following:

Stability: The binding of avidin and biotin is not disturbed by extremes of salt, pH or temperature.

Specificity and sensitivity: Avidin has a very high binding affinity for biotin and so the system avidin-biotin is highly specific; moreover, the rate constant for the avidin-biotin association is also fast; and as a result, assay protocols become rapid and simple.

Speed of the reaction: The solid phase is coated with avidin and the capture antibody is biotinylated, this minimizes the need for the other coating methods and facilitates the use of antibodies with high affinities, reducing overall assay incubation time.

Temperature stability and other problems: Non-bound avidin is very thermostable for the folded-unfolded transition, Tm=85 degree Celsius (pH 7–9). When biotin is bound, the protein acquires greater thermostability (Tm=132 degree celsius).

Thus, the avidin-biotin is more resistant to high temperature. This greater thermostability of avidin-biotin system overcomes/reduces the problems faced during transportation, storage use and handling.

Significance of Coating Streptavidin as Solid Phase

Streptavidin possesses greater electrostatic attraction for the microwell/plastic tubes.

Streptavidin-biotin based IEMA systems use a biotinylated antibody (biotin-labeled 1st antibody/capture). This is because biotin can be attached to the F_c portion of an antibody in relatively high proportion without loss of immunoreactivity.

The binding ratio of avidin to biotin is 1: 4. One molecule of streptavidin, which is a tetramer can bind with four molecules of biotin/biotinylated 1st antibody. In a traditional enzyme immunoassay, a limited space is normally available for coating the capture/1st antibody in the bottom of the microwell/plastic tube. Ideally, if one can increase the number of capture/1st antibodies coated on the microwell. The assay sensitivity goes up because more number of antigen-binding sites becomes available in case of low concentration of analytes (antigens) present in the sample.

Streptavidin biotin based systems coat streptavidin on the microwell/plastic tubes instead of directly coating the capture/1st antibody. Capitalizing the tetrameric valency of streptavidin binds with four molecules of biotinylated capture/1st antibody thus providing an excess of binding sites to the system, which ensures four-fold higher sensitivity of the IEMA system (Fig. 22.14).

Analytes tested by Streptavidin-biotin ELISA

Pituitary hormones: TSH, FSH, LH, PRL
Tumor markers: PSA, CEA, AFP, CA 125, CA 15.3, CA 242, hCG
Antibody estimation: Anti-thyroglobulin, anti-thyroid peroxidase (TPO), anti-*H. pylori.*

Immunocapture ELISA

This is also a type of sandwich ELISA and is commonly known as μ-capture/IgM-capture ELISA. It is mainly used for the identification of IgM class of antibodies. In this, there is an "immunocomplex" (antigen complexed with conjugate) is used. The microwell is coated with anti-human IgM, which is IgG in nature, which is specific against the μ-chain of IgM class antibody (from the patient sample). After binding the conjugate, it is added followed by substrate (Fig. 22.15).

Analytes tested by immunocapture ELISA

Infectious serology IgM panels:
TORCH infections: Toxo, Rubella, CMV, HSV
Hepatitis markers: HAV IgM, HBc IgM, HDV IgM.

Interference Corrected ELISA

It is also one type of sandwich ELISA and is used for infectious diseases immunoassays. This ELISA is best

FIG. 22.15: Immunocapture ELISA

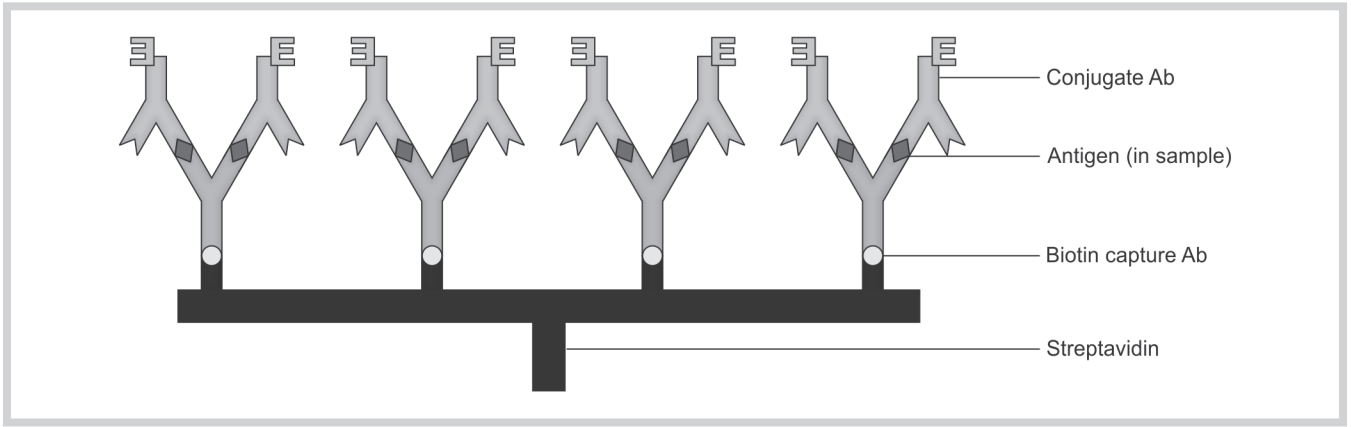


FIG. 22.14: Streptavidin-biotin ELISA

for overcoming all the false positives and false negatives, which affect the correct result. For TORCH (Toxo, Rubella, CMV, HSV) IgM assays, many factors can give false positives and false negatives. This leads to wrong reporting and wrong diagnosis. "Interference correction" is a principle by which one can remove all these factors. A few manufacturers have this feature. For TORCH assays, it is always suggested to use interference corrected ELISA kits. Based on separation steps, ELISA can be classified as:

Homogeneous ELISA

Do not require separation of free and bound label. Bound label selectively separates or label is inactive when bound.

Latex Particle Agglutination Immunoassay (LPAIA)
A large number of latex agglutination immunoassays have been adopted from clinical chemistry. These assays are based on the visualization of antigen-antibody complexes by the attachment of latex particles or gold colloids. Entities of this type with dimensions in the nanometer or micrometer range can be quantified by turbidimetry, nephelometry, light scattering techniques, and particle counters.

Enzyme-Multiplied Immunoassay Technique (EMIT)
In the EMIT, the analyte is covalently bound to the enzyme in spatial proximity to the active site; and consequently, the formation of the antibody-antigen complex inactivates the enzyme; addition of hapten results in a reduction of this inactivation. Over a limited range, the enzyme activity is approximately proportionate to analyte concentration. This method has been widely employed for therapeutic drug monitoring.

Apoenzyme Reconstitution Immunoassay System (ARIS)
If, however, the antigen is covalently bound to the prosthetic group of an enzyme such as glucose oxidase and an aliquot of the coupled antigen to flavin–adenine dinucleotide is added to determine an analyte, free antibodies prevent the reconstitution of the enzyme. The concentration of the free antibody naturally depends on the analyte concentration in the sample. Similar to the EMIT technique, the ARIS is used in automatic analyzer systems in clinical chemistry.

Fluorophore-Labeled Homogeneous Immunoassay (FLHIA)
At first glance, fluorescent labeling appears to have a much higher detection strength compared to colorimetric detection, but this is not the case. First, the affinity constant generally limits the detection strength of a process. Second, fluorophores are exposed to many influences, such as quenching by impurities, or even adsorption of the fluorophore molecule. However, the fact that the detection can be repeated is advantageous, whereas a chemical reaction is irreversible.

Homogeneous Fluorescence Polarization Immunoassay (FPIA)
Direct observation of the formation of a hapten-(fluorescent labeled) antibody complex is also possible in polarized light. The presence of free hapten reduces the antibody-tracer complex concentration, and the degree of polarization is lowered. The detection strength of this test is in the μmol/L range and thus not yet high enough for environmental analysis.

Microparticle Enzyme Immunoassay (MEIA)
There are a number of variations in this method. The enzyme-labeled binder binds to the analyte, which in turn is bound to binder-coated microparticles. Initially, free in solution during the foregoing chemical reactions, the microparticles are immobilized on glass fiber, and the complex of primary binder (capture), ligand (analyte) and labeled binder (conjugate) is exposed to substrate, producing a colored product.

Heterogeneous ELISA

This requires separation of free and bound label. Most ELISAs described above, fall into this category.

Based on the functional results ELISA can be classified as shown in Figure 22.16.

Quantitative ELISA

In this type the concentration of the analyte is measured and expressed in standardized units (ng/dL for T3, ng/mL for PSA). Standards are run and graph is plotted against which the concentration of the analyte is estimated, e.g. T3, T4, TSH, FSH, LH, etc.

Semi-quantitative ELISA

In this type, the controls are used (positive control, negative control, cut-off control). An arbitrary unit is given to express the concentration (EU/mL). Graph may or may not be used, e.g. TORCH, ANA, etc.

Qualitative ELISA

In this type, the controls are used and is formula based. No graphs are required, e.g. HIV, HBsAg, etc.

ELISA: Practical Aspects

The different components of ELISA are packed together. This is commonly known as "Kit". The components are as follows:

Solid Surface

It can be a microwell, coated tube or bead. This can be compared to a plate on which the reaction takes place. The

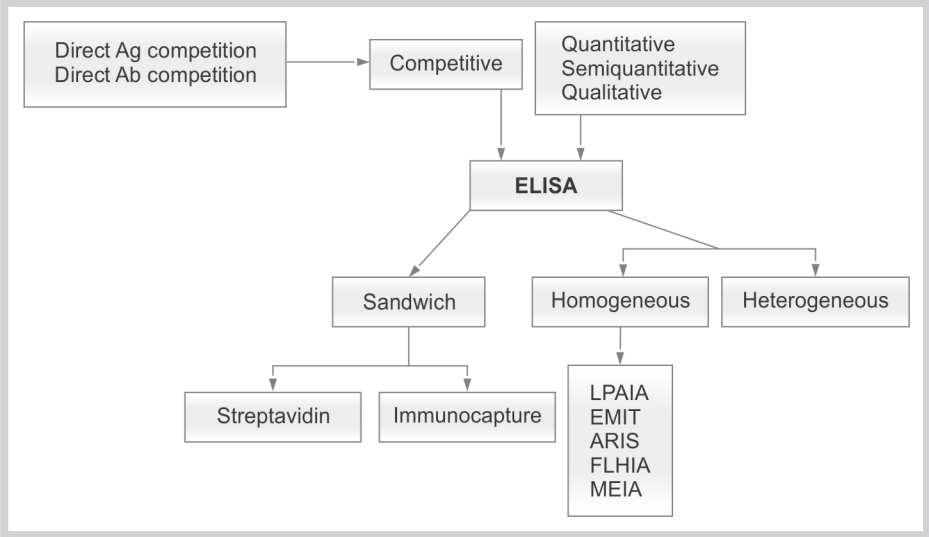


FIG. 22.16: Classification of ELISA

microwell can be breakable or unbreakable. The coated tubes may be of polystyrene or polypropylene in nature. The solid phase may be coated with antigen, antibody or streptavidin. The choice of solid phase influences the measurement of optical density. In the case of Microwell, it is measured with an ELISA reader; and in coated tube it is measured by an analyzer.

The process of fixing onto the solid phase is called "adsorption" and is commonly called coating. Most proteins adsorb to plastic surfaces, probably, as a result of hydrophobic interactions between nonpolar protein structures and plastic matrix. There may be nonspecific binding of unwanted proteins in available free sites. This can be avoided by adding "immunologically inert" proteins so as to block the free sites. These blocking agents may be added during the coating process.

Calibrators/Controls

They are references against which the value of the analyte in the sample is estimated. An important fact is that immunoassays do not actually measure the analyte. They can only provide a quantitative estimate of concentration by direct comparison with standard/calibrator material.

The Features of an Ideal Calibrator

- A prerequisite for standardization is that the standard/calibrator and analyte are identical
- The calibrator should contain the analyte in a form identical to that found in the sample
- Calibrators should ideally be prepared by using a base material identical to that in the test sample
- For clinical applications, human serum is the preferred base matrix.

References

The matrix of a calibrator needs to behave in a similar way to the sample matrix.

For assay of hormones that are bound to serum protein, it is hard to use any other matrix other than human serum.

A prerequisite for standardization is that the standard/calibrator and analyte are identical. In other words, the calibrator should contain the analyte in the form identical to that found in the sample.

Conjugate

It is the binder in the immunoassay system. The analyte in the sample may compete (in case of competitive ELISA) or bind with (in sandwich ELISA) the conjugate. It is either an antigen or antibody tagged with an enzyme (depending upon what it is being detected). The conjugate should have certain characteristics:

- The enzyme must be capable of binding to an antigen or antibody (the enzyme will react with the substrate to give color)
- Should be stable at typical assay temperature
- Should be stable when stored at 2 to 8°C
- It must undergo only low-grade inactivation of reagent and enzyme

- Long-term stability without loss of immunological and enzymatic activities.

Most Commonly Used Enzymes in Immunoassays

Alkaline phosphatase, horseradish peroxidase, acetylcholinesterase, carbonic anhydrase, glucose oxidase, glucamylase, glucose-6 phosphate dehydrogenase, lysozyme, malate dehydrogenase.

Substrate

The confirmation of an antigen-antibody reaction is done by a suitable indicator. In ELISA this is done by the substrate. The substrate reacts with the enzyme (in the conjugate) to give a colored end product. The intensity of the colored product is directly/inversely proportional to the antigen-antibody reaction (in turn to the presence/absence or concentration of the analyte in the sample). The colored end product may be soluble which is measured colorimetrically. This is mainly used in quantitative immunoassays. The end product may also be insoluble which is measured visually. It is suitable for dot blot assays. The end product remains as a permanent record (e.g. Western blot strips, Rapid test cartridges, etc.). The substrate should have the following features:

- It should be able to produce intense colored end product
- Fast reaction rate or rate of conversion of substrate to end product
- Ability to produce a broad range of colored end product in a given time depending upon the amount of conjugate (analyte) it has reacted with.

The Commonly Used Substrates

TMB (tetra-methyl benzidine), OPD (o-phenlyenediamine), DAB [diaminobenzidine (with enzyme HRP)] and BCIP (5-bromo 4-chloro 3-indolyl phosphate), [NBT (Nitroblue tetrazolium)] (with enzyme alkaline phosphatase).

The factors affecting the performance of substrate are: temperature, pH, buffer composition, etc.

Stop Solution

The enzyme substrate reaction needs to be stopped to measure the optical density of the end product. The stop solution acts by destroying the enzyme component. The commonly used stop solutions are 1N HCl, 4N H_2SO_4, NaOH.

Steps in ELISA

There are multiple steps involved in an ELISA procedure. They can be grouped as follows:

Dilution

This is the first step. The reagents like conjugate, controls, sample diluent, wash buffer, stop solution, substrate, etc. are mixed in required proportions. In some cases, sample may also be diluted in given ratios before adding them in the well. Proper calculation of dilution ratios should be made. It is advisable to prepare slight excess of the quantity required to avoid pipetting errors. In some cases, the dilution itself will have excess volumes to offset the pipetting errors.

Addition

This is the pipetting step. It is done by either manual or electronic dispensing systems. The tips used must be compatible with the pipette. Multichannel pipettes can be used for addition of common reagents like conjugates, substrates, stop solution, etc. The advantage of electronic dispensing system is that errors are minimized. During pipetting some bubbles may be formed in the well. They should be burst using a pin. Different pins should be used for breaking different wells, as usage of same pin may lead to carry over.

Incubation

It is time period during which antigen combines with antibody or enzyme reacts with substrate. There are two types of incubation—stationary and rotatory incubation. In stationary—incubation, mixing takes place through diffusion of reagents. Because stationary incubation relies on diffusion of molecules, the role of temperature becomes extremely critical. To ensure complete reaction, longer incubation time is recommended. Rotatory incubation ensures complete mixing of reagents. This leads to increased contact between analyte and the capture/adsorbed reactant. Rotation gives additional kinetic energy to the system and hence, the reaction is less dependent on temperature.

Wash

It is actually a dilution process to optimally dilute the original solution without stripping off the bound/capture protein. It is one of the critical steps in ELISA. The optimal dilution step requires 3–5 cycles. Less than 3 cycles will leave behind residual proteins in the wells. The volume of wash solution dispensed per well should be high enough to cover the entire surface coated with antigen/antibody. The entire well must be filled during the wash cycle. Enough care is needed to prevent well-to-well overflowing of wash solution. During washing, more

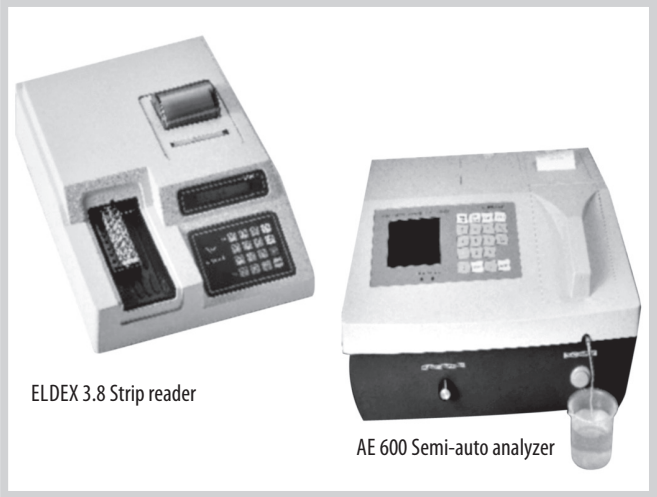


FIG. 22.17: Analyzers (*Courtesy*: Lilac Medicare)

specifically in aspiration step, it is recommended to leave a small amount of wash buffer in the wells. This creates a film on the well and thus, prevents denaturation due to drying effect. The liquid used to wash wells is usually buffered (PBS) in order to maintain isotonicity, since most Ag-Ab reactions are optimal under such conditions. Tap water is not recommended, since tap water varies greatly in composition (pH, molarity and so on).

Estimation

The estimation of color can be done either visually (for rapid tests, Western blots, etc.) or using an ELISA reader. It is an instrument to measure the optical density and give the interpretation according to the program. The instrument can be programmed to do calculation and print the results. In case of coated tubes, the measurement is done by an analyzer (Fig. 22.17).

Interferences in Immunoassays

Despite advances in the design of immunoassays, the problems of unwanted interference have yet to be completely overcome. An ideal immunoassay should have the following attributes:

- The immunochemical reaction behavior should be identical and uniform for both the reference (standard/calibrator) preparation and the analyte in the sample
- The immunochemical reaction of the reagent is uniform from batch to batch
- The immunochemical method is well standardized to ensure that the size of measurement signal is caused only by the antigen-antibody reaction
- For macromolecules, the results declared in arbitrary units (IU—International Units), the conversion to (SI) units is not constant and depends on many factors.

Definition of Interference

Interference may be defined as "the effect of a substance present in an analytical system which causes a deviation of the measured value from the true value, usually expressed as concentration or activity."

The IFCC (International Federation of Clinical Chemistry) offers the following definition: "Analytical interference is the systematic error of measurement caused by a sample component, which does not, by itself, produce a signal in the measuring system."

Assay interference can be "analyte dependent or analyte independent" and can increase or decrease the measured result.

Increase (positive interference) is due to lack of specificity.

Decrease (negative interference) is due to lack of sensitivity.

Assay interference can be of different types:

- Preanalytical errors
- Analytical errors
- Postanalytical errors (Fig. 22.18).

Preanalytical Variables

All factors associated with the procedures before the actual performance of the test are known as preanalytical errors. They can be as follows:

Patient Based

Such as incorrect sampling times and environmental factors such as smoking, etc. may change analyte concentration and consequently interpretation.

Specimen Based

There are many factors that constitute this.

Blood collection procedure and time of collection. Certain hormones are affected by the time of collection.

Nature of the Sample

For all immunoassays, serum is the matrix of choice. Samples collected in to tubes containing sodium fluoride may be unsuitable for some enzymatic immunoassay methods; preservation with sodium fluoride may affect results. Impurities in tracers interfere with direct dialysis methods for free hormones.

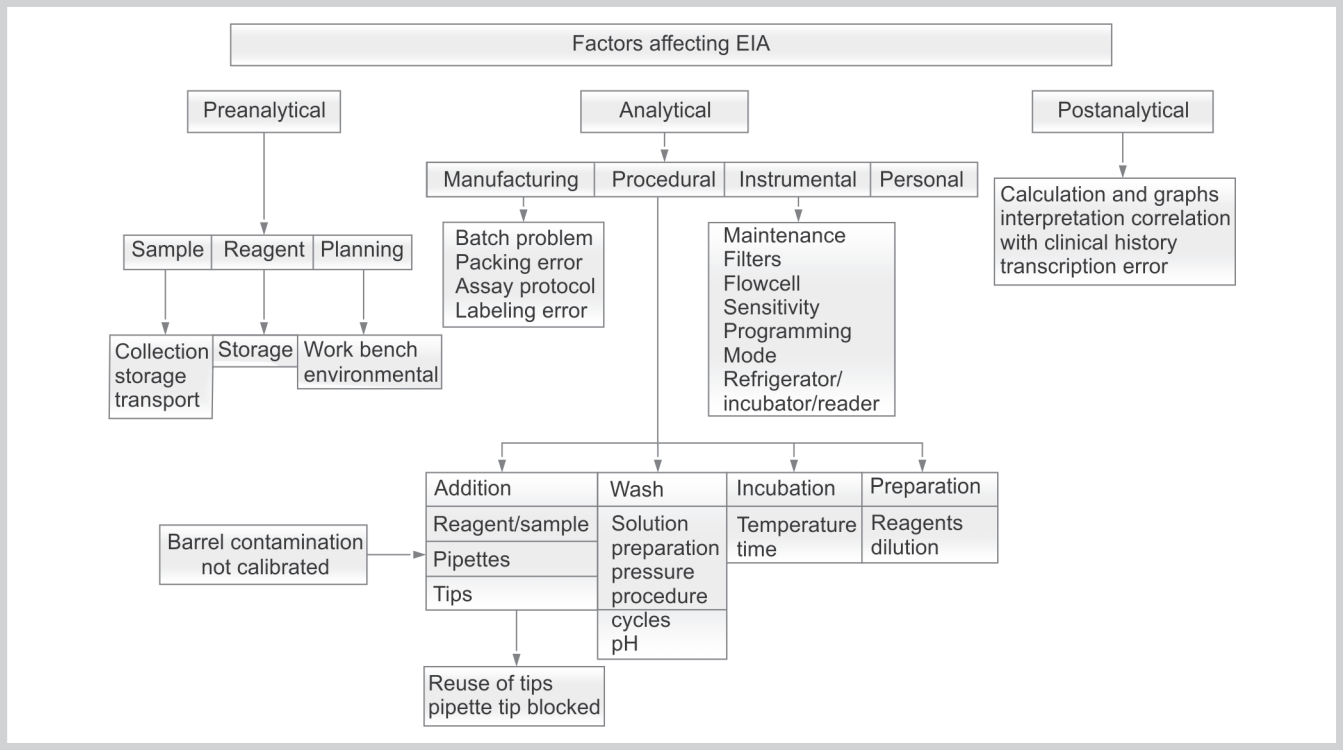


FIG. 22.18: Factors affecting EIA

Hemolysis and Hyperbilirubinemia

Lipemia may cause interference with assays for fat-soluble compounds such as steroids.

Stability and storage of reagents and samples are as follows:

Assay Based

Certain procedures before the test is performed like:

- Bringing the kit to the room temperature
- Checking the incubator temperature
- Checking the room temperature
- Formatting and arranging the workbench
- Planning for assays to be performed
- Proper dilution calculation
- Maintaining the fridge temperature
- Dilution of samples with distilled water instead of zero calibrator.

Manufacturing error:

- Batch problem
- Packing error
- Assay protocol error
- Labeling error
- Wrong pack insert.

Analytical Variables

These form the major part of all errors that affects the results in immunoassays. Mostly, these are overlooked and so attention is not paid to rectify them. These will lead to erroneous results. They are mainly the procedural errors. They are mostly confined to the different steps—addition, washing, incubation, dilution, pipetting, reading the protocol suggested by the manufacturer is not followed. Some of the errors are as follows:

Washing Errors

- High pressure washing
- Carry over during washing
- Drying of wells
- Splashing during washing
- Non-removal of all wash solution from wells or tubes
- Not following soak time (if present) during washing
- Using a syringe to wash
- Leaving bubbles in the well after washing
- Not tapping the well after washing
- Use of contaminated wash buffer
- Wells/tubes falling off while washing.

Pipetting Error

- Reuse of pipette tips
- Pipette tip blocked
- Pipette/dispenser not primed
- Pipette barrel contaminated
- Using tips to break bubbles
- Using same tips to break bubbles.

Equipment Error

- Incubators not maintained at right temperature
- Heating not uniform throughout
- Washer probes blocked, contaminated
- Refrigerator not maintaining right temperature
- Defrost water falling on kits
- Use of dry incubators instead of water bath
- Instrument filters not checked periodically.

Procedural Errors

- Interchange of reagent lots
- Wells not covered during incubation
- Not blanking when required
- Not running all calibrators to plot a graph
- Reuse of pipette tips
- Bubbles in wells
- Use of contaminated or uncleaned tubes to prepare reagents
- Using kits/reagents beyond expiry
- Using negative wells again
- Not mixing after adding stop solution.

Postanalytical Variables

The errors that occur after the performance of the test are called as postanalytical errors. These are like:

- Calculation mistakes
- Choosing a wrong graph scale
- Comparison of result with inappropriate reference interval
- Not correlating the results with clinical history
- Transcription error when report is prepared.

Use of Wrong Reference Values

The reference ranges (normal ranges) of various parameters are different. Manufacturers specify the reference ranges. The units for reference ranges may differ. One should only compare with reference intervals given in the pack insert or should establish their own reference interval.

Comparison with results of different reference intervals will create confusion. Many laboratories make the mistake of comparing results with other labs without knowing the assay conditions and other factors that affect ELISA.

Use of Wrong Units

The units given by manufacturers may be different. For example, T3 ng/dL is different from ng/mL. A kit having a sensitivity of 0.4 ng/dL is more sensitive than 0.4 ng/mL (it is 40 ng/dL).

One should always make note of the units. Reports from different laboratories may differ in this aspect and create confusion.

The errors may be of any kind, but the outcome is that the result is incorrect and hence, a wrong report is given. To overcome this, one should follow all the steps and adhere to the protocol strictly.

ELISA Troubleshooting

ELISA is a technique of multiple steps. The steps must be followed strictly to achieve good results. Errors at any levels will affect the final result. Complete understanding of the process is necessary for troubleshooting.

Practical Tips on ELISA

Factors affecting EIA are shown in Figure 22.18.

Normal Washing

In washing plate manually, the most important factor is that each well receives the washing solution so that, no air bubbles are trapped in the well or a thumb is not placed over corner wells.

Strip/Plate Washers

Various washing cycles can be programmed. Careful maintenance is essential, since they are prone to machine errors, such as having a particular nozzle being blocked.

Washing Tips

- Follow procedure for preparation of wash buffer
- Check washer alignment daily as part of routine instrument start-up procedures
- Ensure that the plate is levelled
- Make certain well is completely filled, when washing, to ensure residual conjugate is removed
- Examine that the plate is levelled
- Make certain well is completely filled, when washing, to ensure residual conjugate is removed
- Examine the fill volume (a slight dome should be observed at the top of the well)
- When washing does not allow wells to overflow
- Reduce pressure in wash system
- Check washers before use to determine they are working properly. Perform routine maintenance

- Be certain to wash the specified number of times
- Allow approximately 20 seconds between the addition of wash solution and subsequent aspiration
- Examine the wells for complete aspiration of contents
- Upon completion of wash cycle, blot to remove residual fluid.

Pipetting Tips

- Calibrate pipettes regularly according to manufacturer's instructions
- Avoid touching sidewall of well with tips
- Avoid splashing of sample and reagents
- Avoid blowing out tip contents
- Use a new tip for each sample/control/reagent addition
- New tips should be used on the multichannel pipettes for each reagent to be added
- Reverse pipette when using the multichannel pipette to add conjugate and substrate solution
- Forward pipette when using the multichannel pipettes to add stop solution
- Check pipette tips are long enough to provide air space between top of tip and pipette barrel
- Check pipette barrel for residual fluid or dried material, remove if present
- Ensure pipettes tips are fitted tightly
- Service pipettes periodically by the manufacturers or authorized person
- Do not open the pipette without proper tools.

Microplates

- Bring microplate pouches to room temperature before opening
- Level microwells evenly in microplate frame as the individual breakaway wells have very flexible plate frames leading to bowing of wells and yield poor washes
- Place plates in dark immediately after addition of substrate solution, provided the substrate is sensitive to light
- Grasp holder on grip marks when tapping to avoid strips slipping from holder
- Rotate strips 180°C and reinsert or use correct holder if strips do not fit in holder
- Seal unused wells in pouches along with the desiccant
- Date the pouches when first opened
- Clean bottom surface of plates with wash buffer to remove fingerprints
- Make sure microwells are at level during washing, reagent addition and plate/strip reading
- Wipe the bottom of the plate with a lint-free cloth/towel before reading
- Do not allow microwells to become dry once the assay has begun.

Substrate Preparation

- Use freshly prepared substrate A and substrate B
- Do not hold substrate solution longer than 1 hour
- Follow procedure of working substrate solution
- The temperature of solution is important because it affects rate of color reaction
- Do not add fresh substrate to reagent bottle containing old substrate
- Clean old substrate solution bottle with H_2SO_4 and thoroughly rinse with distilled water.

Conjugates

- Store at recommended temperature
- Never store exclusively diluted conjugate for use at some later time
- Always make up the working dilution of conjugate just before you need it
- Never leave conjugates on the bench for excessive time.

General Tips

- Plan the assay properly
- Ensure all necessary items are chosen before starting the assay
- Maintain a logbook on calibration and results data
- While performing the assay, do not divert attention.

Matrix Effects

A fundamental problem with the analysis of components in biological materials is the effect of the extremely complex and variable mixture of proteins, carbohydrates, lipids, and small molecules and salts constituting the sample. The effect of these compounds on analytical techniques is termed as matrix effect.

It can be defined as "the sum of the effects of all the components, qualitative or quantitative, in a system with the exception of the analyte to be measured."

The Effect of Reagents

Assay buffers: The ionic strength and pH of buffers are vitally important, particularly in the case of monoclonal antibodies with pH values of 5–9. The use of binding displacers (blockers) may change the binding characteristics of antibodies, particularly those of low affinity. Detergents used in the buffers may contain peroxides, which inhibit antigen-antibody reaction.

Immunoassay Labels
Labels have a profound effect on assays. The structure of most molecules, especially haptens, may be dramatically changed by labeling, e.g. by attachment of a radioactive iodine atom to a steroid. Labeling antibodies with enzymes is less of a problem because of their large size.

Separation of the Antibody-bound and Free Fractions

The proportion of free analyte in the bound fraction and vice versa is known as the "misclassification error". Antibody bound fraction may be efficiently separated from the free analyte using solid-phase systems in which the antibody is covalently linked to an inert support, e.g. the reaction tube, a polystyrene bead, a cellulose or nylon.

Effect of Proteins

Interfering proteins of general relevance include the following:

Albumin
It may interfere as a result of its comparatively huge concentration and its ability to bind as well as to release large quantities of ligands.

Rheumatoid Factors
These are autoantibodies usually IgM class, and directed against the Fc portion of IgG. They are not specific to rheumatoid arthritis and are found in other autoimmune diseases, including systemic lupus erythematosus, scleroderma and chronic active hepatitis.

Complement
These proteins bind to the Fc fragment of immunoglobulins, blocking the analyte specific binding sites.

Lysozyme
Strongly associates with proteins having low isoelectric points (pI). Immunoglobulins have a pI of around 5 and lysozyme may form a bridge between the solid-phase IgG and the signal antibody.

Endogeneous Hormone-binding Proteins
These are present in varying concentrations in all serum and plasma samples and may markedly influence assay performance. For example, **HBG** (sex hormone binding globulin) interferes in immunoassay of testosterone and estradiol **TBG**, (thyroxine binding globulin) and **NEFA** (non-esterified fatty acid) interfere with the estimation of free T4.

Abnormal forms of Endogeneous binding Proteins
These are present in the plasma of some patients. They are present in familial dysalbuminemic hyperthyroxinemia (FDH) in which albumin molecules bind to thyroxine (T4). Individuals with FDH can be diagnosed as thyrotoxic, in spite of being normal.

Heterophilic Antibodies

They may arise as a consequence of intimate contact, either intentional or unintentional, with animals. The most familiar effect of heterophilic antibodies is observed in two-site sandwich reagent—excess assays, in which a 'bridge' is formed between the two antibodies forming the sandwich. Assays that are affected by heterophilic antibodies include CEA, CA 125, hCG, TSH, T3, T4, free T4, prolactin, HBsAg and Digoxin.

Mechanical Interference

Fibrinogen from incompletely clotted samples interferes with sampling procedures on automated immunoassay instruments and may produce spurious results. Paraproteinemia causes interferences in many assays by increasing the viscosity of the sample. They may also nonspecifically bind either analytes or reagents that may affect the result.

Nonspecific Interference

Nonspecific interference may arise from excessive concentrations of other constituents of plasma. Free fatty acids affect some assays for free T4 by displacement of T4 from endogeneous binding proteins.

Hook Effect

The "Hook Effect" is characterized by the production of artefactually low results from samples that have extraordinarily high concentrations of antigen (analyte), far exceeding the concentration of the upper standard in the assay concerned.

The hook effect is most commonly found in single-step immunometric assays, a popular format, chosen for its specificity and speed, particularly with high-throughput immunoassay analyzers. The assays most affected are those that have analyte concentration that may range over several orders of magnitude. For example, alpha fetoprotein (AFP), CA-125, hCG, PSA, TSH, prolactin and ferritin are most affected by Hook effect.

Reduction of Hook Effect

The incidence of Hook effect can be reduced (but not eliminated) by careful assay design—incorporating a wash step prior to addition of the second antibody, thereby avoiding simultaneous saturation of both antibodies.

Despite attempts to eliminate or reduce the Hook effect by careful assay design, the only reliable method of routinely eliminating the effect is to test the samples that are likely to be affected by Hook effect in undiluted and also at a suitable dilution. Such samples should be diluted using either the assay diluent or serum from a normal subject until a stable quantitative response is achieved.

Edge Effect

Sometimes with ELISA performed in a microwell plate unexpectedly higher (or lower) optical densities (OD) are measured in the peripheral wells than in the central wells. This phenomenon is called "edge effect". The most probable causes of this effect are illumination or temperature differences between the peripheral and the central wells.

Light may cause edge effect if the substrate is photosensitive (i.e. converted by light exposure) like the H_2O_2/OPD substrate in the peroxidase system. Thus, if strong light is coming from one side (e.g. sunlight from a window) during the substrate reaction, the peripheral wells closest to the light source may give elevated OD values. Temperature difference, however, is the most common cause of edge effect.

Incubation at 37°C instead of room temperature is often used for shortening incubation time, which is not correct. Also, a common mistake is to use reactant liquids straight from a refrigerator and then incubate in a 37°C incubator (or at room temperature). Temperature changes of these magnitudes may, especially with short incubation times, destroy the assay homogeneity in microwell plates. The peripheral wells will normally be heated up first because of their position closest to the lower edge of the plate, which is in direct contact with the warm incubator shelf, which may result in higher OD values in these wells, other things being equal. The edge effect may be more pronounced if plates are stacked during incubation, especially in plates in the middle of the stack because their central wells are shielded from the warmer surroundings by the plates above and beneath.

To avoid the above-mentioned problems, the following precautions should be taken:

- Incubations should take place in subdued light or in the dark (if protocol requires)
- Reactant liquids (and plates) should be adjusted to the temperature intended for incubation
- Plates should be sealed with adhesive tape or placed in a 100% relative humidity environment during incubation.

Assay Specificity

It is one of the most important requirements of immunoassays. Interference occurs in all situations in which the antibody is not absolutely specific for the analyte. Consequently, assessment of specificity is a vital step in the optimization of every new immunoassay. Poor specificity results in interference from compounds of similar molecular structure or which carry similar immunoreactive epitopes. In determining the overall specificity of an assay, a major factor is the crossreactivity of the antibody.

Some of the major specificity problem areas are related to measurement of steroids and structurally related compounds. All commonly used testosterone assays, cross react in varying degrees with 5 α-dihydrotestosterone, and all cortisol assays cross react with prednisolone.

Assessment of the specificity of immunometric assays is complex and quite different from that used for single-site assays. In most assays, two different antibodies are employed, each having unique specificity for a different epitope on the antigen. It is usual practice to employ at least one monoclonal antibody, which can be selected by epitope mapping to react only with predetermined sites on the antigen molecule. Use of two monoclonal antibodies can introduce extreme specificity.

Assay Sensitivity

The ability of a kit to detect very low concentrations of an analyte (in quantitative ELISA) is mainly understood by the sensitivity of the kit. Many manufacturers mention the sensitivity and specificity after the result interpretation. This is overlooked commonly. One should observe this carefully. Higher sensitivity is a desirable property in any kit. Some doubts have been expressed regarding the value of ultrasensitive assays, which detect very minute amounts of analyte, which may be below the clinically or diagnostically significant values.

Most diagnostic kits are not exhausted overnight. Repeated usage and storage exposes the kit to multiple thermal shocks. This affects the performance of the kit over a period of time due to lowering of sensitivity. This shift in sensitivity affects the ultrasensitive kits lesser than those with less sensitivity.

A good example of ultrasensitive kit is "Third Generation TSH kits" which are very useful in the diagnosis of hypothyroidism.

As compared to low sensitive kits, ultrasensitive kits are more robust, more accurate that improve the reliability of results and provide confidence to the clinicians on the laboratory results.

CHEMILUMINESCENCE: THE TECHNOLOGY

Introduction

"Chemiluminescence" is defined as the production of electromagnetic (ultraviolet, visible or near-infrared) radiation as a result of a chemical reaction. One of the reaction products is in an excited state and emits light on returning to its ground state.

The generation of signal and its estimation varies from technology to technology. In RIA (radioimmunoassay) the radioactive signal is measured in gamma counter. In ELISA, the enzyme and substrate react to produce color, which is measured using an ELISA reader. Fluorescence immunoassays involve a similar principle where enzyme and substrate react to produce a fluorophor, which is measured fluorometrically. In case of chemiluminescence immunoassays, the light is produced which is measured.

Measurement of light from a chemical reaction is highly useful because the concentration of unknown can be inferred from the rate at which light is emitted. The rate of light output is directly related to the amount of light emitted. This type of luminescence is frequently compared with fluorescence, which also involves emission of light as a result of relaxation of excited states. Since, chemiluminescence does not involve initial absorption of light, measurement of chemiluminescence emission are made against a lower background noise that is not possible with conventional fluorescence, thus potentially allowing greater sensitivities of detection in chemiluminescent technology. This lack of inherent background and the ability to easily measure very low and very high light intensities with simple instrumentation provide a large potential dynamic range of measurement. Linear measurement over a dynamic range of 10^6 or 10^7 using purified compounds and standards has become possible with developments in the technology.

Light, as we see it, consists of billions of tiny packets of energy called photons, which are measured in the detection process. There are different factors that affect the emission and measurement of light.

- The efficiency of light emission from a chemiluminescent molecule is expressed as the chemiluminescence quantum yield, ÖCL, which describes the number of moles of photons emitted per mole of reactant
- The signal
- The quantity of signal required to produce the emission
- The duration of emission
- Instrumentation employed for the quantification of emission.

Components of Chemiluminescent System

The Signal

The signal (or substrate) used for generation of light should have optimum stability. There are many signal reagent available—luminol, 1,2 Dioxetanes, Acridinium ester, ruthenium salts, etc. Luminol is preferred of all these because of its stability and its advantage of being enhanced by iodophenol and phenothiazine.

Signal Quantity

The light emission in a chemiluminescent reaction is influenced by the quantity of signal used for generation of light. The manufacturing capabilities are limited globally and hence a prohibitive cost in procuring the signal for use in commercial scale. This limits the volume of signal for generation and also the sensitivity (lesser quantum of light produced, compromising the assay sensitivity).

The solution for this impediment can be achieved by increasing the quantity of signal generated in the reaction process. This is best done by using enhancers, which increase the intensity of signal produced. In 1985, Kircka and co-workers discovered that iodophenol compounds are strong enhancers that intensify luminol chemiluminescence about 1000 times, while also prolonging the duration of chemiluminescence.

Since the appearance of enhanced chemiluminescence, where enzymes like iodophenol, phenothiazine, etc. are employed to improve the light output of reactions, enzyme-sensitive chemiluminescent compounds have been the basis of several new clinical laboratory tests. These compounds increase the duration and quantum of signal produced by the reaction. Both peroxidase (HRP) - and phosphatase-sensitive chemiluminescent tags are commercially available. More tests employing these compounds can be expected to reach the clinical laboratory soon. Also, the recent introduction of enzyme-sensitive chemiluminescent tags with amplified light output has resulted in clinical tests with much-improved sensitivity.

This process of enhancement improves the performance of chemiluminescence immunoassay kits.

Signal Duration

Equally important is the fact that the light produced by the reaction process be measured within a specific time. The chemiluminescent reactions can be of two types depending on the duration of light produced.

Flash

In this, the addition of signal causes the immediate emission of light, typically over milliseconds or seconds. The instrumentations generating this type use a module for injecting the signal into the reaction system (injector module). These systems have moderate efficiencies. These systems have the benefit of a traditional chemiluminescent systems by increased sensitivity and dynamic range, but with its inherent inadequacies like homogenization effect, difficult for photon counting and impossibility of repeat measurements in a reaction. Particularly the repeat measurement is important because, it gives more confidence in reporting. This is not possible by these systems and one has to repeat the entire test for second measurement.

Glow

The emission of light builds and reaches a maximum. The emission is stable for a longer period of time making remeasurement possible. Glow type systems are excellent for quantitative systems such as immunoassays and detection of proteins. In the case of glow reactions, procedure development is relatively simple and the timing of reagent addition and reagent/sample mixing are not critical as in flash reactions.

Instrumentation

The instrumentations perform the function of quantification of emission and read out design. There are many ways of doing this depending on the level of sensitivity and sophistication required. The instrument employs a photomultiplier tube (PMT) for this purpose. These devices can be used in either a current measuring or photon-counting mode. Photon-counting systems are the latest development in chemiluminescence technology and provide greater sensitivity and long-term stability than the traditional current measuring chemiluminescent systems. Different types of PMTs exhibit different sensitivities to different wavelengths and it is, therefore, important to select the PMT with maximum spectral response for maximum sensitivity. There are a very few good manufacturers of PMT present globally.

The instrumentations are available from simple one, which can count photon emissions from a single tube to fully automated systems capable of counting microplates by photon-counting mode. These often carry the software on board to be able to perform data reduction of standards and samples. The PMT count every single electron generated by secondary emission from the system in the form of a pulse and gives the output.

These pulse chemiluminescent systems are better than other chemiluminescent systems.

Comparison with Other Technologies

The detection of antigen-antibody binding can be done by many ways. Methods like RIA, ELISA, and fluorescence immunoassay have been used widely. Of this, ELISA is adopted commonly for many parameters.

Drawbacks of Other Technologies

Radioimmunoassay

- Low sensitivity
- Disposal issues, health hazard pertaining to radioactivity
- Older technology.

Enzyme Immunoassay

- Limitation of photometric measuring range
- Low sensitivity in 2nd generation assays
- Smaller dynamic range and linearity.

Fluorescence Immunoassay

- Compromised sensitivity
- Background fluorescence
- Protein quenching
- Sensitivity to temperature, pH
- Interference from hemoglobin, bilirubin.

References

"Interference from light scattering, background fluorescence and quenching can reduce the potential sensitivity of fluorescence immunoassay by factors between 100 and 1000.

"Fluorescent EIAs are identical to other EIAs. There may be substances in the system that emit fluorescent light. These substances increase the background signal which may interfere with the assay's sensitivity" (Fig. 22.19).

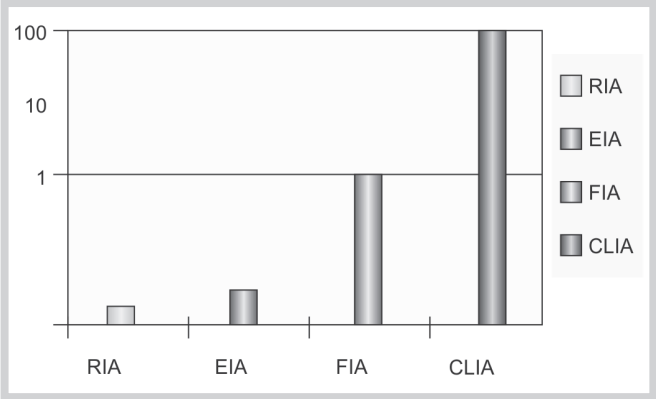


FIG. 22.19: Relative sensitivity

Advantages of Chemiluminescence Technology

1. **Linearity:** In chemiluminescence, since the individual photons are counted, there is very high linearity. Very high values can be obtained without dilution.
2. **Stability:** The signal generated in chemiluminescence is stable for long time making it better than other technologies.
3. **Sensitivity:** The lower detection limit is more in chemiluminescene than other technology.
4. **Convenience:** There is no second incubation in chemiluminescence since there is no substrate incubation step.
5. **Cost:** Since less signal quantity is used in "Enhanced pulse chemiluminescence" systems, the reagent and instrumentation cost are less than the closed chemiluminescent systems.

Overall, enchanced pulse chemiluminescence is favored for the following reasons:

- No excitation source is required
- Chemiluminescent substrates have a shelf-life of about a year, whereas those of fluorescence (which contain a fluorescein molecule) will last only about a week
- The level of detection is also lower with that of chemiluminescence—femtogram level has been well documented
- Fluorescence due to its limited availability is very expensive. Chemiluminescence is much more affordable
- Extraordinary sensitivity; a wide dynamic range; inexpensive instrumentation; and the emergence of novel luminescent assays make this technique very popular
- Superior sensitivity and low background distinguish chemiluminescence from other analytical methods
- Chemiluminescence is up to 100,000 times more sensitive than absorption spectroscopy and is at least 1,000 times more sensitive than fluorometry
- The background light component is much lower in chemiluminescence than in other analytical techniques such as spectrophotometry and fluorometry
- Wide dynamic range and low instrument cost are also distinct advantages of chemiluminescence. Samples can be measured across decades of concentration without dilution or modification of the sample cell.

Enhanced pulse chemiluminescence immunoassays are available in two formats.

1. Impulse 9.0: An open semi automated chemiluminescent immunoassay system (Fig. 22.20).

Advantages

- First of its kind in the category of chemiluminescent instruments in India
- Wide range of assay menu

FIG. 22.20: Impulse 9.0 enhanced pulse chemilunescence system. (*Courtesy*: Lilac Medicare)

FIG. 22.21: Alpha prime LS. (*Courtesy*: Lilac Medicare)

- No protein quenching problem as in fluorescence
- Better sensitivity out of all available immunoassay technologies
- Simple operation, performs single tests
- Robust instrument design. Ideal for distant locations for engineer free operations
- Alpha Prime LS: Fully automated walkaway chemiluminescent immunoassay system (Fig. 22.21).

Advantages

- Fully automated multiparametric immunoassay system
- Can run up to 384 samples at a time
- Can perform 18 different parameters simultaneously
- Can operate in CLIA and EIA technology also (for infectious and autoimmune diseases parameters).

POLYMERASE CHAIN REACTION

PCR stands for the Polymerase Chain Reaction (Fig. 22.22) and was developed in 1987 by Kary Mullis and associates. It is capable of producing enormous amplification (i.e. identical copies) of a short DNA sequence from a single

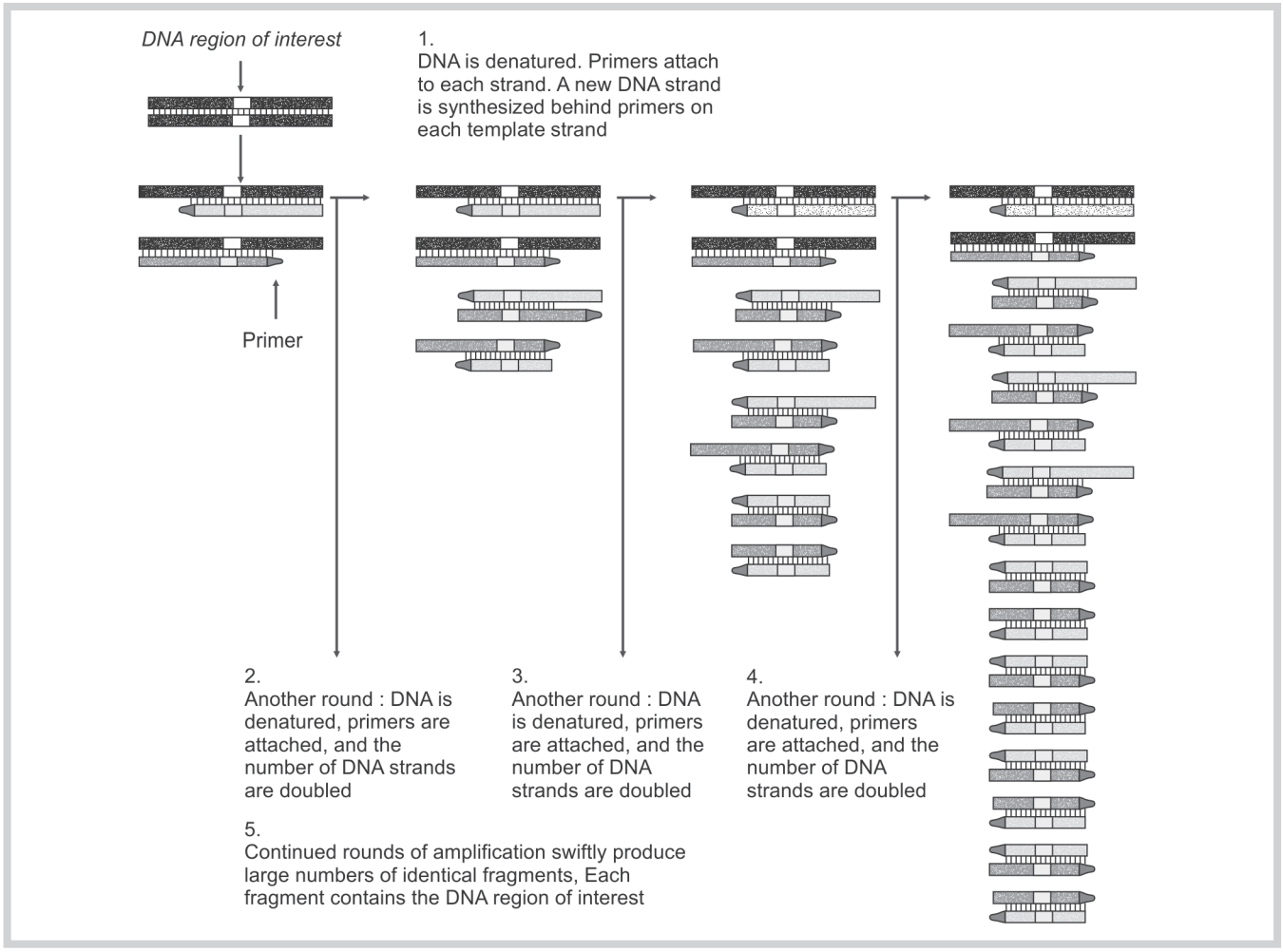


FIG. 22.22: Polymerase chain reaction

molecule of starter DNA. It is used to amplify a specific DNA (**target**) sequence lying between known positions (**flanks**) on a double-stranded (ds) DNA molecule.

The amplification process is mediated by oligonucleotide primers that, typically, are 20–30 nucleotides long. The primers are single-stranded (ss) DNA that have sequences complementary to the flanking regions of the target sequence. Primers anneal to the flanking regions by complementary-base pairing (G≡C and A=T) using hydrogen bonding.

The amplified product is known as an amplicon.

Generally, PCR amplifies smallish DNA targets 100–1000 base pairs (bp) long. (It is technically difficult to amplify targets > 5000 bp long.)

PCR has many applications in research, medicine and forensic science.

One PCR cycle consists of three steps:

- Denaturation
- Annealing
- Extension.

Denaturation by Heat

Heat (usually >90°C) separates double-stranded DNA into two single strands, referred to as "denaturation". Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact.

Annealing Primer Binding to Target

Primers are short, synthetic sequences of single-stranded DNA typically consisting of 20–30 bases, with a biotin-labeled 5' end to aid in detection. They are specific for the target region of the organism. Two primers are included in the PCR, one for each of the complementary single DNA strands that was produced during denaturation. The beginning of the DNA target sequence of interest is marked by the primers that anneal (bind) to the complementary sequence.

Annealing temperature: Annealing usually takes place between 40 and 65°C, depending on the length and base sequence of the primers. This allows the primers to anneal to the target sequence with high specificity.

Extension

Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately 72°C and the enzyme Taq DNA polymerase is used to replicate the DNA strands. Taq DNA polymerase is a recombinant thermostable DNA polymerase from the organism *Thermus aquaticus* and, unlike normal polymerase enzymes is active at high temperatures.

Taq DNA polymerase, begins the synthesis process at the region marked by the primers. It synthesizes new double-stranded DNA molecules, both identical to the original double-stranded target DNA region, by facilitating the binding and joining of the complementary nucleotides that are free in solution (dNTPs). Extension always begins at the 3' end of the primer making a double strand out of each of the two single strands. Taq DNA polymerase synthesizes exclusively in the 5' to 3' direction. Therefore, free nucleotides in the solution are only added to the 3' end of the primers constructing the complementary strand of the targeted DNA sequence.

Following primer extension, the mixture is heated (again at 90–95°C) to denature the molecules and separate the strands and the cycle repeated.

Each new strand then acts as a template for the next cycle of synthesis. Thus amplification proceeds at an exponential (logarithmic) rate, i.e. amount of DNA produced doubles at each cycle. 30–35 cycles of amplification can yield around 1 µg DNA of 2000 bp length from 10^{-6} µg original template DNA. This is a million-fold amplification.

Initially, the 3 different stages at 3 different temperatures were carried out in separate water baths but nowadays, a thermal cycler is used (a machine that automatically changes the temperature at the correct time for each of the stages and can be programed to carry out a set number of cycles).

A typical thermal cycle might be as follows:
Heat denaturation at 94°C for 20 seconds
Primer annealing at 55°C for 20 seconds
Primer extension at 72°C for 30 seconds
Total time for one cycle = approx. 4 minutes.

DNA is denatured
Primers attach to each strand. A new DNA strand is synthesized behind primers on each template strand.

Another round
DNA is denatured, primers are attached, and the number of DNA strands are doubled.

Continued rounds
Continued rounds of amplification swiftly produce large numbers of identical fragments. Each fragment contains the DNA region of interest.

Limitations/Difficulties

While a very powerful technique, PCR can also be very tricky. The polymerase reaction is very sensitive to the levels of divalent cations (especially Mg^{2+}) and nucleotides, and the conditions for each particular application must be worked out. Primer design is extremely important for effective amplification. The primers for the reaction must be very specific for the template to be amplified. Crossreactivity with non-target DNA sequences results in nonspecific amplification of DNA. Also, the primers must not be capable of annealing to themselves or each other, as this will result in the very efficient amplification of short nonsense DNAs. The reaction is limited in the size of the DNAs to be amplified (i.e. the distance apart that the primers can be placed). The most efficient amplification is in the 300–1000 bp range, however, amplification of products up to 4 Kb has been reported. Also, Taq polymerase has been reported to make frequent mismatch mistakes when incorporating new bases into a strand.

The most important consideration in PCR is contamination. If the sample that is being tested has even the smallest contamination with DNA from the target, the reaction could amplify this DNA and report a falsely positive identification. For example, if a technician in a crime lab sets up a test reaction (with blood from the crime scene) after setting up a positive control reaction (with blood from the suspect) cross contamination between the samples could result in an erroneous incrimination, even if the technician changed pipette tips between samples. A few blood cells could volatilize in the pipette, stick to the plastic of the pipette, and then get ejected into the test sample. The powerful amplification of PCR may be able to detect this cross contamination of samples. Modern labs

take account of this fact and devote tremendous effort to avoiding this problem.

Types of PCR

RT-PCR
This is reverse transcriptase-PCR and is a two-stage procedure used for the amplification of RNA. The first stage employs an enzyme called reverse transcriptase, which synthesises a DNA strand complementary to the RNA of interest by using one of the PCR primer as its primer. The complementary DNA is then used in the second stage as the starting material for PCR amplification by a conventional thermostable DNA polymerase.

Nested PCR
It is a PCR done in two steps, a primary PCR reaction and a nested reaction. The primary (or first) reaction uses a set of primers to generate a product that serves as the template for the nested (or second) reaction. The nested reaction uses a set of PCR primers specific for a region within the amplified product from the first reaction. Therefore, the nested reaction often serves as a confirmation for the specificity of the PCR products amplified in the primary reaction.

Real-Time PCR
Combines PCR amplification and detection into a single step. The basic principle of real-time quantitative PCR is the detection of target sequences using a fluorogenic 5' nuclease assay (often called 'TaqMan'). The advantages of this system include high reproducibility, the capability of handling large numbers of samples, the potential for quantitative results, and decreased turnaround time. The disadvantages include high instrument cost and the requirement for technical proficiency.

Multiplex PCR
It is a PCR designed to detect more than one target sequence in a single PCR reaction. The assay uses two or more sets of primers. Each set of primers is specific for a different target sequence. The assay is most commonly used for simultaneous detection of multiple viral genes and differentiation of genotypes or subtypes of related microorganisms.

Differential PCR
Differential PCR can sometimes be used to distinguish closely related targets. Differential PCR is done either in a multiplex format using two or more sets of primers or by running two separate PCR assays.

RIA

Radioimmunoassay (RIA) combines the high specificity of an antigen-antibody reaction with the great sensitivity of detection and quantification of compounds tagged with a radioactive "label" atom.

If there is, in a solution, a mixture of three components, i.e. a "natural", or unlabeled, antigen, the same antigen with one of its atoms carrying a radioactivity label, and a quantity of antibody specific for the antigen that is insufficient to bind all the unlabeled and labeled antigen molecules present, the two forms of the antigen will compete for the available binding sites. Thus, if the number of labeled and unlabeled molecules is the same, each type has an equal chance of finding a free binding site, half the available antibody-binding sites will carry labeled antigen and half will carry unlabeled antigen. If the number of unlabeled antigen molecules is greater than the number of labeled ones, a large number of antibody-binding sites will become occupied by unlabeled antigen molecules. Thus, the larger the number of unlabeled antigen molecules in the mixture, the smaller the fraction of the original quantity of labeled antigen that will become bound by antibody. Since the firmly bound combination of antigen and antibody can be separated from the remaining components of the original mixture and its radioactivity determined and compared with that of the original labeled antigen addition; and since the relative amounts of bound and free labeled antigen will depend upon the number of unlabeled antigen molecules originally present, a calibration curve can be made by adding known amounts of unlabeled antigen to the system of labeled antigen and antibody, separating and determining the ratio of radioactivity of bound to original labeled antigen, and plotting this ratio against the known amounts of added unlabeled antigen. If a sample containing an unknown amount of natural (unlabeled) antigen is then mixed with the same amounts of labeled antigen and antibody as in the calibration curve mixture, the antigen-antibody complex separated and the ratio of its radioactivity determined when compared with that of the original amount of labeled antigen, this ratio, usually expressed as a percentage, when referred to the calibration curve, will give the amount of unlabeled (natural) antigen in the sample.

The unique combination of specificity and sensitivity of the RIA principle makes it particularly suitable for the assay of substances such as insulin, growth hormone, thyroxine, testosterone, progesterone, angiotensin, aldosterone, and drugs such as digoxin in serum or plasma at the level of nanogram per mL.

The procedures involved in RIA differ in the radioactive element used as the label, in the method used to separate the antigen-antibody combination from the unbound

antigen, and in the standardization. The majority of current methods use ^{125}I (radioactive iodine) or ^{3}H (tritium, the radioactive isotope of hydrogen) as the labels. For separation of antigen-antibody combination, charcoal coated with dextran is used. The dextran acts as a molecular sieve that passes only unbound antigen molecules for retention by the charcoal, the antigen-antibody combinations are too large to cross the dextran coating. After centrifuging, the relatively dense charcoal grains with their adsorbed antigen molecules will be packed at the bottom of the tube, and the supernatant containing the antigen-antibody combinations can be separated. Measurement of the ratio of radioactivities of the two components completes the assay. A more sophisticated method of precipitating the antigen-antibody combination is to add a second antibody prepared to react with the protein of the first antibody, usually a gamma globulin. The resultant complex can then be separated either by centrifugation or cellulose acetate filters. Standardization can be done as described in the description of general principles above.

The RIA technique promises to provide reliable data by relatively simple methods about biological substances that present considerable analytical problems when more orthodox procedures are used. In practice, of course, RIA has its own sources of error. These include:

- Lability of compound analyzed
- Antibody cross reaction with related antigens
- Interfering substances in the sample, e.g. urea and bilirubin
- Poor pipetting technique (good pipetting technique is critical, because of the very small volumes)
- Contamination of equipment from extraneous radioactive materials
- Change in the antigen's chemical or immunological identity owing to the process of adding radioactive label to the antigen.

The RIA methods measure the amounts of particular molecular structures, not their biological activity.

Measurement of Radioactivity

The radioactive atoms used as labels produce different types of emitted radiation. ^{125}I emits short-wavelength, high-energy gamma rays; ^{3}H (tritium) produces beta-type radiation, which is actually high-speed particles, positively or negatively charged electrons. Gamma rays are detected by a so-called scintillation counter, which consists of a large sodium iodide crystal that contains thallium as an activator. The crystal is in close contact with a photomultiplier tube; and when an emitted quantum of gamma radiation strikes a sodium iodide molecule in the crystal lattice, it produces a photon of light energy. This light is picked up and amplified by the assopifeted photomultiplier tube and converted to a pulse of electrical energy. The number of pulses is proportional to the quantity of radioactive material in the sample, the power or energy of the pulse is determined by the energy of the original gamma ray. The scintillation counter incorporates "discriminators," which pass through only those pulses whose energy levels correspond to those of the gamma radiation emitted from the particular radioactive atom whose detection is required. Finally, the scintillation counter uses a sealer to count the number of pulses arriving in a preset time or to determine the time required for a preset number of pulses to occur. To detect beta particles, which have less energy than gamma radiation is used.

The preparation of serology reagents and anti-sera is much too complex and beyond the scope of this book. It is, therefore, advised that ready-made kits available commercially be used. Basic principles are mentioned. Product insert must, however, be read and followed strictly.

LIQUID HANDLING SYSTEMS

Pipetting Basics

Human beings are creature of habits. We often seek stability and continuity and are very much wary of damage.

Pipetting in history was carried out most exclusively by suction using a glass pipette. However, inspection, evaluation and subsequent changes are necessary for growth and improvement. Though these methods were convenient and economical, they lack accuracy and precision. Secondly aspirating small volumes of liquid using a glass pipette is not possible.

Classification of Pipettes

There are many ways of classifying pipettes:

Based on the Material

Glass pipettes

It is a traditional old pipette made of long glass tube scaled for different volumes by a marking on its surface. The principle of aspiration of the liquid is by suction. Though this method is convenient and economical, it lacks accuracy and precision.

Plastic pipette

Made of total plastic components and parts. It is the most commonly used pipette. Some pipettes are difficult to

calibrate and are not fully autoclavable. Dissembling and assembly is not possible in most of the pipettes. Also both variable and fixed volume is not in one pipette as compared to "New Third Generation Pipettes". The principle of operation is by suction.

Metal pipettes
They are called as new generation pipettes and are being increasingly used commonly. These pipettes are made of anodized aluminum and the piston made of stainless steel. These come with detachable controllers for variable and fixed volumes with digital volume setting.

Based on Function

Fixed and variable pipette
These may be plastic or partial metal pipettes but serving only one function. They can either be used for aspiration of fixed volume of liquids or a specific range of volumes.

Combined pipettes
These pipettes offer the flexibility and user friendliness of both variable and fixed volume options in the same pipette.

Pipetting Techniques

The first step in pipetting is to choose the pipetting mode best suited to the type of work. These pipetting modes are:

Forward Pipetting

It is the standard technique for pipetting aqueous liquids.

1. Press the operating button to the first step.
2. Dip the tip into the solution to a depth of 1 cm and slowly release the button. Withdraw the tip from liquid, touching it against the edge of the reservoir to remove excess liquid.
3. Dispense the liquid into the receiving vessel by gently pressing the operating button to the first step. After one second, press the button down to the second stop. This action will empty the tip. Remove the tip from the vessel, sliding it along the wall of the vessel.
4. Release the operating button to the ready position.

Reverse Pipetting

This technique is used for pipetting solutions of high viscosity or a tendency to foam. This method is also recommended for dispensing small volumes.

1. Press the operating button to the second stop.
2. Dip the tip into the solution to a depth of 1cm and slowly release the button. This action will fill the tip. Withdraw the tip from the liquid, touching it against the edge of the reservoir to remove excess liquid.
3. Dispense the liquid into the receiving vessel by pressing the button gently and steadily down to the first stop. Hold the button in this position. Some liquid will remain in the tip, and this should not be dispensed.
4. The liquid remaining in the tip can be pipetted back into the original solution or thrown away with the tip.
5. Release the operating button to the ready position.

Repetitive Pipetting

This technique is intended for repeated pipetting of the same volume.

1. Press the operating button down to the second stop.
2. Dip the tip into solution to a depth of 1cm and slowly release the operating button. Withdraw the tip from the liquid, touching it against the edge of reservoir to remove excess liquid.
3. Dispense the liquid in the receiving vessel by gently pressing the operating button to the first stop. Hold the button in this position. Some liquid will remain in the tip and this should not be dispensed.
4. Continue pipetting by repeating steps 2 and 3.

Whole Blood Pipetting

Use forward technique steps 1 and 2 to fill the tip with blood. Wipe the tip carefully with a dry clean cloth.

1. Dip the tip into the reagent and press the operating button down to the first stop. Make sure the tip is well below the surface.
2. Release the button slowly to the ready position. This action will fill the tip with the reagent. Do not remove the tip from the solution.
3. Press the button down to the first stop and release slowly. Repeat this process until the interior wall of the tip is clear.
4. See 3.
5. Press the button down to the second stop and completely empty the tip.
6. Release the operating button to the ready position.

Proper Pipetting Skills

1. Warming up the pipette mechanism: The pipette mechanism should always be warmed up before starting by gently pressing and releasing the plunger 15–20 times.
2. Pre-wet the pipette tip: Aspirate and expel and amount of the sample liquid at least 2–3 times before aspirating a sample for delivery. Pre-wetting the tip influences accuracy by increasing the humidity within the tip, minimizing evaporation of the solution. This is

particularly important for solvents with high vapor pressure.
3. Work at room temperature: Allow liquids to ambient temperature. Make sure the tips and solution are at the same temperature.
4. Use consistent plunger pressure and speed: Depress and release the plunger smoothly and consistently for each sample. The fatigue and strain caused due to this is minimized in new generation pipettes due to their ergonomic design.
5. Use the correct pipette tip: Securely attach a tip designed for use with the pipette. Pick out fresh and uncontaminated tips only. Do not reuse pipette tips. If the shape of the pipette is disfigured, discard and use a fresh one.
6. Storage and maintenance: Always store pipettes in an upright position when not in use. Pipette stands are ideal for this purpose. Check calibrations regularly, and follow instructions of the manufacturer for recalibration. Proper care and maintenance of the pipette will ensure a longer life.

Criteria for Choosing the Right Pipette

In order to maximize the accuracy and reproducibility of volume delivery using micropipette, it is critical to evaluate all of the components comprising the volume delivery system. The choice of pipette, the selection of the most appropriate instrument of the application, should be based on prioritizing various criteria characterizing instrument performance. The choice of pipette tip is also critical to performance. The following is a list of criteria that can be used in choosing a pipette.

Accuracy and Reproducibility

The following are problems to watch out for when reviewing pipette performance:

- Pipettes with advertised ranges that exceed the performance tolerance provided by the manufacturer
- Pipettes whose tolerances are too tightly specified; these will have trouble meeting the manufacturer's specified tolerance limits
- Mulltichannel pipettes with tolerances that are interchannel statistics as opposed to intrachannel statistics.

Durability

A pipettes' durability is primarily a function of the sturdiness of its components. In general, the thicker the plastic the more durable the pipette.

Ergonomics

Whatever pipette an end-user chooses, it is critical that the end-user feels comfortable pipetting for the extended periods of time typical of much of lab use. If a pipette is too large for the end-users hand it is extremely likely that it would cause repeat motion related injury to the hand. In addition, it would be more difficult for the end-user to develop proper technique that would deliver accurate results with that pipette.

Specific Applications

There are several types of pipettes designed for specific applications. For example–autoclavibility. It is important to check for the following information:

- Is the entire pipette autoclavable, or are only some parts autoclavable?
- What are the recommended conditions for autoclaving?
- Can the plastic used for the pipette's body, shaft and tip cones can withstand exposure to UV light?
- What are the chemical compatibilities and incompatibilities of the pipette?

Quality of Product Support

It is important to know:

- How supportive the manufacturer and or the distributor of the pipette are?
- How responsive is customer service on warranty issues?
- How knowledgeable is the technical staff in terms of the mechanics and technical specifications of the pipette?
- How accessible is the manufacturer for visits?

Use of Multiple Brands of Pipettes

There are two major issues:

1. The need to train technical staff on each type of pipette separately. Different brands may use different designs for the pipetting mechanisms requiring differences in pipetting technique. These pipettes may require the application of different amounts of force while pipetting which is a skill that requires training and repetition to acquire.

2. Stocking of variety of pipettes. Many labs try to stock a single tip for all brands. Unfortunately the choice of single tip ends up in a compromise given the variety of shapes and plastic compositions of tips.

STREPTAVIDIN-BIOTIN SYSTEMS

Streptavidin-Biotin Systems, Better than Traditional Antibody Capture Systems

Streptavidin-Biotin Based IEMA Systems use a biotinylated antibody (biotin-labeled 1st antibody/capture). This is because biotin can be attached to the F_C portion of an antibody in relatively high proportion without loss of immunoreactivity.

The binding ratio of Avidin to Biotin is 4:1. One molecule of Streptavidin, which is a tetramer can bind with four molecules of Biotin/Biotinylated 1st Antibody.

In a traditional enzyme immunoassay, a limited space is normally available for coating the Capture/1st Antibody in the bottom of the microwell/plastic tube.

Ideally, if one can increase the number of Capture/1st Antibodies coated on the microwell, the assay sensitivity goes up because more number of Antigen binding sites are available in case of low concentration of analytes (Antigens) present in the sample.

Streptavidin–biotin based systems coat streptavidin on the microwell/plastic tubes instead of directly coating the capture/1st antibody. Capitalizing the tetrameric valency of streptavidin to biotin, each molecule of coated streptavidin binds with four molecules of biotinylated capture/1st antibody thus providing an excess of binding sites to the system, which ensures four fold higher sensitivity of the IEMA system.

In other words, the streptavidin-biotin system helps to increase the number of binding sites and thus increasing the chances and probability of binding an antigen to an antibody by four fold.

Streptavidin possess greater electrostatic attraction for the microwell/plastic tubes.

Streptavidin/avidin is more inert in assay systems.

Why Streptavidin-Biotin Based Lema Systems are a Better Choice for Tropical Laboratories?

Stability: The binding of avidin and biotin is not disturbed by extremes of salt, pH or temperature.

Specificity and sensitivity: Avidin has a very high binding affinity for biotin and so the system avidin-biotin is highly specific moreover the rate constant for the avidin-biotin association is also fast.

Speed of the reaction: The solid phase is coated with avidin and the capture antibody is biotinylated,this minimizes the need for other coating methods and facilitates the use of antibodies with high affinities.

Temperature stability and other problems: Non-bound avidin is very thermostable for the folded-unfolded transition,Tm = 85°C (pH 7–9). When biotin is bound,the protein acquires greater thermostability Tm = 132°C.

Thus, the avidin-biotin system is more resistant to high temperature. This greater thermostability of avidin-biotin system overcomes/reduces the problems faced during:

- Transportation
- Storage
- Use and handling.

Signal Noise Ratio

The sensitivity of any analytical technique is defined as the minimal concentration that can be reliably estimated.

In any immunometric assay the signal measured at the end of the assay consists of two types of signals:

1. The signal seen due to the presence of analyte–what is desired.
2. The signal due to non-specific adsorption of labeled antibody–commonly called background absorbance.

Technically sensitivity can be defined as the minimal concentration of analyte that is statistically unlikely to form part of the range of signals seen in the absence of analyte.

1. Third Generation Ultrasensitive assay designs are based on maximizing the Signal Noise Ratio (S/N).

Streptavidin-biotin based assays offer four fold increase in signal generation, thus making the background noise negligible. Chemiluminescent assays are also based on the principle of generating very high signal in presence of analyte in order to make the background noise negligible.

Primary Calibrators and Matrix Effect

The aim of standardization of laboratory is to improve the accuracy, i.e. the results should be as close to the true value as possible.

Immunoassays do not actually measure the analyte. They can only provide a quantitative estimate of concentration by direct comparison with standard/calibrator material.

A prerequisite for standardization is that the standard/calibrator and analyte are identical. In other words, the calibrator should contain the analyte in a form identical to that found in the sample.

Calibrators should ideally be prepared by using a base material identical to that in the test samples. For clinical applications Human Serum is the preferred base matrix.

The matrix of a calibrator needs to behave in a similar way to the sample matrix.

For assay of hormones that are bound to serum protein, it is hard to use any other matrix other than Human Serum.

Calibration of direct assays for protein bound hormones is complicated because of interference by the binding proteins. The effect of the binding proteins is hard to eliminate completely. Therefore, serum based calibrators need to be used.

Ultrasensitive Assays

Few immunoassays are totally free from interference from the ill-defined composition of biological fluids under test (matrix). Different samples containing the same amount of analyte may give different results due to this Matrix effect. Assays having higher sensitivity are able to better identify and amplify the analyte thereby reducing the matrix effect, and improving the assay accuracy.

Most of the diagnostic immunoassay kits are not exhausted overnight. Repeated usage and the store/use/store cycles, exposes the immunoassay system to multiple thermal shocks. This impacts the analytical performance of the immunoassays due to the lowering of sensitivity. This shift in sensitivity affects the ultrasensitive assays lesser since the percentage change in sensitivity would be proportionally smaller as compared to assays having lower sensitivity.

Due to their higher sensitivity and amplification ability, ultrasensitive assays enable test run on the smaller volume samples, such as capillary blood from children. This not only assures better testing confidence but also minimizes the need for assay reruns.

Ultrasensitive assays have opened up new opportunities in the diagnosis of diseases or clinical conditions which were previously unrecognized or the test for which were unavailable. A good example is the development of Third Generation Thyrotropin (TSH) assays/Ultrasensitive TSH assays which for the first time opened up the possibility of differentiating between the euthyroid and hyperthyroid state.

As compared to low sensitivity assays, ultrasensitive assays also offer an increase in signal ratio as well as improvement in rate of change of the measured signal which in turn offers the immunoassay users greater accuracy from the test system in question.

In conclusion: Ultrasensitive assays are more robust, more accurate, versatile systems that improve reliability of results and provide confidence to the clinicians on the laboratory results.

Epitype Characterization

Epitope: Part of an antigen to which antibody binds.
Epitype: Part of an epitope where the antibody binds.

- Conformational or discontinuous: T3, TSH
- Linear or sequential: T4
- Higher specificity
- Minimal cross reaction
- No nonspecific binding.

REPRESENTATIVE ELISA/CLIA TECHNIQUES

ELISA/CLIA Analyte Determination Principles

Product	Principle
Thyroid stimulating hormone	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Total triiodothyronine (T3)	Competitive
Free-triiodothyronine (FT3)	Competitive
Total thyroxine (T4)	Competitive
Free-thyroxine (FT4)	Competitive
Anti-thyroglobulin	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Anti-thyroperoxidase (TPO)	Immunoenzymometric/Sandwich (Streptavidin Biotin)
T-Uptake	Competitive
Lutropin (LH)	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Follitropin (FSH)	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Prolactin (PRL)	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Prolactin (PRL)	Immunoenzymometric/Sandwich Sequential (Streptavidin Biotin)
Beta-hCG	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Insulin	Immunoenzymometric/Sandwich (Streptavidin Biotin)
C-Peptide	Immunoenzymometric/Sandwich (Streptavidin Biotin)
AFP	Immunoenzymometric/Sandwich (Streptavidin Biotin)
CEA	Immunoenzymometric/Sandwich (Streptavidin Biotin)
PSA	Immunoenzymometric/Sandwich (Streptavidin Biotin)
CA-125	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Free beta hCG	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Free PSA	Immunoenzymometric/Sandwich (Streptavidin Biotin)
CA 19-9	Immunoenzymometric/Sandwich (Streptavidin Biotin)
IgE	Immunoenzymometric/Sandwich (Streptavidin Biotin)

Contd...

Contd...

Ferritin	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Anti-H. pylori IgG	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Anti-H. pylori IgM	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Anti-H. pylori IgA	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Human growth hormone (GH)	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Cortisol	Immunoenzymometric/Sandwich (Streptavidin Biotin)
cTroponin-I	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Myoglobin	Immunoenzymometric/Sandwich (Streptavidin Biotin)
CK-MB	Immunoenzymometric/Sandwich (Streptavidin Biotin)
hs CRP	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Digoxin	Competitive
Neonatal TSH	Immunoenzymometric/Sandwich (Streptavidin Biotin)
Neonatal T4	Competitive
Thyroglobulin	Immunoenzymometric/Sandwich (Streptavidin Biotin)

Parameter	**Principle**
HAV Ab	Competitive ELISA
HAV Ab Ig M	Mu-Capture
HBeAg	Sandwich ELISA
HBeAb	Competitive ELISA
HbeAg/Ab	
HBsAb	Quantitative indirect ELISA
HBcAb	Competitive ELISA
HBclgM	Mu-Capture
HBsAg	One step Sandwich ELISA
HCV Ab	4th generation, indirect ELISA
HCV IgM	Indirect ELISA
HDV Ab	Competitive ELISA
HDV IgG	Indirect ELISA
HDV IgM	Mu-Capture
HEV Ab	
HEV IgG	
HEV IgM	
HIV	Indirect ELISA 3rd gen
TB IgG	Indirect ELISA
TB IgA	Indirect ELISA
TB IgM	Indirect ELISA
Toxo avidity	Avidity, Indirect ELISA
Toxo IgG	Interference corrected
Toxo IgM (Mu)	Mu-Capture
Toxo IgM (IC)	Interference corrected
Rubella IgG	Interference corrected
Rubella IgM (Mu)	Mu-Capture
Rubella IgM (IC)	Interference corrected
Rubella avidity	Indirect ELISA
CMV IgG	Interference corrected
CMV IgM (Mu)	Mu-Capture

Contd...

Contd...

CMV IgM (IC)	Interference corrected
CMV avidity	Avidity, indirect ELISA
HSV 1+2 IgG	Interference corrected
HSV 1+2 IgM	Interference corrected
HSV 1 IgG	Interference corrected
HSV 1 IgM	Interference corrected
HSV 2 IgG	Interference corrected
HSV2 IgM	Interference corrected
TORCH screen IgG	Interference corrected
TORCH screen IgM	Interference corrected

TORCH CHEMI

Toxo IgG	Interference corrected
Toxo IgM (Mu)	Mu-Capture
Rubella IgG	Interference corrected
Rubella IgM (Mu)	Mu-Capture
CMV IgG	Interference corrected
CMV IgM (IC)	Interference corrected
HSV 1+2 IgG	Interference corrected
HSV 1+2 IgM	Interference corrected
TORCH screen IgG	
TORCH screen IgM	
Testosterone	Competitive ELISA
Androstenedione	Competitive ELISA
DHEA-S (Dehydroepi-androsterone sulfate)	Competitive ELISA
Estriol-Free	Competitive ELISA
Estriol-Total	Competitive ELISA
17- Estodiol	Competitive ELISA
17-OH Progestirol	Competitive ELISA
Progesterone	Competitive ELISA
Cortisol	Competitive ELISA
Free Testosterone	Competitive ELISA

TORCH parameters available in ELISA and CLIA formats

Parameter	**Principle**
Rheumatology	
Total ANA screen	Indirect ELISA/Sandwich ELISA
Anti-ds DNA screen	Indirect ELISA/Sandwich ELISA
ANA Combi ELISA (8 Antigen)	Indirect ELISA/Sandwich ELISA
EIA Anti-SS-A	Indirect ELISA/Sandwich ELISA
EIA Anti-SS-B	Indirect ELISA/Sandwich ELISA
EIA Anti-Sm	Indirect ELISA/Sandwich ELISA
EIA Anti-Sm/RNP	Indirect ELISA/Sandwich ELISA
EIA Anti-Scl-70	Indirect ELISA/Sandwich ELISA
EIA Anti-Jo-1	Indirect ELISA/Sandwich ELISA
RNP - 70	Indirect ELISA/Sandwich ELISA
EIA Anti-ssDNA	Indirect ELISA/Sandwich ELISA
ENA screen	Indirect ELISA/Sandwich ELISA
ENA combi	Indirect ELISA/Sandwich ELISA
ENA-4-profile	Indirect ELISA/Sandwich ELISA
ENA-6-profile	Indirect ELISA/Sandwich ELISA
Centomere B	Indirect ELISA/Sandwich ELISA
Anti-histone antibody	Indirect ELISA/Sandwich ELISA
Anti-nucleosome antibody	Indirect ELISA/Sandwich ELISA
Anti-alpha fodrin antibody	Indirect ELISA/Sandwich ELISA

Contd...

Contd...

DNase activity	Indirect ELISA/Sandwich ELISA
Anti-C1q	Indirect ELISA/Sandwich ELISA
Anti-Mutated Citrullinated vimentin (MCV)	Indirect ELISA/Sandwich ELISA
Anti-Rib-P	Indirect ELISA/Sandwich ELISA
SSA 52	Indirect ELISA/Sandwich ELISA
SSA 60	Indirect ELISA/Sandwich ELISA
Rheumatoid factor screen	Indirect ELISA/Sandwich ELISA
Rheumatoid factor IgG	Indirect ELISA/Sandwich ELISA
Rheumatoid factor IgM	Indirect ELISA/Sandwich ELISA
Rheumatoid factor IgA	Indirect ELISA/Sandwich ELISA
DNase activity	Direct ELISA/Sandwich ELISA
Vasculitis	
C-ANCA (PR 3)	Indirect ELISA/Sandwich ELISA
ANCA COMBI (P+C)	Indirect ELISA/Sandwich ELISA
GBM	Indirect ELISA/Sandwich ELISA
Anti-BPI	Indirect ELISA/Sandwich ELISA
Anti-Elastase	Indirect ELISA/Sandwich ELISA
Anti-Cathepsin G	Indirect ELISA/Sandwich ELISA
Anti-Lysozyme	Indirect ELISA/Sandwich ELISA
Anti-Lactoferrin	Indirect ELISA/Sandwich ELISA
ANCA Screen	
Thrombosis	
Anti-Phospholipid Screen	Indirect ELISA/Sandwich ELISA
Phosphatidyl serine	Indirect ELISA/Sandwich ELISA
Anti-Cardiolipin IgA	Indirect ELISA/Sandwich ELISA
Anti-Cardiolipin Screen (IgG/IgM /IgA)	Indirect ELISA/Sandwich ELISA
Anti-β2-Glycoprotein I IgG/IgM	Indirect ELISA/Sandwich ELISA
Anti-β2-Glycoprotein I IgA	Indirect ELISA/Sandwich ELISA
Anti-β2-Glycoprotein I Screen	Indirect ELISA/Sandwich ELISA
Anti-Prothrombin IgG/IgM	Indirect ELISA/Sandwich ELISA
Anti-Prothrombin IgA	Indirect ELISA/Sandwich ELISA
Anti-Prothrombin Screen	Indirect ELISA/Sandwich ELISA
Anti-Annexin V	Indirect ELISA/Sandwich ELISA
Anti-Phospholipid Screen IgG/IgM	Indirect ELISA/Sandwich ELISA
Anti-Phosphatidyl Inositol IgG/IgM	Indirect ELISA/Sandwich ELISA
Anti-Phosphatidic Acid IgG/IgM	Indirect ELISA/Sandwich ELISA
Thyroid	
Thyroglobulin (Tg)	Indirect ELISA/Sandwich ELISA

Contd...

Contd...

Anti-thyroperoxidase (TPO)	Indirect ELISA/Sandwich ELISA
Anti-thyroglobulin	Indirect ELISA/Sandwich ELISA
Gastrointestinal	
Anti-Parietal cell	Indirect ELISA/Sandwich ELISA
Anti-Gliadin IgG	Indirect ELISA/Sandwich ELISA
Anti-Gliadin IgA	Indirect ELISA/Sandwich ELISA
Anti-Gliadin screen	Indirect ELISA/Sandwich ELISA
Anti-Tissue transglutaminase IgA	Indirect ELISA/Sandwich ELISA
Anti-Tissue transglutaminase IgG	Indirect ELISA/Sandwich ELISA
Anti-Tissue transglutaminase screen	Indirect ELISA/Sandwich ELISA
Anti-Mitochondrial Antibody-M2	Indirect ELISA/Sandwich ELISA
Anti-Saccharomyces cerevisiae antibody (ASCA)	Indirect ELISA/Sandwich ELISA
Anti-Intrinsic factor	Indirect ELISA/Sandwich ELISA
Diabetes	
Anti-Insulin	Indirect ELISA/Sandwich ELISA
Miscellaneous	
Ferritin	Indirect ELISA/Sandwich ELISA
Beta-2-microglobulin	Indirect ELISA/Sandwich ELISA
Microalbumin	Competitiive ELISA
Immunoblots	
Gastro-5-Line	Nitrocellulose membrane based indirect immunoassay
ANA-9-Line	
Nucleo-9-Line	

EXAMPLES OF DETAILED ELISA METHODS

Competitive ELISA

Total Triiodothyronine (tT3)

(*Courtesy:* Lilac Medicare)

Intended Use: The quantitative determination of total triiodothyronine concentration in human serum or plasma by a microplate enzyme immunoassay.
Mfd: Monobind Inc.

Principle

Competitive Enzyme Immunoassay

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a

competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubulized binding sites. The interaction is illustrated by the following equation:

$$Enz_{Ag} + Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{C.W.} + {}^{Enz}AgAb_{C.W.}$$

$Ab_{C.W.}$ = Monospecific immobilized Antibody (constant quantity)

Ag = Native antigen (variable quantity)

${}^{Enz}Ag$ = Enzyme-antigen conjugate (constant quantity)

$AgAb_{C.W.}$ = Antigen-antibody complex

${}^{Enz}Ag\ Ab_{C.W.}$ = Enzyme-antigen conjugate - antibody complex

k_a = Rate constant of association

k_{-a} = Rate constant of dissociation

K = ka/k_{-a} = Equilibrium constant

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Immunoenzymometric/Sandwitch (Streptavidin-Biotin) ELISA

Thyrotropin (TSH)

(*Courtesy:* Lilac Medicare)

Intended use: The quantitative determination of thyrotropin concentration in human serum by a microplate immunoenzymometric assay.
mfd: Monobind Inc.

Summary and Explanation of the Test

Measurement of the serum concentration of thyrotropin (TSH), a glycoprotein with a molecular weight of 28,000 daltons and secreted from the anterior pituitary, is generally regarded as the most sensitive indicator available for the diagnosis of primary and secondary (pituitary) hypothyroidism. Increase in serum concentrations of TSH, which is primarily responsible for the synthesis and release of thyroid hormones, is an early and sensitive indicator of decrease thyroid reserve and in conjunction with decreased thyroxine (T4) concentrations is diagnostic of primary hypothyroidism. The expected increase in TSH concentrations demonstrates the classical negative feedback system between the pituitary and thyroid glands. That is, primary thyroid gland failure reduces secretion of the thyroid hormones, which in turn stimulates the release of TSH from the pituitary.

Additionally, TSH measurements are equally useful in differentiating secondary and tertiary (hypothalamic) hypothyroidism from the primary thyroid disease. TSH release from the pituitary is regulated by thyrotropin releasing factor (TRH), which is secreted by the hypothalamus, and by direct action of T4 and triiodothyronine (T3), the thyroid hormones, at the pituitary. Increase levels of T3 and T4 reduces the response of the pituitary to the stimulatory effects of TRH. In secondary and tertiary hypothyroidism, concentrations of T4 are usually low and TSH levels are generally low or normal. Either pituitary TSH deficiency (secondary hypothyroidism) or insufficiency of stimulation of the pituitary by TRH (tertiary hypothyroidism) causes this. The TRH stimulation test differentiates these conditions. In secondary hypothyroidism, TSH response to TRH is blunted while a normal or delayed response is obtained in tertiary hypothyroidism.

Further, the advent of immunoenzymometric assays has provided the laboratory with sufficient sensitivity to enable the differentiating of hyperthyroidism from euthyroid population and extending the usefulness of TSH measurements. This method is a second-generation assay, which provides the means for discrimination in the hyperthyroid-euthyroid range. The functional sensitivity (< 20% between assay CV) of the one-hour procedure is 0.195 μIU/mL while the two-hour procedure has a functional sensitivity of 0.095 μIU/mL.

In this method, TSH calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies are added and the reactants mixed. Reaction between the various TSH antibodies and native TSH forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the antibody bound enzyme thyrotropin conjugate is separated from the unbound enzyme thyrotropin conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyrotropin levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with thyrotropin concentration.

Principle

Immunoenzymometric Assay

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-TSH antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(p)} + Ag_{TSH} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(p)}\text{-}Ag_{TSH}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated monoclonal antibody (excess quantity)

Ag_{TSH} = Native antigen (variable quantity)

$^{Enz}Ab_{(p)}$ = Enzyme-polyclonal antibody (excess quantity)

$^{Enz}Ab_{(p)}$ - Ag_{TSH}-$^{Btn}Ab_{(m)}$= Antigen-Antibodies Sandwich complex

k_a = Rate constant of association

k_{-a} = Rate constant of dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$^{Enz}Ab_{(p)}\text{-}Ag_{TSH}\text{-}{}^{Btn}Ab_{(m)} + Streptavidin_{C.W.} \Rightarrow \text{immobilized complex}$$

$Streptavidin_{C.W.}$ = Streptavidin immobolized on well
Immobilized complex = Sandwich complex bound to the solid surface

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Reagents

Materials Provided

A. **Thyrotropin calibrators—1 mL/vial:** Seven (7) vials of references for TSH Antigen at levels of 0(**A**), 0.5(**B**), 2.5(**C**), 5.0(**D**), 10(**E**), 20(**F**) and 40(**G**) µIU/mL. Store at 2–8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 2nd IRP 80/558.

B. **TSH enzyme reagent—13 mL/vial:** One (1) vial containing enzyme labeled affinity purified polyclonal goat antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2–8°C.

C. **Streptavidin coated microplate—96 wells:** One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2–8°C.

D. **Wash solution concentrate—20 mL:** One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

E. **Substrate A—7 mL/vial:** SA One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2–8°C.

F. **Substrate B—7 mL/vial:** One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2–8°C.

G. **Stop solution—8 mL/vial:** One (1) bottle containing a strong acid (1N HCl). Store at 2–30°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2–8°.

Note 3: Above reagents are for a single 96-well microplate.

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Precautions

All products that contain human serum have been found to be nonreactive for Hepatitis B surface antigen, HIV 1 and 2 and HCV antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control/National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd edition, 1988, HHS.

Specimen Collection and Preparation

The specimens shall be blood serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or gel barrier. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2–8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of –20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100 mL of the specimen is required.

Required but not Provided

1. Pipette(s) capable of delivering 50 µL and 100 µL volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 mL and 0.300 mL volumes with a precision of better than 1.5%.
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Reader with 450 nm and 620 nm wavelength absorbance capability (The 620 nm filter is optional).
5. Adjustable volume (200–1000 µL) dispenser.
6. Container(s) for mixing of reagents (see below).
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. Storage container for storage of wash buffer.
12. Distilled or deionized water.
13. Quality control materials.

Reagent Preparation

1. **Wash Buffer**
   Dilute contents of Wash Concentrate to 1000 mL with distilled or deionized water in a suitable storage container. Store at room temperature 20–27°C for up to 60 days.
2. **Working Substrate Solution**
   Pour the contents of the vial labeled Solution 'A' into the vial labeled Solution 'B'. Mix and store at 2–8°C. Use within 60 days. Or for longer periods of usage determine the amount of reagent needed and prepare by mixing equal portions of Substrate A and Substrate B in a suitable container. For example, add 1 mL of A and 1 mL of B per two (2) eight well strips (A slight excess of solution is made. Discard the unused portion).
   Note: Do not use the working substrate if it looks blue.

Test Procedure

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20–27°C).

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2–8°C.
2. Pipette 0.050 mL (50 µL) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 mL (100 µL) of the TSH Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.
4. Swirl the microplate gently for 20–30 seconds to mix and cover.
5. Incubate 60 minutes at room temperature.**
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 300 µL of wash buffer (see Reagent Preparation Section) decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.
8. Add 0.100 mL (100 µL) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.
   Do not shake the plate after substrate addition.
9. Incubate at room temperature for fifteen (15) minutes.
10. Add 0.050 mL (50 µL) of stop solution to each well and mix gently for 15–20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.
11. Read the absorbance in each well at 450 nm (using a reference wavelength of 620–630 nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

** For better low-end sensitivity (< 0.5 µIU/mL). Incubate 120 minutes at room temperature. The 40 µIU/mL calibrator should be excluded since absorbance over 3.0 units will be experienced. Follow the remaining steps.

Quality Control

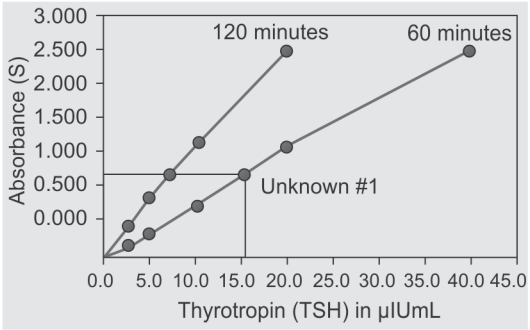
Each laboratory should assay controls at levels in the low, normal, and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable asssay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the dose response curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

Results

A dose response curve is used to ascertain the concentration of thyrotropin in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in following example (An example of the 120-minute incubation is presented in italic type).
2. Plot the absorbance for each duplicate serum reference versus the corresponding TSH concentration in µIU/mL on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of TSH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µIU/mL) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.019) intersects the dose response curve at (15.3 µIU/mL) TSH concentration (Fig. 22.23).

Sample ID.	Well number	Abs (A)	Mean Abs (B)	Value (ng/mL)
Cal A	A1	0.001	0.010	0
	B1	0.009		
Cal B	C1	0.034	0.033	0.5
	D1	0.033		
Cal C	E1	0.175	0.176	2.5
	F1	0.177		
Cal D	G1	0.377	0.371	5.0
	H1	0.366		
Cal E	A2	0.743	0.745	10
	B2	0.743		
Cal F	C2	1.452	1.442	20
	D2	1.431		
Cal G	E2	2.633	2.581	40
	F2	2.530		
Control	G2	0.382	0.385	5.2
	H2	0.388		
Patient	A3	1.028	1.019	15.3
	B3	1.011		



*The data presented above are for illustration only and should not be used in lieu of a dose response curve prepared with each assay.

FIG. 22.23: Example showing average absorbance intersects dose response curve at TSH concentration

QC Parameters

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0 ng/dL should be > 1.3.
2. Four out of 6 quality control pools should be within the established ranges.

Limitations of Procedure

A. *Assay performance*

1. It is important that the time of reaction in each well is held constant for reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
4. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time-deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.

B. *Interpretation*

1. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
2. Serum TSH concentration is dependent upon a multiplicity of factors: Hypothalamus gland function, thyroid gland function, and the responsiveness of pituitary to TRH. Thus, thyrotropin concentration alone is not sufficient to assess clinical status.
3. Serum TSH values may be elevated by pharmacological intervention. Domperiodone, amiodazon, iodide, phenobarbital, and phenytoin have been reported to increase TSH levels.
4. A decrease in thyrotropin values has been reported with the administration of propranolol, methimazol, dopamine and d-thyroxine.
5. Genetic variations or degradation of intact TSH into subunits may affect the biding characteristics of the antibodies and influence the final result. Such samples normally exhibit different results among various assay systems due to the reactivity of the antibodies involved. The interpretation of FT4 is complicated by a variety of drugs that can affect the binding of T4 to the thyroid hormone carrier proteins or interfere in its metabolism to T3.

"Not intended for newborn screening."

Expected Ranges of Values

A study of euthyroid adult population was undertaken to determine expected values for the TSH ELISA Microplate Test System. The number and determined range are given in Table 22.1. A nonparametric method (95% Percentile Estimate) was used.

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: The specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

Immunoenzymometric/Sandwich Sequential (Streptavidin-Biotin) ELISA

Prolacting Hormone (PRL) Sequential Method

(*Courtesy:* Lilac Medicare)

Intended Use: The quantitative determination or prolactin hormone concentration in human serum by a microplate sequential immunoenzymetric assay.

TABLE 22.1: Expected values for the TSH ELISA test system (in µIU/mL)

Number	139
Low normal range	0.39
High normal range	6.16
70% Confidence intervals for	2.5 Percentile
Low range	0.28–0.53
High range	5.60–6.82

Principle

Immunoenzymometric Sequential Assay (Type 4)

The essential reagents required for an immuno-enzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-prolactin antibody.

Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen, reaction results between the native antigen and the antibody, forming an antibody-antigen complex. The interaction is illustrated by the following equation;

$$Ag_{(prl)} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\Leftarrow}} Ag_{(prl)}\text{-}\,{}^{Btn}Ab^{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated monoclonal antibody (excess quantity)

$Ag_{1(prl)}$ = Native antigen (variable quantity)

$Ag_{1(prl)}{}^{-\,Btn}\,Ab_{(m)}$ = Antigen–antibody complex (variable quantity)

k_a = Rate constant of association

k_{-a} = Rate constant of dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$Ag_{(prl)}\text{-}\,{}^{Btn}Ab_{(m)\,+}$ $Streptavidin_{cw} \Rightarrow$ Immobilized complex (IC)

$Streptavidin_{cw}$ = Streptavidin immobilized on well

Immobilized complex (IC) = Ag-Ab bound to the well

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation of aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells. Excess enzyme is washed off via a wash step. A suitable substrate is added to produce color measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

$$(IC) + {}^{Enz}Ab_{(x\text{-}prl)} \underset{k_{-b}}{\overset{k_b}{\Leftarrow}} {}^{Enz}Ab_{(x\text{-}prl)}\text{-}\,IC$$

$^{Enz}Ab_{(x\text{-}prt)}$ = Enzyme labeled antibody (excess quantity)

$^{Enz}Ab_{(x\text{-}prt)}$- IC = Antigen-antibodies complex

k_b = Rate constant of association

k_{-b} = Rate constant of dissociation

"Mu Capture" Immunocapture ELISA

HAV-IgM

Courtesy: Lilac Medicare
Mfd: Equipar

Enzyme "Capture" Immunoassay for the qualitative determination of IgM class antibodies to Hepatitis A virus in human serum and plasma.

For in vitro diagnostic use only.

Principle of the Assay

Microplates are coated with a monoclonal anti-IgM antibody that in the first step captures specifically this class of antibodies. After washing out all the other components of the sample, bound anti-HAV specific IgM are detected by the addition of a preformed immunocomplex, made of HAV antigens and a virus specific antibody, labeled with peroxidase (HRP). The captured enzyme, acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the presence of anti-HAV IgM in the sample.

Direct ELISA

DNase Activity

(*Courtesy:* Lilac Medicare)
Mfd: Orgentec

DNase Activity is a solid phase enzyme immunoassay (ELISA) for the quantitative screening of DNase in human serum or EDTA-plasma. The assay is intended for in vitro diagnostic use only.

Principle of the Test

Specific DNase substrate is bound to microwells. Any present DNase activity reacts with the specific immobilized DNase substrate for 60 minutes at 37 °C. Washing of the microwells removes nonreactive serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-DNase substrate immunologically detects the remaining DNase substrate immobilized on the microplate. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm. The amount of color is inversely proportional to the DNase activity. Pathologic samples exhibit a higher activity reduction (%AR).

CLIA—Immunoenzymometric/Sandwich (Streptavidin-Biotin) ELISA

Thyrotropin (TSH)

Intended Use: The quantitative determination of thyrotropin concentration in human serum by a microplate (CIA) chemiluminescence immunoassay.
Courtesy : Lilac Medicare.
Mfd: Monobind Inc.

Summary and Explanation of the Test

Measurement of the serum concentration of thyrotropin (TSH), a glycoprotein with a molecular weight of 28,000 Daltons and secreted from the anterior pituitary, is generally regarded as the most sensitive indicator available for the diagnosis of primary and secondary (pituitary) hypothyroidism. The structure of human TSH is similar to that of the pituitary and placental gonadotropins, consisting of an 89 amino acid a subunit which is similar or identical between these hormones and a 115 amino acid β-subunit, which apparently confers hormonal specificity. The production of the 2 subunits is separately regulated with apparent excess production of the a subunit. The TSH molecule has a linear structure consisting of the protein core with carbohydrate side chains; the latter accounts for 16% of the molecular weight.

Increase in serum concentrations of TSH, which is primarily responsible for the synthesis and release of thyroid hormones, is an early and sensitive indicator of decrease thyroid reserve and in conjunction with decreased thyroxine (T4) concentrations is diagnostic of primary hypothyroidism. The expected increase in TSH concentrations demonstrates the classical negative feedback system between the pituitary and thyroid glands. That is, primary thyroid gland failure reduces secretion of the thyroid hormones, which in turn stimulates the release of TSH from the pituitary.

Additionally, TSH measurements are equally useful in differentiating secondary and tertiary (hypothalamic) hypothyroidism from the primary thyroid disease. TSH release from the pituitary is regulated by thyrotropin releasing factor (TRH), which is secreted by the hypothalamus, and by direct action of T4 and triiodothyronine (T3), the thyroid hormones, at the pituitary. Increase levels of T3 and T4 reduces the response of the pituitary to the stimulatory effects of TRH. In secondary and tertiary hypothyroidism, concentrations of T4 are usually low and TSH levels are generally low or normal. Either pituitary TSH deficiency (secondary hypothyroidism) or insufficiency of stimulation of the pituitary by TRH (tertiary hy-pothyroidism) causes this. The TRH stimulation test differentiates these conditions. In secondary hypothyroidism, TSH response to TRH is blunted while a normal or delayed response is obtained in tertiary hypothyroidism.

Further, the advent of immunoenzymometric assays has provided the laboratory with sufficient sensitivity to enable the differentiating of hyperthyroidism from euthyroid population and extending the usefulness of TSH measurements. This method is a second generation assay, which provides the means for discrimination in the hyperthyroid-euthyroid range.

In this method, TSH calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (Abs) are added and the reactants mixed. Reaction between the various TSH antibodies and native TSH forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the antibody bound enzyme-thyrotropin conjugate is separated from the unbound enzyme-thyrotropin conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce light.

The employment of several serum references of known thyrotropin levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with thyrotropin concentration.

Principle

Immunoenzymometric Assay

The essential reagents required for an immuno-enzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-TSH antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or stearic hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(p)} + Ag_{TSH} + {}^{Btn}AB_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(p)} - Ag_{TSH} - {}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Ab (excess quantity)

Ag_{TSH} = Native Ag (variable quantity)

$^{ENZ}Ab_{(p)}$ = Enzyme labeled polyclonal Ab (excess quantity)

$^{ENZ}Ab_{(p)}$-Ag_{TSH}-$^{Btn}Ab_{(m)}$ ⇒ Antigen-antibodies sandwich complex

k_a = Rate constant of association

k_{-a} = Rate constant of dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$^{Enz}Ab_{(p)}\text{-}Ag_{TSH}\text{-}^{Btn}Ab_{(m)} + Streptavidin_{C.W.} \Rightarrow$$

Immobilized complex

$Streptavidin_{C.W.}$ = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the solid surface.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Materials Provided for 96-well Microplate

A. **Thyrotropin calibrators — 1.0 mL/vial:** Seven (7) vials of references for TSH Antigen at levels of 0(**A**), 0.5(**B**), 2.5(**C**), 5.0(**D**), 10(**E**), 20(**F**) and 40(**G**) μIU/mL. Store at 2-8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 2nd IRP 80/558.

B. **TSH Tracer Reagent—13 mL/vial:** One (1) vial containing enzyme labeled affinity purified polyclonal goat antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2–8°C.

C. **Streptavidin Reaction Wells—96 wells:** One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2–8°C.

D. **Wash Solution Concentrate—20 mL:** One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2–30°C.

E. **Signal Reagent A—7.0 mL/vial:** One (1) bottle containing luminol in buffer. Store at 2–8°C.

F. **Signal Reagent B—7.0 mL/vial:** One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2–8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2–8°C.

Materials [Required But Not Provided]

1. Pipette(s) capable of delivering 50 μL and 100 μL volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 mL and 0.300 mL volumes with a precision of better than 1.5% (optional).
3. Microplate washer or a squeeze bottle (optional).
4. Microplate luminometer.
5. Adjustable volume (200–1000 μL) dispenser.
6. Container(s) for mixing of reagents (see below).
7. Absorbent paper for blotting the microplate wells.
8. Plastic wrap or microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. Storage container for storage of wash buffer.
12. Distilled or deionized water.
13. Quality control materials.

Precautions

For in vitro diagnostic use
Not for internal or external use in humans or animals

All products that contain human serum have been found to be nonreactive for Hepatitis B surface antigen, HIV 1 and 2 and HCV antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control/National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd edition, 1988, HHS.

Specimen Collection and Preparation

The specimens shall be blood serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain red-top venipuncture tube with or without gel

barrier. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2–8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of –20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100 mL of the specimen is required.

Reagent Preparation

1. **Wash buffer**
   Dilute contents of Wash Concentrate to 1000 mL with distilled or deionized water in a suitable storage container. Store at room temperature (20–27°C) for up to 60 days.
2. **Working signal reagent solution**
   Mix equal volumes of Solution 'A' and Solution 'B' in a clean container. Use within 60 minutes. For example, add 1 mL of A and 1 mL of B for two (2) eight well strips (A slight excess of solution is made. Discard the unused portion).

Test Procedure

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20–27°C).

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2–8°C.
2. Pipette 0.050 mL (50 μL) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 mL (100 μL) of the TSH Tracer Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.
4. Swirl the microplate gently for 20–30 seconds to mix and cover.
5. Incubate 45 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 350 μL of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.
8. Add 0.100 mL (100 μL) of working signal reagent solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.
9. Incubate for five minutes at room temperature in the dark.
10. Read the RLU's (Relative Light Units) in each well in a microplate luminometer for at least 0.2 seconds/well. The results can be read within 30 minutes of adding the substrate solution.

Quality Control

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the dose response curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

Results

A dose response curve is used to ascertain the concentration of TSH in unknown specimens.

1. Record the RLU's obtained from the printout of the microplate reader as outlined in following Example.
2. Plot the RLU's for each duplicate serum reference versus the corresponding TSH concentration in μIU/mL on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of TSH for an unknown, locate the average RLU's for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in μIU/mL) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU's (15032) of the unknown intersects the calibration curve at (6.04 μIU/mL) TSH concentration (Fig. 22.24).

Note 1: Computer data reduction software designed for chemiluminescence assays may also be used for the data reduction. Duplicates of the unknown may be averaged as indicated (Fig. 22.24).

Note 2: Monobind can assist the laboratory in the purchase and implementation of equipment/software to measure and interpret chemiluminescence data.

QC Parameters

1. The Dose Response Curve should be within established parameters.
2. Four out of six quality control pools should be within established ranges.

Limitations of Procedure

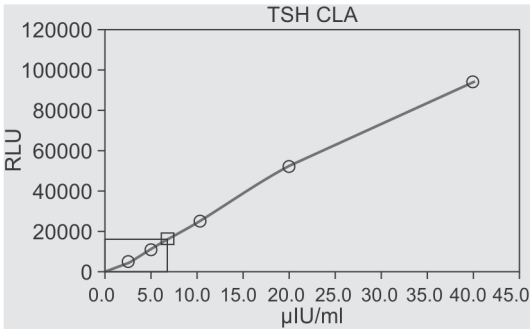
A. Assay performance

1. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift. If more than one (1) plate is used, it is recommended to repeat the dose response curve. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
2. Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemeic or hemolyzed specimen(s) should similarly not be used.
3. Patient specimens with TSH concentrations above 40 µIU/mL may be diluted with the zero calibrator and reassayed. The sample's concentration is obtained by multiplying the result by the dilution factor.
4. Each component in one assay should be of the same lot number and stored under identical conditions.

B. Interpretation

1. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
2. Serum TSH concentration is dependent upon a multiplicity of factors: Hypothalamus gland function, thyroid gland function, and the responsiveness of pituitary to TRH. Thus, thyrotropin concentration alone is not sufficient to assess clinical status.
3. Serum TSH values may be elevated by pharmacological intervention. Domperiodone, amiodazon, iodide, phenobarbital, and phenytoin have been reported to increase TSH levels.

Sample ID.	Well number	RLU's (A)	Mean Abs (B)	Value (ng/mL)
Cal A	A1	25	37	0
	B1	50		
Cal B	C1	372	434	0.5
	D1	495		
Cal C	E1	5757	5954	2.5
	F1	6140		
Cal D	G1	12425	12453	5.0
	H1	12481		
Cal E	A2	25972	25976	10.0
	B2	25980		
Cal F	C2	52810	53052	20.0
	D2	53294		
Cal G	E2	95658	96361	40.0
	F2	97054		
Control 1	G2	329	293	*0.31*
	H2	267		
Control 2	A3	14823	15032	*6.04*
	B3	15241		
Patient 1	C3	1255	1281	*1.51*
	D3	1305		



* The data presented above is for illustration only and should not be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to approximately 100,000 RLU's for the A calibrator (greatest light output). This conversion eliminates differences caused by efficiency of the various instruments that can be used to measure light output.

FIG. 22.24: Example showing average RLU intersects calibration curve at TSH concentration

4. A decrease in thyrotropin values has been reported with the administration of propranolol, methimazol, dopamine and d-thyroxine.
5. Genetic variations or degradation of intact TSH into subunits may affect the biding characteristics of the antibodies and influence the final result. Such samples

normally exhibit different results among various assay systems due to the reactivity of the antibodies involved.

"Not intended for newborn screening"

Expected Ranges of Values

A study of euthyroid adult population was undertaken to determine expected values for the TSH CIA Microplate Test System. The number and determined range are given in Table 22.2. A nonparametric method (95% Percentile Estimate) was used.

It is important to keep in mind that expected values for normal population is dependent upon a multiplicity of factors: The specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

TABLE 22.2: Expected values for the TSH CIA test system (in µIU/mL)

Number	139
Low normal range	0.39
High normal range	6.16
70% confidence intervals for 2.5 percentile	
Low range	0.28–0.53
High range	5.60 – 6.82

TESTS FOR SYPHILIS

VDRL Test

Conventional VDRL Test

Serological testing, for the diagnosis of syphilis, traditionally has been based on the detection of "Reagin" by the use of antigen, prepared from normal tissues, most commonly beef heart. VDRL slide test can be used both qualitatively and quantitatively for the detection of "Reagin" in serum. As other better methods are available, this method is hardly even used.

Principle

A phospholipid viz. cardiolipin, derived from beef heart muscle together with cholesterol and lecithin, is used as an antigen. After mixing the antigen with patient's serum, the reaction is accelerated by rotatory agitation either on a mechanical shaker or by hand. The antigen reacts with reagin and forms floccules. These floccules can be observed with naked eye, hand lens or under a low power objective of a microscope when the reaction is weak.

Equipment Required

1. VDRL Test Slide: A 2" × 3" glass slide with 12 paraffin or ceramic rings of approximately 14 mm inside diameter.
2. Hypodermic needles without bevels (18, 19 gauge).
3. Syringe (1–2 mL).
4. Thirty mL flat or concave inner bottomed glass stoppered, narrow mouth bottle, approximately 35 mm in diameter (bottles with convex inner bottom surface are unsatisfactory).

Precautions

1. Use clean and dry glassware.
2. Allow all reagents and samples to reach room temperature before starting the test.
3. Carry out the test at room temperature (preferably between 23 and 29°C).

Sample

Serum (0.05 mL is required for qualitative test and 0.1 mL is needed for quantitative test.

Preparation of Patient's Serum (Inactivation)

Heat clear serum sample in a water bath at 56°C for 30 minutes. Examine all serum samples after removing from the water bath and those found to contain particulate debris should be recentrifuged. Serum samples to be tested more than 4 hours after inactivation should be reheated at 56°C and allowed to cool to room temperature.

Reagents Usually Supplied

- Reagent 1: VDRL antigen
- Reagent 2: Buffered saline diluent
- Reagent 3: Positive control serum
- Reagent 4: Negative control serum.

Preparation of Working Solutions

Working Antigen Suspension

a. Pipette 0.4 mL of Buffered Saline Diluent into a glass stoppered bottle (with concave or flat inner bottom) and make the reagent completely cover the inner bottom as a thin layer.
b. Add 0.5 mL of VDRL Antigen from the lower half of a 1.0 mL pipette, which is graduated to the tip, directly

to the Buffered Saline Diluent while continuously but gently rotating the bottle. Add the antigen drop by drop but rapidly, allowing 6 seconds for completing the addition of 0.5 mL of the antigen. The pipette tip should remain in the upper third of the bottle and rotation should not be vigorous enough to splash Buffered Saline Diluent on to the pipette. The proper speed of rotation is obtained when the center of the bottle circumscribes a 5 cm diameter circle approximately three times a second.

c. The last drop of the antigen should be blown from the pipette tip so that the pipette tip does not touch the surface of the contents of the bottle.
d. Continue rotation of the bottle for 10 more seconds.
e. Now add 4.1 mL of Buffered Saline Diluent to the bottle. Close the bottle with the stopper and shake it for approximately 10 seconds. The working antigen suspension is now ready for preliminary testing.

Note

There is some indication that maturation of the antigen increases the sensitiveness and this is almost complete within 15 to 30 minutes after preparing Working Antigen suspension. This may then be used within 24 hours.

Under condition of high temperature and low humidity, the Working Antigen should be stored in a refrigerator, but should be brought to room temperature before use. Mix the working antigen suspension gently each time it is used. Do not mix it by forcing it back and forth through the syringe and needle as this may lead to breakdown of antigen particles which results in loss of activity.

Preliminary Testing of the Working Antigen Suspension

1. Each time the Working Antigen Suspension is prepared, it has to be tested with negative and positive control sera by means of slide qualitative test method described under Procedure, using the controls in place of test sera.
2. The Working Antigen Suspension should give expected typically reactive and nonreactive results respectively. Also, the size and number of antigen particles per microscopic field in the nonreactive serum should be optimum. If the antigen particles in nonreactive serum appear too large, the fault will usually be in the preparation of the Working Antigen Suspension although other factors are sometimes responsible.
3. The antigen control (suspension in saline) should be smooth in appearance with antigen particles well dispersed.
4. Do not use Working Antigen Suspension if it does not give satisfactory performance in the preliminary testing.

Storage and Stability

The VDRL Antigen and Buffered Saline Diluent are to be stored exclusively in a cool and dark place at room temperature (preferably at 23–29°C); and at these conditions, the reagents are stable till the expiry date mentioned.

The control sera are stable at 2 to 8°C till the expiry date mentioned.

Working Antigen Suspension prepared for any day must NOT under any circumstances be kept and used for the subsequent days.

Procedure

Qualitative Test

1. Pipette 0.05 mL of patient's inactivated serum into the concavity of the VDRL slide.
2. Pipette 0.05 mL each of positive and negative control sera into other two concavities of the VDRL slide.
3. Add one drop (1/75 mL) of the *Working Antigen Suspension* to each of the above concavities, with a calibrated 23-gauge needle without bevel.
4. Rotate the slide for 4 minutes with hand on a flat surface (this movement should circumscribe roughly about 5 cm diameter circle 120 times per minute) or on a VDRL rotator.
5. Read the tests immediately under a low power objective of a microscope.

Quantitative Test

For quantitative test with sera reacting strongly in qualitative test, the following procedure should be followed:

1. Prepare different dilutions of test serum, in test tubes, in the range of 1:2, 1:4, 1:8, 1:16, 1:32 or more with normal saline.
2. Transfer 0.05 mL of each of the above diluted sera into separate concavities of the VDRL slide.
3. With the help of a 23-gauge needle and syringe, add one drop (1/75 mL) of Working Antigen Suspension to each of the above concavities.
4. Rotate the slide for 4 minutes with hand on a flat surface (this movement should circumscribe roughly about 5 cm diameter circle 120 times per minute) or on a VDRL rotator.
5. Read the test immediately under a low power objective of a microscope.

Note

The results of controls should be satisfactory for validating the results of tests.

Interpretation of Test Results

The antigen particles are seen as small fusiform needles which remain more or less evenly dispersed in case of a nonreactive serum and aggregate into clumps with reactive serum. The conclusions can be drawn from the observations as follows:

	Observation	*Conclusion*
i.	No clumps or very slight roughness	Nonreactive
ii.	Small clumps	Weakly reactive
iii.	Medium/large clumps	Reactive

Notes

1. Zonal reactions occasionally occur in serological tests. In such cases, a strongly reactive serum may show a weak or atypical reaction when undiluted serum is tested (prozone phenomenon). A completely negative reaction with very strongly reactive sera is extremely rare.
2. A reactive or weakly reactive test result indicates the presence of reagin, which almost invariably is formed in *Treponema* infection, but which may be produced by a variety of other conditions. In actual practice, a reactive result in the presence of clinical symptoms is considered as a confirmatory evidence of syphilitic infection. However, in the absence of clinical findings, test reactivity can represent any of the following: (a) Latent syphilis, (b) A biological false-positive reaction, either temporary or chronic or (c) A technical or clinical error.

Limitation of the Test

1. Acute or chronic infections such as malaria, leprosy, infectious mononucleosis and upper respiratory diseases as well as collagen and immunologic diseases such as rheumatoid arthritis and lupus erythematosus can produce false-positive reagin tests.
2. Other less well-known conditions include tissue regeneration, pregnancy, heroin addiction and the use of certain drugs for hypertension.
3. Reliable test results require strict attention to details of technique, including proper identification of specimens, accurate measurement, temperature control, correct timing and observation of principles of quality control.

MODIFIED VDRL REAGENT TREPOLIPIN®

(Courtesy: Tulip Group of Companies)

Reagent

1. TREPOLIPIN® reagent A ready to use stabilised emulsion of cardiolipin, lecithin and cholesterol.
2. Positive control, reactive with TREPOLIPIN® reagent.
3. Negative control, nonreactive with TREPOLIPIN® reagent.

The TREPOLIPIN® detects antilipoidal antibodies in serum, plasma and cerebrospinal fluid (CSF). As against the conventional VDRL reagents, test samples do not require heat inactivation.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of reagent is as per the expiry date mentioned on the reagent vial label. Avoid exposure to elevated temperature and air as the reagent is highly sensitive to denaturation and drying.

Principle

When serum, plasma or cerebrospinal fluid (CSF) containing antilipoidal antibodies is reacted with TREPOLIPIN® reagent, a flocculation reaction is produced.

Flocculation is a positive test result and indicates presence of antilipoidal antibodies in the sample. No flocculation is a negative test result and indicates absence of antilipoidal antibodies in the sample.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
3. The antigen suspension should be gently but thoroughly mixed by swirling before testing to homogenize the reagent and improve test readability.
4. Performance of the reagent must be verified with positive and negative controls and it is recommended that controls be run with each test series.
5. Accessories provided with the kit only must be used for optimum results.

Sample Collection and Storage

1. No special preparation of the patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples.
2. Fresh serum, plasma or CSF should be used for testing.
3. Hematogenous CSF should not be used for testing. For cloudy samples, centrifuge and use the clear supernatant for testing.

Material Provided with the Kit

1. Stabilized cardiolipin suspension.
2. Reagent dropper assembly for dispensing the antigen suspension.
3. Positive control, reactive with reagent.
4. Negative control, nonreactive with reagent.

Additional Material Required

Conventional VDRL cavity slide (glass), microscope (with magnification of 100 x), Pasteur pipettes, mechanical rotor (180 rpm), isotonic saline.

Note: For TREPOLIPIN® 5 × 5 mL kit. Known reactive and nonreactive samples would be required additionally.

Test Procedure

Bring reagent and samples to room temperature before testing.

1. Thoroughly mix the TREPOLIPIN® reagent suspension by gentle agitation before testing.
2. With cerebrospinal fluid, the test specimen volume is 0.01 mL.
3. For use with cerebrospinal fluid, each drop of TREPOLIPIN® reagent should be diluted with 0.02 mL of good isotonic saline before testing.

Qualitative Method

1. Pipette 0.05 mL of serum or plasma to the VDRL slide cavity.
2. Dispense one drop of TREPOLIPIN® reagent to the surface of the test sample in the same cavity using the reagent dropper provided.
3. Rotate the slide continuously at 180 rpm for 4 minutes, observing for flocculation.
4. Read the results macroscopically or microscopically at 4 minutes.
5. All positive test results may be further tested by the quantitative test procedure.

Quantitative Method

1. Pipette 0.1 mL of isotonic saline into seven test tubes.
2. Pipette 0.1 mL of the test sample into the first test tube.
3. Transfer 0.1 mL of the diluted test sample from the first tube to the second tube.
4. Continue the serial dilution of the test sample till dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 are achieved.
5. Transfer 0.05 mL each dilution of the test sample from tubes 1 to 7 to a conventional VDRL slide.
6. Dispense one drop of TREPOLIPIN® reagent to each dilution of the sample on the VDRL slide.
7. Rotate the slide continuously at 180 rpm for four minutes.
8. Observe for flocculation macroscopically or microscopically at 4 minutes.

Interpretation of Results

Qualitative Method

Flocculation is a positive test result and indicates presence of antilipoidal antibodies in the test sample.

No flocculation is a negative test result and indicates absence of antilipoidal antibodies in the test sample. The strength of flocculation may vary, depending upon the degree of positivity of the test sample.

Quantitative Method

The antilipoidal antibody titer is the highest dilution of the test sample giving a positive test result (flocculation).

Remarks

1. Quantitative procedure must be performed to determine the response to treatment and detect reinfection.
2. False positive reactions occur not infrequently and have been attributed to a variety of acute and chronic conditions.
3. In the absence of supporting clinical, historical or epidemiological evidence reactive results must be confirmed with more specific *Treponema* tests.
4. It is recommended that results of the test should be correlated with clinical findings to arrive at the final diagnosis.
5. Microscopic evaluation of test results requires well-trained and experienced professional. It is recommended that a few known negatives should be run with each batch of the tests so as to familiarize and differentiate effectively the appearance of nonreactive samples from the reactive ones.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Exposure of reagents to high temperature	Exposure to high temperatures of the reagents can cause autoflocculation of reagents. Also since the reagent is alcohol based prolonged exposure to high temperature may lead to evaporation. Therefore, avoid exposure of the reagents to elevated temperatures
2. Cloudy CSF used for testing	Hematogenous CSF should not be used for testing. For cloudy samples, centrifuge at 1000 rpm for 1 minute and use the clear supernatant for testing
3. Serum is allowed to dry prior to addition of antigen	To avoid drying of the serum, pipette 50 µL of the serum to the VDRL slide cavity and immediately add one drop of Trepolipin reagent to the test sample
4. Slide was rotated for too long. Drying has taken place	Rotate the slide continuously at 180 rpm for 4 minutes, observing for flocculation.
	Do not perform the test directly under a fan
5. False positives may occur due to overspill from one cavity to another while rotating	Care must be taken to see that there is no overspill of the test mixture during rotation of the slide
6. False positive reactions can also be attributed to a variety of acute and chronic conditions like leprosy, malaria, infectious mononucleosis, hepatitis, systemic lupus erythematosus and rheumatoid arthritis	Check the history of the patient. The test results must be correlated with clinical findings and all positive results must be further confirmed by using Treponemal tests

Problem: False negative results

Possible causes	*Solutions*
1. Excess serum dispensed (prozoning)	Pipette exactly 50 µL of serum or positive or negative control to VDRL slide cavity
2. Excess antigen dispensed (postzoning)	Dispense exactly one drop of Trepolipin reagent using the reagent dropper provided with the kit
3. Serum, plasma or CSF stored for a long period of time is used for testing	Fresh serum, plasma or CSF should be used for testing
4. The volume of CSF used for testing is incorrect. In CSF testing, the Trepolipin reagent has not been diluted	Test specimen volume in CSF is 10 mL. Each drop of the Trepolipin reagent has to be diluted with 20 mL of isotonic saline for CSF testing
5. Hemolyzed samples may have been used for testing	Do not use hemolyzed samples
6. Antigenic suspension has settled down in the vial	Shake the Trepolipin reagent vial well before use to disperse the reagent particles uniformly and improve test readability
7. Cold reagents are used for testing	Bring all reagents and samples to room temperature before commencing the testing procedure
8. Expired reagents	Check the expiry date of the reagents before use
9. Error in interpreting the test results	Flocculation results should be interpreted carefully after comparing with positive and negative controls. The strength of flocculation may vary, depending upon the degree of positivity of the test sample
10. False negatives are obtained in the tertiary stage of the disease	The test should be confirmed with FTA (fluorescent treponemal antibody) or TPHA (treponema pallidum hemagglutination) tests

TOLUIDINE RED UNHEATED SERUM TEST FOR RAPID SERODIAGNOSIS OF SYPHILIS REDGEN®

(Courtesy: Tulip Group of Companies)

Reagent

1. REDGEN reagent: A particulate suspension containing a red micronised dye coated with lipid complexes.
2. Positive control, reactive with the REDGEN latex reagent.
3. Negative control, non-reactive with the REDGEN latex reagent.

REDGEN detects antilipoidal antibodies in serum or plasma. As against the conventional VDRL reagents, test samples do not require heat inactivation.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label.

Avoid exposure to elevated temperature and air as the reagent is highly sensitive to denaturation and drying.

Principle

During the test procedure, the specimen, serum or plasma is mixed with REDGEN reagent and allowed to react for eight minutes. If antilipoidal antibodies are present in the specimen, they will react with REDGEN reagent forming visible red floccules against the white background of the reaction card. If antilipoidal antibodies are not present in the specimen, there will be no flocculation, resulting in an even pink mat on the reaction circle.

Note

1. In vitro diagnostic reagent for laboratory or professional use only. Not for medicinal use.
2. The reagent contains thiomersal 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
3. The REDGEN reagent suspension should be gently but thoroughly mixed before testing to disperse the dye particles uniformly and improve test readability.
4. Performance of the reagent must be verified with positive and negative controls and it is recommended that controls be run with each test series.
5. Accessories provided with the kit should be used for optimum results.

Sample Collection and Storage

1. No special preparation of the patient is required prior to sample collection by approved techniques. Hemolyzed or lipemic samples are not suitable for testing. In case of oxalated blood samples, it is advisable to avoid excess of oxalate as it may interfere with the test results.
2. Fresh serum or plasma should be used for testing.
3. Samples not tested immediately may be stored at 2 to 8°C for up to 48 hours.
4. Hazy samples should be centrifuged. Use the clear supenatant for testing.

Material Provided with the Kit

The TRUST antigen, positive control reactive with the reagent, negative control nonreactive with the reagent, disposable slides with eight reaction circles, disposable sample/control dispensing pipettes, mixing sticks, rubber teats, reagent dropper for dispensing the REDGEN reagent suspension.

Additional Material Required

Stopwatch, high intensity light source, isotonic saline, pipettetes, test tubes, mechanical rotor at 180 rpm circumscribing a circle 2 cm in diameter on a horizontal plane.

Test Procedure

Bring all reagents and samples to room temperature before testing.

Thoroughly mix the REDGEN reagent suspension by gentle agitation before testing.

Qualitative Method

1. Place one drop of the test sample, positive and negative controls onto separate reaction circles of the disposable slide using a sample dispensing pipette.
2. Add one drop of well mixed REDGEN reagent next to the test sample or controls by using the reagent dropper provided with the kit. Do not let the dropper tip touch the liquid on slide.
3. Using a mixing stick, mix the test sample and REDGEN reagent thoroughly, spreading uniformly over the entire reaction circle.
4. Immediately start a stopwatch. Rotate the slide gently and continuously either manually or on a mechanical rotor at 180 rpm.
5. Observe for flocculation macroscopically at 8 minutes.

Quantitative Method

1. Using isotonic saline, prepare serial dilutions of the test sample positive in the qualitative method 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and so on.
2. Perform the qualitative test procedure using each dilution as a specimen.
3. The titer is reported as reciprocal of the highest dilution which shows a positive test result.

Interpretation of Test Results

Qualitative Method

1. Large and medium RED colored floccules against white background: Reactive
2. Small RED colored floccules against white background: Weakly reactive
3. No floccules, smooth pink background : Nonreactive.

Flocculation is a positive test result and indicates presence of antilipoidal antibodies in the test sample. No flocculation is a negative test result and indicates absence of antilipoidal antibodies in the test sample.

Quantitative Method

The titer of antilipoidal antibodies is the highest dilution of test sample giving a positive test result.

Remarks

1. Quantitative procedure must be performed to deter-mine response to treatment and detect reinfection.
2. False-positive reactions occur not infrequently and have been attributed to a variety of acute and chronic conditions.
3. In the absence of supporting clinical, historical or epidemiological evidence, reactive result must be confirmed with more specific *Treponema* tests.
4. It is strongly recommended that results of the test should be correlated with clinical findings to arrive at the final diagnosis.
5. Dispose all used and contaminated material as per Standard Biohazard Safety Guidelines.
6. The reagent dropper provided for dispensing the REDGEN antigen should be thoroughly cleaned with distilled water and air dried after use, to ensure that it does not contaminate the reagent during subsequent use.
7. Very slight roughness should be interpreted as a negative test result.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Reagent is contaminated because of unclean dropper and hence not suitable for subsequent use	The reagent dropper provided for dispensing the Redgen reagent should be thoroughly cleaned with distilled water and air dried after use, to ensure that it does not contaminate the reagent during subsequent use
2. Drying of the reagent on the slide	Do not interpret test results beyond 8 minutes. Testing should not be carried out under the fan or under conditions where drying is enhanced
3. False positives may occur due to overspill from one circle to another while rotating	Care must be taken to see that there is no overspill during rotation of the slide
4. False positive reactions can also be attributed to a variety of acute and chronic conditions like leprosy, malaria, infectious mononucleosis, hepatitis, systemic lupus erythematosus and rheumatoid arthritis	Check the history of the patient. The test result must be correlated with clinical findings and all positive results must be further confirmed by using Treponemal tests

Problem: False negative results

Possible causes	*Solutions*
1. The reagent may be exposed to elevated temperatures, air and direct sunlight, as it is highly sensitive to denaturation, drying and microbial contamination	Check the performance of the reagent with positive and negative controls provided with the kit. Avoid exposure of the reagent to light
2. Hemolyzed or lipemic samples may have been used for testing	Avoid using hemolyzed or lipemic samples for testing
3. Antigenic suspension has settled down in the vial	Shake the reagent vial well before use to disperse particles uniformly and improve test readability
4. Weak flocculation may be interpreted as negative	The antigen should be gently but thoroughly mixed before testing to disperse the dye particles uniformly and improve test readability. The serum and reagent should be mixed properly. Small red floccules against a white background indicate a weakly reactive test result
5. Cold reagents are used for testing	Bring all reagents and samples to room temperature before commencing the testing procedure
6. Expired reagents are used for testing	Check the expiry date of the reagents before use
7. Error in interpreting the test results	Flocculation results should be interpreted carefully after comparing with positive and negative controls Note: Very slight roughness should be interpreted as a negative result
8. The reagent used for testing is in a frozen condition	The reagent should not be frozen. It should be stored at 2–8°C
9. False negatives are obtained in the tertiary stage of the disease	The test should be confirmed with FTA (Fluorescent treponemal antibody) or TPHA (Treponema pallidum hemagglutination) tests

Problem: Negative control giving false positive reaction

Possible causes	*Solutions*
1. Negative control contaminated with positive control/positive sample	Validate the antigen by using known negative (saline) and positive control. If proper results are obtained, with known negative (saline) and positive controls, then the negative control is contaminated and should not be used for further testing

LATEX SLIDE TEST FOR VDRL SYPHFINAL®

(Courtesy: Tulip Group of Companies)

Reagent

Syphfinal reagent is a ready-to-use, uniform suspension of polystyrene latex particles coated with cardiolipin, suspended in a suitable buffer of proprietary composition.

Though Syphfinal reagent, in performance, corresponds to the other USRs, it accords better readability to the test results, thereby giving better confidence in reporting results. As against the conventional VDRL tests, the samples do not require heat inactivation.

Each batch of reagent undergoes rigorous quality control at various stages of its manufacture for its sensitivity, specificity and performance.

Reagent Storage and Stability

Store the reagent at 2–8°C. Do not freeze. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial labels. Avoid exposure to elevated temperatures, air and direct sunlight as the reagents are highly sensitive to denaturation, drying and microbial contamination.

Principle

Syphfinal, latex VDRL reagent, is based on the principle of agglutination. The test specimen, serum or plasma is mixed with Syphfinal latex VDRL reagent and allowed to react for 6 minutes. If antilipoidal antibodies are present in the specimen, they will react with the latex reagent forming a visible agglutination. If antilipoidal antibodies are not present in the specimen, then no agglutination is observed.

Note

1. *In vitro* diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
3. All reagents derived from human source are tested for HBsAg and anti-HIV antibodies and found to be nonreactive. However, handle the material as if infectious.
4. Syphfinal latex VDRL reagent should be gently but thoroughly mixed before testing to disperse the latex particles uniformly and improve test readability.
5. Performance of the reagents must be verified with positive and negative controls provided with the kit and it is recommended that controls be run with each test series.

Sample Collection and Storage

No special preparation of the patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples. Fresh serum or plasma should be used for testing. Samples not tested immediately may be stored at 2 to 8°C for up to 48 hours. Hazy samples should be centrifuged. Use clear supernatant for testing.

Material Provided with the Kit

Latex VDRL reagent, positive control reactive with the reagent, negative control nonreactive with the reagent, disposable slides with eight reaction circles, disposable sample/control dispensing pipettes, mixing sticks, rubber teats.

Additional Material Required

Stopwatch, high intensity direct light source, isotonic saline, pipettes, test tubes.

Test Procedure

Bring all reagents and samples to room temperature before testing.

Qualitative Method

1. Pipette one drop of test sample onto one of the reaction circles of the disposable slide using a sample dispensing pipette. The disposable slides and the sample dispensing pipettes are provided with the kit.
2. Repeat the procedure with positive and negative controls.
3. Add one drop of well-mixed Syphfinal latex reagent to the test sample, positive control and negative control respectively. Do not let the dropper tip touch the liquid on the slide.
4. Using a mixing stick, mix the test sample and Syphfinal reagent thoroughly spreading uniformly over the entire reaction circle.
5. Immediately start a stopwatch. Rotate the slide gently and continuously, observing for agglutination macroscopically at 6 minutes.

Quantitative Method

1. Using isotonic saline prepare serial dilutions of the test sample, e.g. 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, etc.
2. Perform qualitative test procedure using each dilution as test specimen.
3. The titre is reported as the reciprocal of the highest dilution which shows a positive test result.

Interpretation of Test Result

Qualitative Method

Agglutination is a positive test result and indicates the presence of antilipoidal antibodies in the test sample. No agglutination is a negative test result indicating the absence of detectable levels of antilipoidal antibodies in the test specimen.

Quantitative Method

The titre of antilipoidal antibodies is the highest dilution of the test sample giving a positive test result (i.e. agglutination).

Remarks

1. Quantitative procedure must be performed to determine the response to treatment and detect reinfection.
2. False positive reactions occur not infrequently and have been attributed to a variety of acute and chronic conditions.
3. In the absence of supporting clinical, historical or epidemiological evidence, reactive results must be confirmed with more specific *Treponema* tests.
4. It is recommended that the results of the test should be correlated with the clinical findings to arrive at the final diagnosis.
5. Dispose all used and contaminated materials as per Standard Biohazard Safety Guidelines.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Reagent is contaminated because of unclean dropper and hence not suitable for subsequent use	The reagent dropper provided for dispensing the reagent should be thoroughly cleaned with distilled water and air-dried after use, to ensure that it does not contaminate the reagent during subsequent use. Care must be taken to ensure that the reagent dropper tip does not touch the liquid on the slide while dispensing
2. Drying of the reagent on slide	Do not interpret test results beyond 6 minutes. Testing should not be carried out under the fan or under conditions where drying is enhanced
3. False positive reactions can also be attributed to a variety of acute and chronic conditions like leprosy, malaria, infectious mononucleosis, hepatitis, systemic lupus erythematosus and rheumatoid arthritis	Check the history of the patient. The test result must be correlated with clinical findings
4. False positives may occur due to overspill from one circle to another while rotating	Care must be taken to see that there is no overspill during rotation of the card

Problem: Negative control giving positive reaction

Possible causes	*Solutions*
1. Negative control contaminated with positive control/positive sample	Validate the latex reagent by using known negative (saline) and positive controls. If proper results are obtained, with known negative (saline) and positive controls then the negative control is contaminated and should not be used for further testing

Problem: False negative results

Possible causes	*Solutions*
1. The reagent may be damaged by exposure to elevated temperatures, air and direct sunlight, as it is highly sensitive to denaturation, drying and microbial contamination	Check the performance of the reagent with positive and negative controls provided with the kit. Avoid exposure of the reagent to light. The vial must be closed properly after use
2. The reagent is in a frozen condition	The reagent should not be frozen. It should be stored at 2–8°C
3. Weak agglutination may be interpreted as negative	The latex reagent should be gently but thoroughly mixed before testing to disperse the latex particles uniformly and improve test readability. The serum and the reagent should be mixed thoroughly
4. Excess sample dispensed/reagent dispensed leading to prozoning/postzoning.	Pipette exactly one drop of test sample using the sample-dispensing pipette. Similarly, dispense one drop of well-mixed latex reagent using the reagent dropper provided with the kit
5. Hemolyzed or lipemic samples may have been used for testing	Avoid using hemolyzed or lipemic samples for testing
6. Reagent is contaminated because of unclean dropper and hence not suitable for subsequent use	The reagent dropper provided for dispensing the reagent should be thoroughly cleaned with distilled water and air-dried after use, to ensure that it does not contaminate the reagent during subsequent use

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Possible causes	*Solutions*
7. Serum or plasma stored for a long period of time is used for testing	Fresh serum or plasma should be used for testing. Samples not tested immediately may be stored at 2–8°C for upto 48 hours. Hazy samples should be centrifuged. Use clear supernatant for testing
8. Antigenic suspension has settled down in the vial	Shake the reagent vial well before use to disperse latex particles uniformly and improve test readability
9. Cold reagents are used for testing	Bring all the reagents and samples to room temperature before commencing the testing procedure
10. Expired reagents are used for testing	Check the expiry date of the reagents before use
11. Error in interpreting the test results	Agglutination results should be interpreted carefully after comparing with positive and negative controls

RAPID PLASMA REAGIN (RPR) CARD TEST/ CARBON ANTIGEN FOR SYPHILIS TESTING (CARBOGEN®)

(Courtesy: Tulip Group of Companies)

Summary

The Rapid Plasma Reagin (RPR)/Carbon Antigen test is a macroscopic non-*Treponema* flocculation test for the detection and quantitation of antilipoidal antibodies. Non-*Treponema* tests like CARBOGEN are of great value when used for screening and follow-up of therapy.

Reagent

1. CARBOGEN reagent: A particulate carbon suspension coated with lipid complexes.
2. Positive control, reactive with the CARBOGEN reagent.
3. Negative control, nonreactive with the CARBOGEN reagent.

The CARBOGEN detects antilipoidal antibodies in serum or plasma. As against the conventional VDRL reagents, test samples do not require heat inactivation.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

Store the reagent at 2 to 8°C. Do not freeze. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label. Avoid exposure to elevated temperatures and air as the reagent is highly sensitive to denaturation and drying.

Principle

During the test procedure, the specimen, serum or plasma is mixed with CARBOGEN reagent and allowed to react for 8 minutes. If antilipoidal antibodies are present in the specimen, they will react with CARBOGEN reagent forming visible black floccules. If antilipoidal antibodies are not present in the specimen, there will be no flocculation.

Note

1. In vitro diagnostic reagent for laboratory or professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
3. CARBOGEN RPR/Carbon antigen should be gently but thoroughly mixed before testing to disperse the carbon particles uniformly and improve test readability.
4. Performance of the reagent must be verified with positive and negative controls and it is recommended that controls be run with each test series.
5. Accessories provided with the kit only must be used for optimum results.

Sample Collection and Storage

1. No special preparation of the patient is required prior to sample collection by approved techniques. Hemolyzed or lipemic samples are not suitable for testing.
2. Fresh serum or plasma should be used for testing.
3. Samples not tested immediately may be stored at 2 to 8°C for up to 48 hours.
4. Hazy samples should be centrifuged. Use the clear supernatant for testing.

Material Provided with the RPR Kit

1. Carbon antigen
2. Positive control, reactive with the reagent
3. Negative control, nonreactive with the reagent
4. Disposable slides with eight reaction circles
5. Disposable sample/control dispensing pipettes
6. Mixing sticks
7. Rubber teats
8. Reagent dropper for dispensing the carbon antigen.

Additional Material Required

Stop watch, high intensity light source, isotonic saline, pipettes, test tubes, mechanical rotor at 180 rpm circumscribing a circle 2 cm in diameter on a horizontal plane.

Note: For CARBOGEN Carbon Antigen: Item Nos. 2 to 7 listed above under RPR kit, would be required additionally.

Test Procedure

Bring reagent and samples to room temperature before testing.

Thoroughly mix the CARBOGEN reagent suspension by gentle agitation before testing.

Qualitative Method

1. Place one drop of the test specimen, positive and negative controls onto separate reaction circles of the disposable slide using a sample dispensing pipette.
2. Add one drop of well-mixed CARBOGEN reagent next to the test specimen, positive control and negative control by using the reagent dropper provided with the kit. Do not let the dropper tip touch the liquid on the slide.
3. Using a mixing stick, mix the test specimen and the CARBOGEN reagent thoroughly spreading uniformly over the entire reaction circle.
4. Immediately start a stopwatch. Rotate the slide gently and continuously either manually or on a mechanical rotor at 180 rpm.
5. Observe for flocculation macroscopically at 8 minutes.

Quantitative Method

1. Using isotonic saline, prepare serial dilutions of the test specimen positive in the qualitative method 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and so on.
2. Perform the qualitative test procedure using each dilution as a test specimen.
3. The titer is reported as the reciprocal of the highest dilution which shows a positive test result.

Interpretation of Test Results

Qualitative Method

1. Large and medium black floccules against white background : Reactive
2. Small black floccules against white background: Weakly reactive
3. No floccules, even grey background: Nonreactive.

Flocculation is a positive test result and indicates presence of antilipoidal antibodies in the test specimen.

No flocculation is a negative test result and indicates absence of antilipoidal antibodies in the test specimen.

Quantitative Method

The titre of antilipoidal antibodies is the highest dilution of the test specimen giving a positive test result.

Remarks

1. Quantitative procedure must be performed to determine response to treatment and detect reinfection.
2. False-positive reactions occur not infrequently and have been attributed to a variety of acute and chronic conditions.
3. In absence of supporting clinical, historical or epidemiological evidence, reactive result must be confirmed with more specific *Treponema* tests.
4. It is strongly recommended that results of the test should be correlated with clinical findings to arrive at the final diagnosis.
5. Dispose all used and contaminated material as per Standard Biohazard Safety Guidelines.
6. The reagent dropper provided for dispensing the carbon antigen should be thoroughly cleaned with distilled water and air dried after use, to ensure that it does not contaminate the reagent during subsequent use.
7. Very slight roughness should be interpreted as a negative test result.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Carbogen antigen contaminated with positive control/positive test sample	Care must be taken to ensure that the dropper tip does not touch the liquid on the slide while dispensing
2. Drying of the reagent on the slide.	Do not interpret test results beyond 8 minutes. Testing should not be carried out under the fan or under conditions where drying is enhanced
3. False positives may occur due to overspill from one back circle to another while rotating	Care must be taken to see that there is no overspill during rotation of the card
4. False positive reactions can also be attributed to a variety of acute and infectious mononucleosis, hepatitis, systemic lupus erythematosus and rheumatoid arthritis	Check the history of the patient. The test results must be correlated with clinical findings and all positive results must be further confirmed by using Treponemal tests

Problem: Negative control giving false positive reaction

Possible causes	*Solutions*
1. Negative control contaminated with positive control/positive sample	Validate the carbon antigen by using known negative (saline) and positive control. If proper results are obtained, with known saline and positive controls then the negative control is contaminated and should not be used

Problem: False negative results

Possible causes	*Solutions*
1. Excess serum dispensed (prozoning)	Pipette exactly 50 µL of serum or positive or negative control to black circle of the RPR card
2. Excess antigen dispensed (postzoning)	Dispense exactly one drop of Carbogen reagent using the reagent dropper provided with the kit
3. Reagent is contaminated because of unclean dropper and hence not suitable for subsequent use	The reagent dropper provided for dispensing the Carbon antigen should be thoroughly cleaned with distilled water and air dried after use, to ensure that it does not contaminate the reagent during subsequent use
4. The reagent may be exposed to elevated temperatures, air and direct sunlight, as it is highly sensitive to denaturation, drying and microbial contamination	Check the performance of the reagent with positive and negative controls provided with the kit. Avoid exposure of the reagent to light
5. Serum, plasma stored for a long period of time is used for testing	Fresh serum or plasma should be used for testing. Samples not tested immediately may be stored at 2–8°C for up to 48 hours
6. Hemolyzed or lipemic samples may have been used for testing	Avoid using hemolyzed or lipemic samples for testing
7. Antigenic suspension has settled down in the vial	Shake the Carbogen reagent vial well before use to disperse carbon particles uniformly and improve test readability

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8. Weak flocculation may be interpreted as negative	The carbon antigen should be gently but thoroughly mixed before testing to disperse the carbon particles uniformly and improve test readability. The serum and the reagent should be mixed properly. Small black floccules against a white background indicate a weakly reactive test result
9. Cold reagents are used for testing	Bring all reagents and samples to room temperature before commencing the testing procedure
10. Expired reagents are used for testing	Check the expiry date of the reagents before use
11. Error in interpreting the test results	Flocculation results should be interpreted carefully after comparing with positive and negative controls. Note: Very slight roughness should be interpreted as a negative result
12. The reagent used for testing is in a frozen condition	The reagent should not be frozen. It should be stored at 2–8°C
13. False negatives are obtained in the tertiary stage of the disease	The test should be confirmed with FTA (Fluorescent treponemal antibody) or TPHA Treponema pallidum hemagglutination) tests

ONE-STEP TEST FOR SYPHILIS: DIPSTICK SYPHICHECK®

(Courtesy: Tulip Group of Companies) (can also be in device form)

Introduction

Syphicheck is a one-step; rapid, self-performing, qualitative, two-site double antigen sandwich immunoassay for the detection of syphilis.

Summary

Syphilis is a sexually transmitted (venereal) disease caused by the spirochete *Treponema pallidum.* The disease can also be transmitted congenitally thereby attaining its importance in antenatal screening. After infection, the host forms non-*Treponema* antilipoidal antibodies (reagins) to the lipoidal material released from the damaged host cells as well as *Treponema* specific antibodies. Serological tests for non-*Treponema* antibodies such as VDRL, RPR, TRUST, etc. are useful as screening tests. Tests for *Treponema* specific antibodies such as TPHA, FTA-ABS, rapid *Treponema* antibody tests are gaining importance as screening as well as confirmatory tests because they detect the presence of antibodies specific to *Treponema pallidum.*

Syphicheck qualitatively detects the presence of IgM and IgG class of *Treponema* specific antibodies during syphilis in serum or plasma specimen within 15 minutes.

Principle

Syphicheck utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flows through the membrane assembly of the dipstick, the recombinant *Treponema* antigen-colloidal gold conjugate forms a complex with *Treponema* specific antibodies in the sample. This complex moves further on the membrane to the test region where it is immobilized by the recombinant *Treponema pallidum* antigen coated on the membrane leading to the formation of a pink to deep purple colored band at the test region which confirms a positive test result. Absence of this colored band in test region indicates a negative test result. The unreacted conjugate and the unbound complex if any move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies coated on the membrane at the control region, forming a pink to deep purple colored band. The control band serves to validate the test results.

Reagents and Materials Supplied

Each individual pouch contains:

1. **Dipstick:** Membrane assembly predispensed with recombinant *Treponema pallidum* antigen-colloidal gold conjugate, recombinant *Treponema pallidum* antigen and anti-rabbit antiserum coated at the respective regions.
2. **Desiccant pouch.**

Additional Material Required

12 × 75 mm test tubes.

Storage and Stability

The sealed pouches in the test kit may be stored between 4 and 30°C for the duration of shelf-life as indicated on the pouch.

Note

1. For in vitro diagnostic use only. Not for medicinal use.
2. Do not use beyond expiry date.
3. Read the instructions carefully before performing the test.
4. Handle all specimens as potentially infectious.
5. Follow standard biosafety guidelines for handling and disposal of potentially infective material.

Specimen Collection and Preparation

No special preparation of patient is necessary prior to specimen collection by approved techniques. Though fresh serum/plasma is preferable, serum/plasma specimens may be stored at 2 to 8°C for up to 24 hours, in case of delay in testing. Do not use hemolyzed or contaminated specimens. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Testing Procedure and Interpretation of Results

Bring kit components, specimen to room temperature prior to testing.

1. Collect serum/plasma in a clean test tube (approximately 0.5–1 mL may be required). Ensure that only sufficient quantity of the specimen is collected to allow submerging the red area on the dipstick (about 1 cm high).
2. Bring the sealed pouch to room temperature, open the pouch and remove the dipstick. Once opened, the dipstick must be used immediately.
3. Dip the dipstick in serum/plasma specimen submerging only the red area.
4. The dipstick should be left submerged for the entire duration of the test ensuring only the red area is submerged in the specimen.
5. At the end of 15 minutes read the results as follows:
   Negative: Only one pink to deep purple colored band appears on the dipstick (Fig. 22.25)
   Positive: Two distinct pink to deep purple colored bands appear on the dipstick
6. The test should be considered invalid if neither the test band nor the control band appears. Repeat the test with a new dipstick.

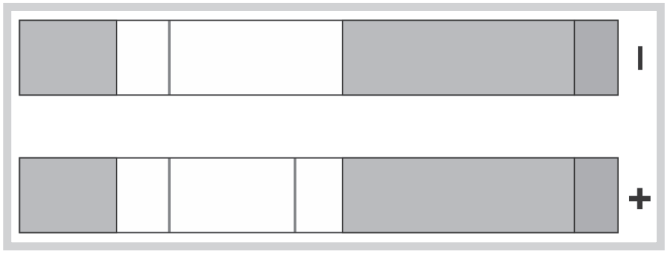


FIG. 22.25: Syphicheck reading

7. Although depending on the concentration of *Treponema* antibodies in the specimen, positive results may appear as early as 2 minutes, negative results must be confirmed only at the end of 15 minutes.

Performance Characteristics

1. Syphicheck detects the presence of *Treponema* antibodies; thus, a positive result indicates a past or present infection. Positive results should be evaluated in correlation with the clinical condition before arriving at a final diagnosis.
2. Low levels of antibodies to *Treponema pallidum* such as those present at a very early primary stage of infection can give a negative result. But a negative result does not exclude the possibility of exposure to or infection with *Treponema pallidum.* Retesting is indicated after two weeks if clinically syphilis is still suspected.
3. In order to assess the clinical response to treatment, it is advisable to use a reagin test such as VDRL, RPR.
4. Syphicheck detects *Treponema* antibodies in serum/plasma; other body fluids may not give accurate results.
5. In immunocompromised patients the test results must be interpreted with caution.

ONE-STEP TEST FOR SYPHILIS (DEVICE) SYPHICHECK®

(Courtesy: Tulip Group of Companies) (can also be in dipstick form)

Introduction

Syphicheck is a one-step; rapid, self-performing, qualitative, two-site double antigen sandwich immunoassay for the detection of syphilis.

Summary

Syphilis is a sexually transmitted (venereal) disease caused by the spirochete *Treponema pallidum.* The disease can

also be transmitted congenitally thereby attaining its importance in antenatal screening. After infection the host forms non-*Treponema* antilipoidal antibodies (reagins) to the lipoidal material released from the damaged host cells as well as *Treponema* specific antibodies. Serological tests for non-*Treponema* antibodies such as VDRL, RPR, TRUST, etc. are useful as screening tests. Tests for *Treponema* specific antibodies such as TPHA, FTA-ABS, rapid *Treponema* antibody tests are gaining importance as screening as well as confirmatory tests because they detect the presence of antibodies specific to *Treponema pallidum.*

Syphicheck qualitatively detects the presence of IgM and IgG class of *Treponema* specific antibodies during syphilis in serum or plasma specimen within 15 minutes.

Principle

Syphicheck utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flows through the membrane assembly of the test device, the recombinant *Treponema* antigen-colloidal gold conjugate forms a complex with *Treponema* specific antibodies in the sample. This complex moves further on the membrane to the test region where it is immobilized by the recombinant *Treponema pallidum* antigen coated on the membrane leading to the formation of a pink to deep purple colored band at the test region 'T' which confirms a positive test result. Absence of this colored band in test region 'T' indicates a negative test result. The unreacted conjugate and the unbound complex if any move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies coated on the control region 'C' of the membrane assembly, forming a pink to deep purple colored band. The control band serves to validate the test results.

Reagents and Materials Supplied

Each individual pouch contains:

1. Test device: Membrane assembly predispensed with recombinant *Treponema pallidum* antigen-colloidal gold conjugate, recombinant *Treponema pallidum* antigen and anti-rabbit antiserum coated at the respective regions.
2. Disposable plastic dropper.
3. Desiccant pouch.

Storage and Stability

The sealed pouches in the test kit may be stored between 4 and 30°C for the duration of shelf-life as indicated on the pouch.

Note

1. For in vitro diagnostic use only. Not for Medicinal use.
2. Do not use beyond expiry date.
3. Read the instructions carefully before performing the test.
4. Handle all specimens as potentially infectious.
5. Follow standard biosafety guidelines for handling and disposal of potentially infective material.

Specimen Collection and Preparation

No special preparation of patient is necessary prior to specimen collection by approved techniques. Though fresh serum/plasma is preferable, serum/plasma specimens may be stored at 2 to 8° C for up to 24 hours, in case of delay in testing. Do not use hemolyzed or contaminated specimens. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Testing Procedure and Interpretation of Results

Bring kit components, specimen to room temperature prior to testing.

1. Bring the sealed pouch to room temperature, open the pouch and remove the device. Once opened, the device must be used immediately.
2. Dispense two drops of serum/plasma specimen into the sample well 'S' using the dropper provided.
3. At the end of 15 minutes, read the results as follows:
   Negative: Appearance of only one pink to deep purple colored band at the control region 'C' (Fig. 22.26).
   Positive: In addition to the control band, a distinct pink to deep purple colored band also appears on the test region 'T' (Fig. 22.26).

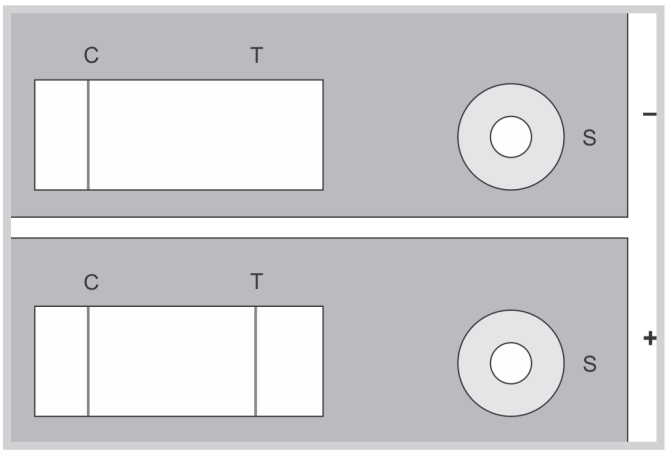


FIG. 22.26: Syphicheck reading

4. The test should be considered invalid if neither the test band nor the control band appear. Repeat the test with a new device.
5. Although, depending on the concentration of *Treponema* antibodies in the specimen, positive results may appear as early as 2 minutes, negative results must be confirmed only at the end of 15 minutes.

Remarks

1. Syphicheck detects the presence of *Treponema* antibodies; thus, a positive result indicates a past or present infection. Positive results should be evaluated in correlation with the clinical condition before arriving at a final diagnosis.
2. Low levels of antibodies to *Treponema pallidum* such as those present at a very early primary stage of infection can give a negative result. But a negative result does not exclude the possibility of exposure to or infection with *Treponema pallidum.* Retesting is indicated after two weeks if clinically syphilis is still suspected.
3. In order to assess the clinical response to treatment, it is advisable to use a reagin test such as VDRL, RPR.
4. Syphicheck detects *Treponema* antibodies in serum/plasma; other body fluids may not give accurate results.
5. In immunocompromised patients the test results must be interpreted with caution.

THIRD GENERATION DOUBLE ANTIGEN SANDWICH ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES TO TREPONEMA PALLIDUM IN HUMAN SERUM OR PLASMA TREPOLISA 3.0

(Courtesy: Tulip Group of Companies)

Trepolisa 3.0

Trepolisa 3.0 is intended to be used for the detection of total antibodies (i.e. IgG, IgM, IgA, etc.) to *Treponema pallidum* in human serum or plasma.

Summary and Explanation

Syphilis is a sexually transmitted (venereal) disease caused by the spirochete *Treponema pallidum*. The disease can also be transmitted congenitally thereby attaining its importance in antenatal screening. After infection the host forms non-treponemal anti-lipoidal antibodies (regains) to the lipodal material released from the damaged host cells as well as treponema specific antibodies. Serological tests for non-treponema antibodies such as VDRL, RPR, TRUST, etc. are useful as screening test. Test for treponema specific antibodies such as TPHA, FTA-ABS, rapid treponema antibody tests and ELISA are gaining importance as screening as well as confimatory tests because they detect the presence of antibodies specific to *Treponema pallidum.*

Principle of the Assay

Microwell strips are coated with recombinant 47 Kd and 17 Kd antigens. The same antigens are conjugated to HRP. Samples along with positive and negative controls are added in the coated wells and incubated simultaneously with antigen HRP conjugate. The wells are washed to remove unbound components. Captured antibodies are detected by adding substrate. The reaction is stopped after specified time with acid and absorbance is determined for each well at 450 nm with an ELISA reader. The cutoff value is calculated by the given formula and absorbances of all the wells are compared with the cutoff value. Any sample having absorbance more than the cutoff value is considered reactive.

TESTS FOR TYPHOID/ENTERIC FEVER WIDAL ANTIGEN SET/ANTIGENS FOR TUBE TESTS (TYPHOCHEK®)

(Courtesy: Tulip Group of Companies)

Summary

Enteric fever occurs when pathogenic microorganisms like *S. typhi, S. paratyphi A, S. paratyphi B* infect the human body. During the course of disease, the body responds to this antigenic stimulus by producing antibodies whose titer rises slowly in early stages, to a maxima and then slowly falls till it is undetectable. Antibodies to *Salmonella* organisms may be detected in the patient serum from the second week after onset of infection. Information regarding the titers and whether or not they are rising or falling can be obtained by performing serological tests using Typhochek widal antigen suspensions.

Reagent

Typhochek contains ready-to-use colored, smooth antigen suspensions of the bacilli; *S. typhi 'O', S. typhi 'H', S. paratyphi 'AO', S. paratyphi 'BO', S. paratyphi 'AH', S. paratyphi 'BH'.*

Typhochek reagents are versatile and standardized for use in a standard tube test procedure for the detection of *S. typhi* and *S. paratyphi* antibodies in the patient's serum.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity and performance.

Reagent Storage and Stability

1. Store the reagents at 2 to 8°C. Do not freeze.
2. The shelf-life of reagents is as per the expiry date mentioned on the reagent bottle labels.

Principle

When the colored, smooth suspension of attenuated Typhochek antigen suspensions are incubated with patient serum, anti-*Salmonella* antibodies present in the patient's serum react with the antigen suspensions to produce an agglutination. Agglutination is a positive test result, indicating presence of *Salmonella* antibodies in the patient's serum. No agglutination is a negative test result indicating absence of *Salmonella* antibodies in the patient's serum.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The *S. typhi 'O'* reagent contains phenol 0.5%, *S. typhi 'H', S. paratyphi 'AH', S. paratyphi 'BH'* reagents contain formaldehyde 0.3% and *S. paratyphi 'AO', S. paratyphi 'BO'* reagents contain ethanol 0.7% as preservatives. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.

Sample Collection and Storage

1. No special preparation of the patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples.
2. Clean and dry glassware free from detergents must be used for sample collection.
3. Do not heat/inactivate the serum.
4. Though freshly collected serum is preferable, store samples at 2 to 8°C in case of delay in testing, for up to 72 hours.

Material Provided with the Kit

Reagent Pack

Typhochek 4 × 50 mL set contains item Nos. 1, 2, 5 and 6 mentioned below.

1. Typhochek *S. typhi 'O'* Antigen suspension
2. Typhochek *S. typhi 'H'* Antigen suspension
3. Typhochek *S. paratyphi 'AO'* Antigen suspension
4. Typhochek *S. paratyphi 'BO'* Antigen suspension
5. Typhochek *S. paratyphi 'AH'* Antigen suspension
6. Typhochek *S. paratyphi 'BH'* Antigen suspension.

Note: Item Nos. 1 to 6 each is available as individual reagent packs.

Additional Material Required

Timer, Kahn tubes/test tubes, pipettes (0.1 mL, 1 mL), isotonic saline, incubator (37°C), test tube rack.

Procedure

a. Bring reagents to room temperature before testing.
b. Shake and mix antigens well before dispensing.
c. Carefully label test tubes for sample and reagent identity when more than one antigens is used during test procedure.

a. Standard Tube Test Method

1. Take appropriate number of sets (as required; one set for each antigen suspension) of 8 Kahn tubes/test tubes and label them 1 to 8.
2. Pipette into tube No. 1 of all sets 0.9 mL of isotonic saline.
3. To each of the remaining tubes (2 to 8 of each set), add 0.5 mL of isotonic saline.
4. To tube No. 1 of all sets, add 0.1 mL of serum sample to be tested and mix well.
5. Transfer 0.5 mL of the diluted serum sample from tube No. 1 to tube No. 2 and mix well.
6. Transfer 0.5 mL of the diluted serum sample from tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 7 in each set.
7. Discard 0.5 ml of the diluted serum from tube No. 7 of each set.
8. Tube No. 8 in all the sets serves as a saline control.
9. To all the tubes of the respective sets, add 0.5 mL of the respective Typhochek antigen suspensions and mix well.
10. This will give final dilutions in tube 1 to 7 as 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280.
11. Cover and incubate at 37°C overnight (approximately 18 hours).
12. Dislodge the sedimented button gently and observe for agglutination macroscopically.

Interpretation of Results

The titer of the patient serum using Typhochek antigen suspensions is the highest dilution of the serum sample that gives a visible agglutination.

Remarks

1. TAB vaccinated patients may show a high titer of antibodies to each of the antigens.
2. 'O' being a somatic antigen brings about a coarse, compact, granular agglutination, whereas 'H' being a flagellar antigen brings about larger, loose, flocculant agglutination.
3. Apart from the pattern of sedimented antigens, in the tube test method a decrease in opacity as compared to the saline control must also be considered while judging the degree of agglutination.
4. While the 'O' antigen is species specific, the 'H' antigen is specific to the serotype.
5. Turbid and contaminated sera should not be used for testing.
6. Generally, antibody titers of 180 or more are considered clinically and diagnostically significant. However, the significant titer may vary from population to population and needs to be established for each area.
7. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
8. Since techniques and standardization vary from lab to lab one tube difference in tube titers can be expected.
9. Do not interchange reagent caps.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Past history of immunization, inapparent infection or prior disease	Positive reaction will be seen in all three 'H' antigens in such cases whereas in case of infection, antibodies will be seen only against the infecting species. Also the patient's history
2. Anamnestic response to other vaccines and unrelated fevers in the case of persons who have had a prior infection or immunization	The anamnestic fever can be differentiated from enteric fever by repetition of the test after a week. The anamnestic response shows only a transient rise, while in enteric fever the rise is sustained
3. If reagents have been exposed to excessively high temperatures, precipitates could be formed	Ensure that the reagents are not exposed to high temperatures and are stored properly at 2–8°C
4. Contamination of serum could lead to false positives	Ensure that clean and dry glassware free from detergents are used for sample collection. If samples are not tested immediately, store them at 2–8°C. Turbid serum should not be used for testing

Problem: False negative results

Possible causes	*Solutions*
1. Infection is in very early stages, when antibody titer is very low	The agglutinin titer depends on the stage of the disease. Agglutinins usually appear by the end of the 1st week, so that the blood taken earlier may give a negative test result. The testing should be repeated after a week in such cases. Demonstration of a rise in titer of antibodies by testing two or more serum samples is more meaningful than a single test
2. Patients on antibiotic therapy during the testing phase	Check the history of the patient for administration of the antibiotics.
3. Insufficient quantity of serum is used for testing hence leading to postzone effect	Pipette one drop of patient serum on the four reaction circles, in case of slide test. In case of tube test, carry out the dilutions carefully and correctly as per the instructions given in the pack insert
4. Hemolyzed samples may be have been used	Avoid using hemolyzed samples for testing
5. Reagents not brought to room temperature. Cold reagents could give false negative results	All reagents must be brought to room temperature prior to commencing the testing procedure
6. The tube is shaken very vigorously while observing for agglutination	Shake the tube gently after incubation and observe for agglutination. 'O' antigen will show coarse, compact, granular agglutination whereas 'H' antigen will show large, loose, flocculant agglutination
7. Insufficient reagent present in the vial	Ensure sufficient reagent is present in the vial before retrieving

WIDAL ANTIGEN SET/ANTIGENS FOR SLIDE AND TUBE TESTS (TYDAL)®

(*Courtesy:* Tulip Group of Companies)

Reagent

The TYDAL contains ready-to-use concentrated, vitally stained, smooth antigen suspensions of the bacilli; *S typhi 'O', S typhi 'H', S paratyphi 'AO', S. paratyphi 'BO', S paratyphi 'AH', S paratyphi 'BH'* and/or polyspecific positive control reactive with these antigens. Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity and performance.

Reagent Storage and Stability

1. Store the reagents at 2 to 8°C. Do not freeze.
2. The shelf-life of reagents is as per the expiry date mentioned on the reagent vial labels.

Principle

When the colored, smooth, attenuated TYDAL antigen suspensions are mixed/incubated with patient serum, anti-*Salmonella* antibodies present in the patient serum react with the antigen suspensions to give agglutination. Agglutination is a positive test result, indicating presence of anti-*Salmonella* antibodies in the patient serum. No agglutination is a negative test result indicating absence of anti-*Salmonella* antibodies.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The *S typhi 'O'* reagent contains phenol 0.5%, *S. typhi 'H', S. paratyphi 'AH', S. paratyphi 'BH'* reagents contain formal dehyde 0.3% and *S paratyphi 'AO', S paratyphi 'BO'* reagents contain ethanol 0.7% as preservatives. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
3. Only a clean and dry glass slide must be used. Clean the glass slide with distilled water and wipe dry.
4. Accessories provided with the kit only must be used for optimum results, (applicable only for TYDAL 4 × 5 mL set and 4 × 10 mL set).

Sample Collection and Storage

1. No special preparation of the patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples.
2. Clean and dry glassware free from detergents must be used for sample collection.
3. Do not heat inactivate the serum.
4. Though freshly collected serum is preferable, store samples at 2 to 8°C in case of delay in testing, for upto 72 hours.

Material Provided with the Kit

Reagent Pack

The TYDAL 4 × 5 mL set and TYDAL 4 × 10 mL set contain item Nos 1, 2, 5, 6, 7 and 8 mentioned below. TYDAL 2 × 5 mL set contains item Nos. 1, 2 and 7 mentioned below:

1. *S. typhi 'O'* Antigen suspension
2. *S. typhi 'H'* Antigen suspension
3. *S. paratyphi 'AO'* Antigen suspension
4. *S. paratyphi 'BO'* Antigen suspension
5. *S. paratyphi 'AH'* Antigen suspension
6. *S. paratyphi 'BH'* Antigen suspension
7. Polyspecific positive control (Goat)
8. Glass slide with six reaction circles, mixing sticks, disposable sample dispensing pipettes with rubber teats.

Note: Item Nos. 1 to 6 each is available as individual reagent packs.

Additional Material Required

Slide test method: Stop watch, variable micropipettes.
Note: Item No. 8 from reagent pack is additionally required for TYDAL 2 × 5 mL set. Item No. 7 and 8 from reagent pack are additionally required for individual reagent packs of TYDAL 'O', TYDAL 'H', TYDAL 'AO', TYDAL 'BO', TYDAL 'AH', TYDAL 'BH' antigens.

Quantitative Method

Timer, Kahn tubes/test tubes, pipettes (0.1 mL, 1 mL), isotonic saline, incubator (37°C), test tube racks.

Procedure

a. Bring reagents to room temperature before testing.
b. Shake and mix antigens well before dispensing.

Slide Screen Method

1. Place one drop of positive control onto a reaction circle of the glass slide.
2. Place one drop of isotonic saline onto the next reaction circle of the glass slide.
3. Place one drop of patient serum to be tested onto each of the required number of reaction circles.
4. Add one drop of appropriate TYDAL antigen suspensions to the reaction circles containing positive control and isotonic saline.

5. Add one drop of appropriate TYDAL antigen suspensions to the reaction circles containing the patient serum.
6. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
7. Rock the slide gently back and forth, and observe for agglutination macroscopically at one minute.

Slide Semiquantitative Method

1. Using a pipette, place 80 µL, 40 µL, 20 µL, 10 µL, and 5 µl of patient serum to be tested on 5 different reaction circles on the glass slide. The corresponding titers obtained will be 1:20, 1:40, 1:80. 1:160, and 1:320 respectively.
2. Follow step No. 5 to 7 of slide screen method.

Note: This method is recommended for obtaining quick approximate titres only.

Quantitative Method

Tube Test Procedure

1. Take appropriate number of sets (as required; one set for each antigen suspension) of 8 Kahn tubes/test tubes and label them 1 to 8.
2. Pipette into tube No. 1 of all sets 1.9 mL of isotonic saline.
3. To each of the remaining tubes (2 to 8), add 1 mL of isotonic saline.
4. To tube No. 1 of all sets add 0.1 mL of serum sample to be tested and mix well.
5. Transfer 1 mL of the diluted serum sample from tube No. 1 to tube No. 2 and mix well.
6. Transfer 1 mL of the diluted serum sample from tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 7 in each set.
7. Discard 1.0 mL of the diluted serum from tube No. 7 of each set.
8. Now the dilutions of the serum sample achieved from tube No. 1 to 7 respectively in each set is as follows 1:20, 1:40, 1:80. 1:160, 1:320, 1:640, 1:1280. Tube No. 8 in all the sets serves as a saline control.
9. To all the tubes (1 to 8) of each set, add one drop of the respective well mixed TYDAL antigen suspensions from the reagent vials and mix well.
10. Cover and incubate at 37°C overnight (approximately 18 hours).
11. Dislodge the sedimented button gently and observe for agglutination.

Interpretation of Results

Slide Screen Method

Agglutination is a positive test result and indicates presence of the corresponding antibody in the patient serum.

No agglutination is a negative test result and indicates absence of the corresponding antibody in the patient serum.

Slide Semiquantitative Method

Agglutination is a positive test result. The titer of the patient serum corresponds to the visible agglutination in the test circle with the smallest amount of serum sample.

Quantitative Method

The titer of the patient serum using TYDAL antigen suspensions is the highest dilution of the serum sample that gives a visible agglutination.

Remarks

1. Positive results obtained in the slide test should be confirmed with the tube test to establish whether the titers are diagnostically significant or not.
2. TAB vaccinated patients may show a high titer of antibodies to each of the antigens.
3. 'O' being a somatic antigen brings about a coarse, compact, granular agglutination whereas 'H' being a flagellar antigen brings about larger, loose, flocculant agglutination.
4. While the 'O' antigen is species specific, the 'H' antigen is specific to the serotype.
5. Turbid and contaminated sera should not be used for testing.
6. Generally antibody titers of 1:80 or more are considered clinically and diagnostically significant. However, the significant titer may vary from population to population and needs to be established for each area.
7. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
8. Since techniques and standardization vary from lab to lab one tube difference in tube titers can be expected.
9. The performance of the reagents should be validated occasionally using know positive control. Good physiological saline may be used as a negative control.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Past history of immunization inapparent infection or prior disease	Positive reaction will be seen in three flagellar antigens: H, AH, BH in such cases whereas in case of infection, antibodies will be seen only against the infecting species. Also check the patient's history
2. Anamnestic response to other vaccines and unrelated fevers in the case of persons who have had a prior infection or immunization	The anamnestic fever can be differentiated from enteric fever by repetition of the test after a week. The anamnestic response shows only a transient rise, while enteric fever the rise is sustained
3. Prolonged rocking of the slide causes drying of the test material	Agglutination should be observed within one minute.
4. If reagents have been exposed to excessively high temperature, precipitates could be formed	Ensure that the reagents are not exposed to high temperatures and are stored properly at 2–8°C
5. Contamination of serum could lead to false positives	Ensure that clean and dry glassware free from detergents are used for sample collection. If serum samples are not test immediately, store at 2–8°C. Turbid serum should not be used for testing
6. Error in interpreting results. Granularity mistaken for clumping	The results should be interpreted properly besides by comparing with the polyspecific positive control provided with the kit

Problem: False negative results

Possible causes	*Solutions*
1. Infection is in very early stages, when antibody titer is very low	The agglutinin titer depends on the stage of the disease. Agglutinins usually appear by the end of the 1st week, so that the blood taken earlier may give a negative test result. The testing should be repeated after a week in such cases Demonstration of a rise in titer of antibodies by testing two or more serum samples 4–6 days apart is more meaningful than single test
2. Patients on antibiotic therapy during the testing phase	Check the history of the patient for administration of antibiotics
3. Insufficient serum dispensed leading to postzone effect	Pipette exactly one drop of patient serum on each of the four reaction circles, in case of slide test In case of tube test carry out the dilutions carefully and correctly as per the instructions given in the package insert
4. Hemolyzed, turbid or contaminated samples may have been used	Avoid using hemolyzed, turbid or contaminated samples for testing
5. Serum stored for a long time is used for testing	Fresh serum should be used for testing. However, in case of delay in testing, the sample can be stored up to a maximum of 72 hours
6. Rotation of the slide too fast may break up agglutinating clumps, which can lead to false negative in borderline cases	Rock the slide gently back and forth and observe for agglutination macroscopically within one minute
7. Reagents not brought to room temperature. Cold reagents could give false negative results	Bring all reagents and samples to room temperature before commencing the testing procedure
8. Insufficient reagent present in the vial	Ensure that sufficient reagent is present in the vial before retrieving the amount required for testing
9. Expired reagents are used for testing	The performance of the reagents should be validated occasionally using the positive control provided. Good physiological saline may be used as a negative control

REDUCED WIDAL ANTIGEN SET: O AND H FOR TUBE TESTS (VITAL WIDAL®)

(Courtesy: Tulip Group of Companies)

Reagent

Vital Widal contains ready-to-use colored, smooth antigen suspensions of the bacilli; *S. typhi O* and *S. typhi H* along with a polyspecific positive control reactive with these antigens. Vital Widal reagents are versatile and standardized for use in a modified tube test procedure for the detection of *S. typhi* antibodies in the patient's serum.

Each batch of regents undergoes rigorous quality control at various stages of manufacture for its specificity and performance.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of the reagent is as per the expiry date mentioned on the reagent bottle labels.

Principle

When the colored, smooth suspension of attenuated Vital Widal antigen suspensions are incubated with the patient's serum, anti-*Salmonella* antibodies if present in the patient's serum react with the antigen suspension to produce an agglutination. Agglutination is a positive test result, indicating presence of *Salmonella* antibodies in the patient's sample. No agglutination is a negative test result indicating absence of *Salmonella* antibodies in the patient's sample.

Note

In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.

The reagent contains 0.5% Phenol/0.3% Formaldehyde as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.

Sample Collection and Storage

1. No special preparation of the patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples.
2. Clean and dry glassware free from detergents must be used for sample collection.
3. Do not heat/inactivate the serum.
4. Though freshly collected serum is preferable, store samples at 2 to 8°C in case of delay in testing.

Material Provided with the Kit

- Antigen suspension, *S. typhi 'O'*
- Antigen suspension, *S. typhi 'H'*
- Polyspecific positive control (goat)
- Color coded vial top squeeze droppers.

Additional Material Required

1. Test tubes/Kahn tubes (preferably)
2. Pipettes 0.1 mL, 1.0 mL
3. lncubator (37°C)
4. Pasteur pipettes
5. Isotonic saline.

Procedure

a. Tear off aluminium seals from the antigen vials. Fit on to each antigen vial, vial top squeeze dropper.
b. Bring all reagents to room temperature before testing.
c. Shake antigens well before dispensing.
d. Carefully label test tubes for sample and reagent identity.
e. Ensure squeeze dropper tips are wiped dry with clean tissues, before recapping.

Tube Test Method

1. Take two sets of 8 Kahn tubes and test tubes and label them as 1 to 8 for O and H antibody detection.
2. Pipette into tube No. 1 of all sets 1.9 mL of isotonic saline.
3. To each of the remaining tubes (2 to 8 each set), add 1.0 mL of isotonic saline.
4. To the tube No. 1 of all sets, add 0.1 mL of serum sample to be tested and mix well.
5. Transfer 1.0 mL of the diluted serum from the tube No. 1 to tube No. 2 and mix well.
6. Transfer 1.0 mL of the diluted serum from the tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 7.
7. Discard 1.0 mL of the diluted serum from tube No. 7.
8. This will give final dilutions in tubes 1 to 7 as 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280.
9. Tube No. 8 serves as saline control.
10. To all the tubes (1 to 8) of each set add one drop of well mixed Vital Widal suspension of the respective specificity. Mix well.
11. Incubate at 37°C overnight (approximately 18 ± 2 hours).
12. Dislodge the sedimented button gently and observe for agglutination macroscopically.

Interpretation of Results

The titer of the patient serum using Vital Widal is the highest dilution of serum that gives a visible agglutination.

Generally antibodies having titers of 1:80 or more are considered diagnostically significant.

Apart from the pattern of sedimented antigens, in the tube test method a decrease in opacity as compared to the saline control must also be considered while judging the degree of agglutination.

Remarks

1. Serum from individuals vaccinated with TAB may also show moderately elevated titers of 'H' agglutinin.
2. 'O' being a somatic antigen, brings about coarse, compact and granular agglutination, whereas 'H' being a flagellar antigen, brings about larger, loose, flocculant agglutination.
3. 'H' antigen, being species specific, is more reliable in determining the type of infection.
4. Turbid and contaminated serum should not be used for testing.
5. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
6. Do not interchange vial top squeeze dropper cap.

POSITIVE CONTROL FOR WIDAL TEST

Summary

Enteric fever occurs when pathogenic microorganisms like *S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C* infect the human body. During the course of disease, the body responds to this antigenic stimulus by producing antibodies. Antibodies to *Salmonella* organisms may be detected in the patient serum from the second week after onset of infection. Information regarding the titers and whether or not they are rising or falling can be obtained by performing serological tests using Widal antigen suspensions. The performance of the Widal antigen suspensions can be validated with the help of positive control for Widal test.

Reagent

The Widal Positive Control contains ready-to-use standardized goat antiserum with polyspecific antibodies having specific reactivity towards *S. typhi 'O' and 'H'* antigens, S, *paratyphi 'AH'* and *'BH'* antigens, S *paratyphi 'AO'* and *'BO'* antigens and *S. paratyphi 'CO'* and *'CH'* antigens and is useful in the validation of the performance of Widal reagents.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of the reagent is as per the expiry date mentioned on the reagent bottle label.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Principle

The positive control is mixed with the Widal antigen suspensions to be tested and allowed to react. Specific reactivity of *Salmonella* antigens if present in the antigen suspensions will produce an agglutination reaction. No agglutination indicates the deterioration of the performance of the antigen suspensions.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.

Additional Material Required

Stopwatch, isotonic saline, glass slide with clear/white background, appropriate pipettes/micropipettes, mixing sticks and a high intensity direct light source.

Procedure

a. Bring reagent to room temperature before testing.
b. Shake and mix the positive control for Widal test well before dispensing.

Slide Test Method

1. Place one drop of positive control onto the reaction circle of the glass slide.
2. Place 50 µL of saline onto the next reaction circle of the glass slide.
3. Add one drop of test reagent *(Salmonella* antigen suspensions) in each of the above circles.
4. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
5. Gently rock the slide back and forth, observe for agglutination macroscopically at one minute against a white background.

Interpretation of Results Slide Test Method

Agglutination is a positive test result and indicates that the test reagent *(Salmonella* antigen suspension) are performing satisfactorily.

No agglutination is a negative test result and indicates the deterioration of the test reagent *(Salmonella* antigen suspension).

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Past history of immunization, inapparent infection or prior disease	Positive reaction will be seen in all three 'H' antigens in such cases whereas in case of infection, antibodies will be seen only against the infecting species. Also check the patient's history
2. Anamnestic response to other vaccines and unrelated fevers in the case of persons who have had a prior infection or immunization	The anamnestic fever can be differentiated from enteric fever by repetition of the test after a week
3. If reagents have been exposed to excessively high temperatures, precipitates could be formed	The anamnestic response shows only a transient rise, while in enteric fever the rise is sustained Ensure that the reagents are not exposed to high temperatures and are stored properly at 2–8°C
4. Contamination of serum could lead to false positives	Ensure that clean and dry glassware free from detergents are used for sample collection. If samples are not tested immediately, store them at 2–8°C. Turbid serum should not be used for testing

Problem: False negative results

Possible causes	*Solutions*
1. Infection is in very early stages, when antibody titre is very low	In enteric fever specific agglutinins are detectable in the patient's blood only after the first week hence blood taken earlier for testing may give a negative result. Testing should be repeated after a week in such cases. Demonstration of a rise in titer of antibodies by testing two or more serum samples is more meaningful than a single test
2. Patients on antibiotic therapy during the testing phase	Check the history of the patient for administration of the antibiotics
3. Insufficient serum could give postzone effect	Perform the dilutions as mentioned in the pack insert
4. Hemolyzed samples may be have been used	Do not use hemolyzed samples for testing
5. Reagents not brought to room temperature. Cold reagents could give false negative results	All reagents must be brought to room temperature before use
6. The tube is shaken very vigorously while observing for agglutination	Shake the tube gently after incubation and observe for agglutination. 'O' antigen will show coarse, compact, granular agglutination whereas 'H' antigen will show large, loose, flocculant agglutination
7. Insufficient reagent present in the vial	Ensure sufficient reagent is present in the vial before retrieving

RAPID TEST FOR DETECTION IGM ANTIBODIES TO S. TYPHI IN SERUM/PLASMA/WHOLE BLOOD (DEVICE) ENTEROCHECK – WB®

(Courtesy: Tulip Group of Companies)

Enterocheck-WB

Enterocheck-WB is a rapid, qualitative, sandwich immunoassay for the detection of IgM antibodies to *S.Typhi* in human serum/plasma or whole blood specimen.

Summary

A febrile condition, Typhoid fever, is a bacterial infection caused by *Salmonella* serotypes including *S. Typhi, S. paratyphi A, S. paratyphi B* and *Salmonella sendai*. The symptoms of the illness include high fever, headache, abdominal pain, constipation and appearance of skin rashes. Accurate diagnosis of typhoid fever at an early stage is not only important for etiological diagnosis but to identify and treat the potential carriers and prevent acute typhoid fever

outbreaks. The conventional WIDAL Test usually detects antibodies to *S. typhi* in the patient serum from the second week of onset of symptoms. However, the detection may be earlier if specific IgM antibodies are detected instead of IgG or both IgG and IgM. **Enterocheck-WB** qualitatively detects the presence of IgM class of antibodies to Lypopolysaccharide (LPS) specific to *S. typhi* in human serum/plasma or whole blood specimens.

Principle

Enterocheck-WB utilizes the principle of Immunochromatography, a unique two-site immunoassay on a nitrocellulose membrane. The conjugate padcontains two components – Anti-human IgM antibody conjugated to colloidal gold and rabbit IgG conjugated to colloidal gold. As the test specimen flows through the membrane test assembly, the highly specific anti-human IgM antibody-colloidal gold conjugate complexes with the *S. typhi* specific IgM antibodies in the specimen and travels on the membrane due to capillary action alongwith the rabit IgG-colloidal gold conjugate. This complex moves further on the membrane to the test region (T) where it is immobilized by the *S. typhi* specific LPS antigen coated on the membrane leading to formation of a pink to deep purple colored band. The absence of this colored band in the test region indicates a negative test result.

The unreacted conjugate and unbound complex, if any, move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies coated on the membrane at the control region (C), forming a pink to deep purple colored band. This control band acts as a procedural control and serves to validate the results.

Reagents and Materials Supplied

Each kit contains.

A. Individual pouches, each containing a -

1. Membrane test assembly: Membrane assembly pre-dispensed with anti Human IgM–colloidal gold conjugate, *S. typhi* LPS antigen and anti-rabbit antiserum coated at the respective regions.
2. Desiccant pouch

B. Sample Running Buffer

C. Package Insert.

Optional Material Required

5 µL precision pipette.

Storage and Stability

The sealed pouches in the test kit and the kit components may be stored between 4–30°C for the duration of the shelf life as indicated on the pouch.

Note

1. For in vitro diagnostic use only. Not for medicinal use.
2. Do not use beyond expiry date.
3. Read the instructions carefully before performing the test.
4. Handle all specimen as if potentially infectious.
5. Follow standard biosafety guidelines for handling and disposal potentially infectious material.
6. If desiccant color at the point of opening the pouch has turned from blue to pink or colorless, another test device must be run.

Specimen Collection and Preparation

1. **Enterocheck-WB** uses human serum/plasma/whole blood as specimen.
2. No special preparation of the patient is necessary prior to specimen collection by approved techniques.
3. For whole blood, collect blood with a suitable anticoagulant such as EDTA or Heparin or Oxalate and use the freshly collected blood.
4. Whole blood should be used immediately and should not be frozen.
5. Though fresh specimen is preferable, incase of delay in testing, it may be stored at 2–8°C for maximum up to 24 hours.
6. If serum is to be used as specimen, allow blood to clot completely. Centrifuge to obtain clear serum.
7. Repeated freezing and thawing of the specimen should be avoided.
8. Do not use turbid, lipemic and hemolyzed serum/plasma.
9. Do not use hemolyzed, clotted, contaminated, viscous/turbid specimens.
10. Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only used for testing.
11. Refrigerated specimens must be brought to room temperature prior to testing.

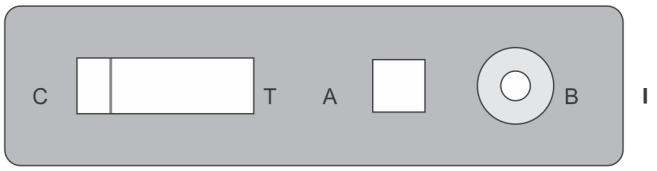
Testing Procedure and Interpretation of Results

1. Bring the kit components of Enterocheck-WB device to room temperature before testing.
2. Open a foil pouch by tearing along the “notch”.

3. Remove the testing device and the specimen dropper. Once opened, device must be used immediately.
4. Label the device with specimen identity.
5. Place the testing device on a flat horizontal surface.
6. Carefully dispense 5 µL of whole blood/serum /plasma into the specimen port "A" using a micropipette or the sample loop provided. Dip the sample loop in the sample container and blot the sample in the sample port "A".
7. After 30 sec, add five drops of sample running buffer into the port "B".
8. Observe the development of visible colored band at Test window (T).
9. Positive results may be observed within 15 minutes, depending on the concentration of IgM antibodies in the tested specimen.
10. The test should be considered invalid if the control band does not appear. Repeat test with a new **Enterocheck-WB** device.

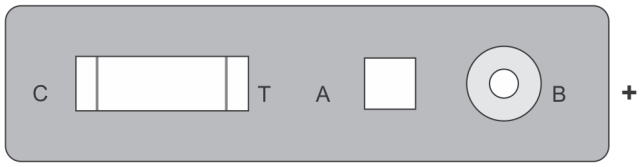
Negative

If IgM antibodies to *S. typhi* are not present, only one colored band at Control (C) would appear.



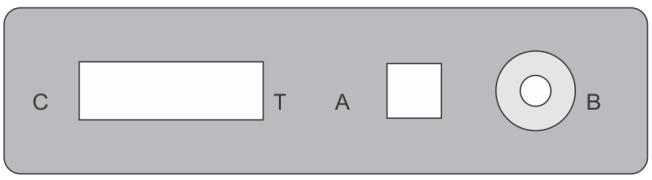
Positive

If IgM antibodies to *S. typhi* are not present, two colored bands appear at Test (T) and Control (C) regions. The intensity of the test band may be more or less than the control band, depending upon the concentration of IgM antibodies in specimen.



Invalid

The test is invalid if the Control band is not visible at 15 minutes. Verify the test procedure and repeat the test with a new Enterocheck-WB device.



11. The test should be considered invalid if neither the control band 'C' nor the test band 'T' appears. Repeat the test with a new device.

Remarks

1. In the studies it has been reported that IgM antibodies to *S. typhi* persist for about 4 months post infection. Therefore, results within four months from an endemic area should be interpreted with caution.
2. The following chart would explain the IgM seroresponse in *S. typhi* infected subjects after onset of fever.

Detectable IgM Response

Onset of fever	*Percent positive*
4–6 days	43.50%
6–9 days	92.90%
> 9 days	100%

3. A negative result, i.e. the absence of detectable IgM antibody does not rule out recent or current infection. However, if *S.typhi* infection is still suspected, obtain a second specimen 5–7 days later and repeat the testing.
4. Specific IgG may compete with the IgM for sites and may result in a false negative. Conversely, rheumatoid factor in the presence of specific IgG may result in a false positive reaction.
5. The membrane is laminated with an adhesive tape to prevent surface evaporation. Air pockets or patches may appear, which do not interfere with the test results. Presence of a band at the test region even if low in intensity or formation is a positive result.
6. The deliberate slow reaction kinetics of Enterocheck-WB is designed to maximize and enhance reaction time between sample capture and tracer elements to improve test sensitivity.
7. Most positive results develop within 15 minutes. However, certain sera sample may take a longer time to flow. Therefore, negatives should be confirmed only at 30 minutes. Do not read results after 30 minutes.
8. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the result of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.
9. Enterocheck-WB should be used as a screening test in clinically suspected cases only, and its results should be confirmed by other supplemental method before taking clinical decisions.

SLIDE AND TUBE TEST FOR DETECTION OF ANTIBODIES TO BRUCELLA ABORTUS/ MELITENSIS BRUCEL A/M®

(Courtesy: Tulip Group of Companies)

Summary

Human brucellosis (diurnal, or undulant fever) is a common febrile illness caused by infection with bacteria of some of the *Brucella* species (*abortus, melitensis)*. This undulant fever is associated with symptoms, which are often variable and nonspecific with chills, fever, sweats and anorexia. On exposure, the body responds to this antigenic stimulation by producing specific antibodies whose titers rise slowly at early stages and then increases. Specific antibodies to the *Brucella* species are detectable a few weeks after exposure and are of considerable importance in the diagnosis of brucellosis. Information regarding the titer of antibodies can be obtained by using specific Tulip Brucel antigen suspensions.

Reagent

The Brucel-A/Brucel-M reagents contain ready-to-use standardized, attenuated, stained, smooth specific antigen suspensions of *Brucella* having specific reactivity towards antibodies to *Brucella abortus* (Brucel-A), and *Brucella melitensis* (Brucel-M).

Reagent Storage and Stability

Store the reagent at 2 to 8°C. Do not freeze.

The shelf-life of the reagents is as per the expiry date mentioned on the reagent vial labels.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity, and performance.

Principle

The smooth, attenuated, stained Brucella antigen suspensions are mixed with the patient's serum. Specific antibodies to *Brucella* antigens if present in the patient serum will react with the antigen suspension to produce an agglutination reaction. No agglutination indicates the absence of specific antibodies to *Brucella* antigens.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains thimerosal 0.01% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.

Sample Collection and Storage

1. No special preparation of patient is required prior to sample collection by approved techniques. Do not use hemolyzed serum samples.
2. Clean and dry glassware free from detergents must be used for sample collection.
3. Do not heat/inactivate the serum.
4. Though freshly collected serum is preferred, samples can be stored at 2 to 8°C, for 24 hours or frozen for 8 days should a delay in testing occur.

Materials Provided with the Kit

Stained Brucel-A/Brucel-M antigen suspensions.

Additional Material Required

Slide Test Method

Stop watch, positive control, isotonic saline, and glass slide with clear/white background, appropriate pipettes/ micropipettes, mixing sticks and a high intensity direct light source.

Quantitative Method

Timer, test tubes (12 mm × 75 mm), test tube rack, appropriate pipettes/micropipettes, isotonic saline/0.25% phenol saline, incubator (37°C).

Procedure

Bring all reagents to room temperature.

Shake and mix the *Bruce* antigen suspensions well before dispensing.

The procedure for Brucel-A and Brucel-M is identical.

Slide Test Method

Qualitative Method

1. Place one drop of positive control onto the reaction circle of glass slide.
2. Place 80 µL of saline onto the next reaction circle of the glass slide.
3. Place 80 µL of patient serum to be tested onto the next reaction circle.
4. Add one drop of the appropriate Brucel antigen suspensions in each of the above circles (containing positive control, saline, and the patient serum to be tested).
5. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
6. Gently rock the slide back and forth, observe for agglutination macroscopically, at one minute against white background.

Semiquantitative Method

1. Using a pipette place 80 µL, 40 µL, 20 µL, 10 µL, and 5 µL of patient serum to be tested on 5 different circles on the glass slide. The corresponding titers obtained will be 1:20, 1:40, 1:80, 1:160, and 1:320 respectively.
2. Place one drop of appropriate Brucel antigen suspension to each circle.
3. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
4. Gently rock the slide back and forth, observe for agglutination macroscopically at one minute against a white background.

Tube Test Method

1. Take 8 test tubes and label them 1 to 8.
2. Pipette 1.9 mL of isotonic saline or preferably 0.25% phenol saline to tube No. 1.
3. To each of the remaining tubes (2–7), add 1.0 mL of isotonic saline or preferably 0.25% phenol saline.
4. To the tube No. 1, add 0.1 mL of serum sample to be tested. Mix well.
5. Transfer 1.0 mL of the diluted serum from tube No. 1 to tube No. 2 and mix well.
6. Transfer 1.0 mL of the diluted serum from tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 7.
7. Discard 1.0 mL of the diluted serum from tube No. 7.
8. Pipette 1.0 mL of isotonic saline in tube No. 8, which serves as a negative control.
9. To all the tubes add 1 drop of appropriate Brucel antigen suspensions and mix well.
10. Cover the tubes and incubate at 37°C for 24 hours.
11. Observe for agglutination macroscopically in each tube of the dilution series.

Interpretation of Results

Slide Test Method

Qualitative Method

Agglutination is a positive test result and indicates the presence of specific antibodies to *Brucella* in the patient serum.

No agglutination is a negative test result and indicates absence of specific antibodies to *Brucella* in the patient serum.

Semiquantitative Method

Agglutination is a positive test result. The titer of patient serum corresponds to the visible agglutination in the test circle with the minimum amount of serum sample.

Tube Test Method

The titer of patient serum is the reciprocal of the last dilution of the serum sample that gives a granular agglutination.

In negative reaction, the appearance of the suspension remains unchanged, which shows a typical swirl when the tube is flicked.

Remarks

1. Turbid and contaminated serum should not be used for testing.
2. In the semiquantitative test the reactions obtained are roughly equivalent to those which would occur in a tube test.
3. Agglutinins are found in high proportion of normal individuals and titers less than 1:80 are of doubtful significance. A rising titer is more significant than a single high titer.
4. False positive reactions may occur in sera of patients infected with *Pasteurella tularensis* or vaccinated with *Vibrio cholerae.*
5. False positive results are likely if the test is read more than 1 minute after mixing on slide test.
6. It is recommended that results of the tests should be correlated with the clinical findings to arrive at the final diagnosis.
7. Prozoning may sometimes be encountered in serum containing very high titers on slide test.
8. Since techniques and standardization vary from laboratory to laboratory one tube difference in titers can be expected.

SLIDE SCREENING TEST FOR BRUCELLA ANTIBODIES (BRUCEL-RB)®

(Courtesy: Tulip Group of Companies)

Reagent

The BRUCEL-RB reagent contains smooth, killed buffered suspensions of *Brucella abortus* strain 99, colored with rose bengal, standardized against the 2nd International preparation, having specific reactivity towards antibodies to *Brucella.*

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of the reagents is as per the expiry date mentioned on the reagent vial labels.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity, and performance.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Past history of immunization, inapparent infection or prior disease	False positive reactions may occur in sera of patients infected with *Pasteurella tularensis* of vaccinated with *Vibrio cholerae* Also check the patient's history
2. Prolonged rocking of the slide causes drying of the test material	Agglutination should be observed within 1 minute
3. If reagents have been exposed to excessively high temperatures, precipitates could be formed	Ensure that the reagents are not exposed to high temperatures and are stored properly at 2–8°C
4. Contamination of serum could lead to false positives	Ensure that clean and dry glassware free from detergents are used for sample collection. If samples are not tested immediately, store temperature at 2–8°C Turbid and contaminated serum should not be used for testing
5. Error in interpreting results. Any debris or dirt in the slide/test tube could be mistaken for agglutination	Clean and dry glassware should be used for testing
6. Single high titer is interpreted as positive	Agglutinins are found in high proportion of normal individuals and titers less than 1:80 are of doubtful significance. A rising titer is more significant than a single titer

Problem: False negative results

Possible causes	*Solutions*
1. Infection is in very early stages, when antibody titer is very low	The agglutinin titer depends on the stage of the disease. Agglutinins usually appear by the end of the 1st week, so that blood taken earlier may give a negative test result. The testing should be repeated after a week in such cases Demonstration of a rise in titer of antibodies by testing two or more serum samples is more meaningful than a single test
2. Patients on antibiotic therapy during the testing phase	Check the history of the patient for administration of the antibiotics
3. Prozoning effect	In serum with very high titers, prozoning may be observed
4. Hemolyzed samples may have been used	Avoid using hemolyzed samples for testing
5. Rotation of the slide too fast may break up agglutinating clumps, which lead to false negative in borderline cases	Rock the slide gently back and forth and observe for agglutination macroscopically within 1 minute
6. Reagents not brought to room temperature. Cold reagents could give false negative results	All reagents must be brought to room temperature before commencing the testing procedure
7. Insufficient reagent present in the vial	Ensure sufficient reagent is present in the vial before retrieving

Principle

The smooth, colored, killed BRUCEL-RB antigen suspension is mixed with the patient serum. Specific antibodies to *Brucella* antigens if present in titres ≥ 120, in the patient serum will react with the antigen suspension to produce an agglutination reaction. No agglutination indicates the absence of detectable levels of specific antibodies to *Brucella.*

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains 0.01% thimerosal as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.

Sample Collection and Storage

1. No special preparation of patient is required prior to sample collection by approved techniques. Do not use hemolyzed serum samples.
2. Clean and dry glassware free from detergents must be used for sample collection.
3. Do not heat/inactivate the serum.

4. Though freshly collected serum is preferred, samples can be stored at 2 to 8°C, for 24 hours, or frozen for 8 days should a delay in testing occur.

Material Provided with the Kit

BRUCEL-RB *Brucella* rose bengal colored antigens.

Additional Material Required

Stop watch, positive control, isotonic saline, glass slide with clear/white background, appropriate pipettes/ micropipettes, mixing sticks and a high intensity direct light source.

Procedure

Bring all reagents to room temperature.

Shake and mix the BRUCEL-RB antigen suspension well before dispensing.

Slide Test Method

Qualitative Method

1. Place one drop of positive control onto the reaction circle of glass slide.
2. Place 80 µL of saline onto the next reaction circle of the glass slide.
3. Place 80 µL of patient serum to be tested onto the next reaction circle.
4. Add one drop of well mixed BRUCEL-RB antigen suspension in each of the above circles containing positive control, isotonic saline and the patient serum to be tested.
5. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
6. Gently rock the slide back and forth, observe for agglutination macroscopically, at one minute against a white background.

Semiquantitative Method

1. Using a pipette, place 80 µL, 40 µL, 20 µL, 10 µL, and 5 µL of patient serum to be tested on 5 different circles on the glass slide. The corresponding titers obtained will be 1:20, 1:40, 1:80, 1:160, and 1:320 respectively.
2. Place one drop of BRUCEL-RB antigen suspension to each circle.
3. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
4. Gently rock the slide back and forth, observe for agglutination macroscopically at 1 minute against a white background.

Interpretation of Results

Qualitative Method

Agglutination is a positive test result and indicates the presence of antibodies to *Brucella* in titers ≥ 1:20 in the patient serum.

No agglutination is a negative test result and indicates absence of antibodies to *Brucella* in titers 1:20 in the patient serum.

Semiqualitative Method

Agglutination is a positive test result. The titer of patient serum corresponds to the visible agglutination in the test circle with the minimum amount of serum sample.

Remarks

1. Turbid and contaminated serum should not be used for testing.
2. Agglutinins are found in high proportion of normal individuals and titers less than 1:80 are of doubtful significance. A rising titer is more significant than a single high titer.
3. False positive reactions may occur in sera of patients infected with *Pasteurella tularensis* or vaccinated with *Vibrio cholerae.*
4. False positive results are likely if the test is read more than 1 minute after mixing on the slide.
5. It is recommended that results of the test should be correlated with the clinical findings to arrive at the final diagnosis.
6. Prozoning may sometimes be encountered in serum containing very high titers on slide test.
7. Since techniques and standardization vary from laboratory to laboratory a difference of titer corresponding to next or previous titer can be expected.

BRUCELLOSIS POSITIVE CONTROL

(Courtesy: Tulip Group of Companies)

Summary

Human brucellosis (diurnal, or undulant fever) is a common febrile illness caused by infection with bacteria of some of the *Brucella* species *(abortus, melitensis).* This undulant fever is associated with symptoms, which are often variable and nonspecific with chills, fever, sweats and anorexia. On exposure, the body responds to this antigenic stimulation by producing specific antibodies whose titers rise slowly at early stages and then increases. Specific antibodies to the *Brucella* species are detectable a few weeks after exposure and are of considerable importance in the

diagnosis of Brucellosis. Information regarding the titer of antibodies can be obtained by using specific Brucel antigen suspensions.

The performance of the Brucel-A/Brucel-M antigen suspensions can be validated with the help of Brucellosis Positive Control.

Reagent

The Brucellosis Positive Control contains ready-to-use standardized goat antiserum with polyspecific antibodies having specific reactivity towards *Brucella abortus* and *Brucella melitensis* antigens and is useful in the validation of the performance of Brucel-A/Brucel-M reagents.

Reagent Storage and Stability

Store the reagent at 2 to 8°C. Do not freeze.

The shelf-life of the reagent is as per the expiry date mentioned on the reagent bottle label.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity, and performance.

Principle

The Brucellosis Positive Control is mixed with the *Brucella* antigen suspensions to be tested and allowed to react. Specific reactivity of *Brucella* antigens if present in the antigen suspensions will produce an agglutination reaction. No agglutination indicates the deterioration of the performance of the antigen suspensions.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagent contains thimerosal 0.01% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.

Additional Material Required

Stopwatch, isotonic saline, glass slide with clear/white background, appropriate pipettes/micropipettes, mixing sticks and a high intensity direct light source.

Procedure

Bring all reagents to room temperature. Shake and mix the Brucellosis Positive Control well before dispensing.

Slide Test Method

1. Place one drop of Brucellosis Positive Control onto the reaction circle of glass slide.
2. Place 80 µL of saline onto the next reaction circle of the glass slide.
3. Add one drop of test reagent *(Brucella* antigen suspensions) in each of the above circles.
4. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
5. Gently rock the slide back and forth, observe for agglutination macroscopically at 1 minute against a white background.

Interpretation of Results

Slide Test Method

Agglutination is a positive test result and indicates that the *Brucella* antigen reagents are performing satisfactorily. No agglutination is a negative test result and indicates the deterioration of *Brucella* antigen reagents.

RAPID TEST FOR IgM AND IgG ANTIBODIES TO DENGUE VIRUS: DENGUE FEVER (DENGUECHECK-WB®) (DEVICE)

(*Courtesy:* Tulip Group of Companies)

Introduction

Denguecheck-WB is a rapid immunochromatographic test for the simultaneous detection of IgM and IgG antibodies to Dengue virus in human serum/plasma/whole blood. The test can be used as a screening test for Dengue viral infection and as an aid for differential diagnosis of the self-limiting primary Dengue infections and the potentially fatal secondary Dengue infections in conjunction with other criteria.

Summary

Dengue fever virus (serotypes 1–4) belong to the family of *Flaviviridae*, which is widely distributed in the epidemic and endemic areas throughout tropical and subtropical regions of the world. Dengue virus infection is considered significant in terms of morbidity, mortality and economic cost associated with it, an estimated 100 million cases of dengue fever occurring throughout the world yearly. Dengue virus is transmitted in nature principally by the day-biting *Aedes aegypti* and *Aedes albopictus* mosquitoes. The mosquito vector is highly domesticated and an urban species. Dengue presents typically as a fever of sudden onset with headache, retro-orbital pain, pain in the back and limbs (break-bone fever), lymphadenopathy and maculopapular rash. Patients diagnosed with dengue infection in endemic areas generally have secondary infection, whereas patients in nonendemic

areas are usually diagnosed with primary infection. Specific antibody response to Dengue virus enables serodiagnosis and differentiation between primary and secondary dengue infections and detection of potentially life-threatening conditions such as Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DS*S).*

Denguecheck-WB is a new generation rapid immunochromatographic test using highly specific and purified immunodominant, Recombinant Dengue 'Env.' antigens. It is a simple test for the differential diagnosis of Dengue virus infection.

Principle

Denguecheck-WB utilizes the principle of immuno-chromatography, a unique two-site, self-performing immunoassay on a membrane. Specific human IgM and human IgG antibody-binding proteins are immobilized on the nitrocellulose membrane as two individual test bands (IgM and IgG) in the test window "T" of the test device at region "M" and region "G" respectively. The IgM band in the test window "T" is closer to the sample well and the IgG band is close to the control window "C". As the test sample flows through the membrane assembly within the test device, the colored-Dengue specific recombinant antigen-colloidal gold conjugate complexes with specific antibodies (IgM and IgG) to Dengue virus, if present in the sample. This complex moves further on the membrane to the test region where it is immobilized by the specific human IgM antibody and/or human IgG antibody binding proteins coated on the membrane leading to formation of a colored band which confirms a positive test result. Absence of these colored bands in the test window "T" indicates a negative test result. A built-in control band in the control window "C" appears when the test has been performed correctly, regardless of the presence or absence of anti-Dengue virus antibodies in the specimen and serves to validate the test performance.

Reagents and Materials Supplied

Each kit contains:

A. lndividual pouches, each containing:
   1. Denguecheck-WB (Device) Membrane test assembly predispensed with recombinant Dengue virus specific antigen colloidal gold conjugate, streptavidin gold conjugate, anti-human IgM at test region 'M' Protein A at the test region 'G' and Biotin at the control region 'C'.
   2. Desiccant pouch
   3. 5 µL sample loop.
B. Sample running buffer.
C. Package insert.

Storage and Stability

The sealed pouches in the test kit and the kit components may be stored between 4 and 30°C for the duration of the shelf-life as indicated on the pouch.

Optional Material Required: 5 µL precision micropipettes.

Note

1. For in vitro diagnostic use only. Not for medicinal use.
2. Do not use beyond expiry date.
3. Read the instructions carefully before performing the test.
4. Handle all specimen as potentially infectious.
5. Follow standard biosafety guidelines for handling and disposal of potentially infective material.

Specimen Collection and Preparation

1. No special preparation of the patient is necessary prior to specimen collection by approved techniques.
2. Though fresh serum/plasma is preferable, specimen may be stored at 2–8° C for up to 24 hours, in case of delay in testing.
3. Whole blood samples collected with a suitable anticoagulant such as EDTA or Heparin or Oxalate can also be used.
4. Do not use turbid, lipemic, icteric and hemolyzed specimen.
5. Repeated freezing, thawing of the specimen should be avoided.
6. Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only should be used for testing.

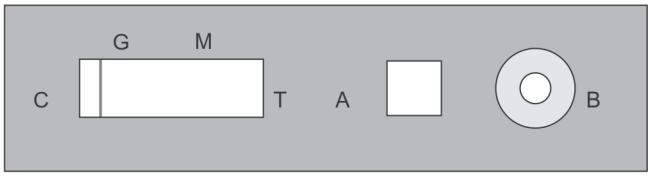
Testing Procedure and Interpretation of Results

1. Bring the kit components to room temperature before testing.
2. Open the pouch and retrieve the test device. Once opened, the device must be used immediately.
3. Label the test device with patient identity.
4. Add 5 µL of serum/plasma/whole blood, with the micropipette into the sample port 'A', or using the 5 µL sample loop provided with the-kit, dip the loop

into the sample and then blot into the sample port 'A'. Ensure that the loop does not retrieve clots or debris from the sample.
5. Add five drops of sample running buffer in the reagent port 'B'.
6. Exactly at the end of 15 minutes, read the test results.

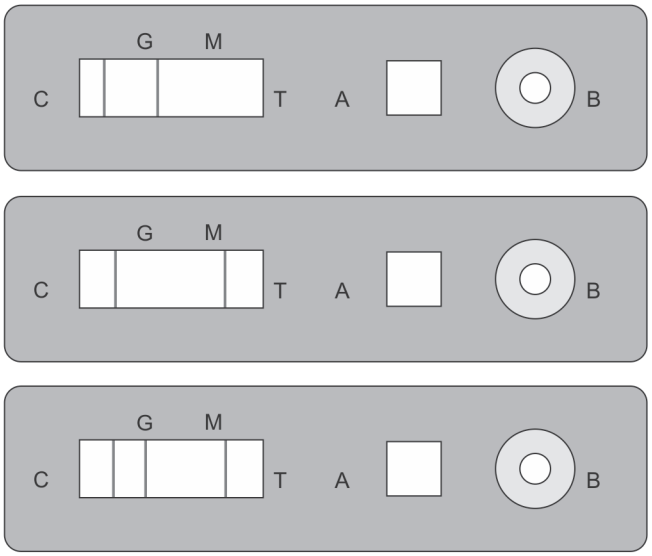
Interpretation of Results

Negative Test Result



The presence of only the single red/purple colored band in the control window "C" indicates the absence of specific antibodies against dengue virus or that the amount of antibodies is below the detection limit of the test.

Positive Test Result



1. In addition to the band in the control window 'C', appearance of two red/purple colored bands in the test window at region 'M' and region 'G' indicates the presence of Dengue virus specific IgM and IgG antibodies (acute secondary infection).
2. In addition to the control band in the control window 'C', appearance of a red/purple colored band in the test window at region 'M' indicates the presence of Dengue virus specific IgM antibodies (acute primary infection).
3. In addition to the control band in the control window 'C', the appearance of a red/purple colored band in the test window at region 'G' indicates the presence of Dengue virus specific IgG antibodies (acute secondary infection).

Invalid Result: If, after 15 minutes, no band is visible either in the test or control window, the result is considered invalid. The test should be repeated with a new device.

Remarks

1. Do not use test kit beyond expiration date.
2. While sample should be collected as soon as possible after onset of illness, it is recommended that follow-up of testing should be done on day 10 after the first sample to allow seroconversion, especially when the test is negative and Dengue virus infection is clinically suspected.
3. Though *Denguecheck*-WB does provide evidence to distinguish the past (secondary) infection from current (primary) ongoing infection, a negative result does not preclude the possibility of infection with Dengue virus.
4. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test but should rather be made by a clinician after all clinical findings have been evaluated.
5. The DHF is primarily the disease of children under 15 years in hyperendemic areas. Impending DSS symptoms include suspected abdominal pain, persistent vomiting, change in the level of consciousness, hypothermia and sudden decrease in platelet counts.
6. Eighty percent of the patients may have detectable levels of IgM antibody by day 5 of illness and 99% by day 10.
7. IgM levels rise quickly and peak by two weeks after onset of symptoms and then fall to become undetectable over 2 to 3 months. IgG antibodies rise quickly and peak at about two weeks postinfection and then decline slowly over 3 to 6 months.

Troubleshooting

Dengue Check WB and Leptocheck-WB

Problem: False positive results

Possible causes	*Solutions*
1. The flow properties of the nitrocellulose membrance are partially affected leading to the movement of partially aggregated gold-sol particles	Check the pouch for pinholes and also observe the desiccant for any color change. The results of the test should be correlated with clinical findings

Problem: Faint lines observed in control and test region

Possible causes	*Solutions*
1. Hemolyzed blood samples were used for testing	Do not use hemolyzed blood samples for testing
2. Reading taken after 15 min	Read results exactly at 15 minutes

Problem: Delayed results and altered flow

Possible causes	*Solutions*
1. Whole blood samples having microclots or fibrin	Ensure that the whole blood collected directly from fingerprick (without anticoagulant) should be free from microclots to avoid altered flow and delayed reaction time

Problem: False negative results

Possible causes	*Solutions*
1. Inadequate quantity of sample used for performing the test	The exact number of drops of the sample as mentioned in the pack insert should be dispensed for performing the test using the dropper provided with the kit
2. The kit is exposed to very high temperatures leading to deterioration of the antibodies coated on the device	Store at recommended temperature when not in use
3. Turbid or contaminated serum samples were used for testing	Do not use turbid or contaminated serum samples for testing
4. Sample tested early after infection	Specific antibodies reach detectable levels about one week after onset of disease; hence follow-up testing after day 10 is recommended to allow seroconversion

Problem: Invalid results

Possible causes	*Solutions*
1. Pinholes/defect in the pouch. The nitrocellulose membrane has lost its flow properties due to absorbance of moisture	Check the pouch for pinholes and also check the color of the desiccant (silica gel). A change in color form deep blue to white indicates absorbance of moisture
2. The device is removed from the refrigerator and tested immediately leading to hydration of the sites on the nitrocellulose membrane hence adversely affecting its flow properties	The test pouch should be brought to room temperature before being tested

TEST FOR INFECTIOUS MONONUCLEOSIS (IMMUTEX®)

(Courtesy: Tulip Group of Companies)

Summary

Infectious mononucleosis is a self-limited prolonged illness strongly associated with Epstein-Barr Virus. Though specific treatment is rarely required since the disease is usually asymptomatic, potential complications, such as inflammation of the liver, enlargement of the spleen, pericarditis, myocarditis and encephalitis as well as hemolytic anemia associated with this disease, require physician's attention.

In individuals with suppressed or abnormal immunodeficiency disorders, cancer or those with recent organ transplant, infectious mononucleosis occurs with severe complications.

Studies have cited the presence of heterophile antibodies during the course of infection with infectious mononucleosis.

Reagent

Immutex is a ready-to-use, uniform suspension of stabilized, specially treated horse erythrocytes highly specific for heterophile antibodies associated with infectious mononucleosis. The reagent does not react with normal Forssman antibodies.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

Store the reagents at 2 to 8°C. Do not freeze. The shelf-life of the reagents is as per the expiry date mentioned on the reagent vial labels.

Principle

Immutex is a rapid slide hemagglutination test for the detection of heterophile antibodies. Immutex IM reagent will agglutinate when mixed with serum containing heterophile antibodies. No agglutination indicates absence of heterophile antibodies.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the components derived from human source have been tested for HBsAg and Anti-HIV antibodies and found to be nonreactive. However, handle the material as if infectious.
3. The reagents contain thimerosal 0.01% as preservative. Avoid contact with skin or mucosa. On disposal, flush with large quantities of water.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of reagents should be verified with known positive and negative controls provided with the kit.
5. Ensure resuspension of the stabilized erythrocyte reagent before use to improve test readability by gently inverting the vial.
6. Do not interchange vial droppers.
7. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry.

Sample Collection and Preparation

No special preparation of the patient is necessary prior to specimen collection by approved techniques. Though fresh serum is preferable, serum specimens stored at 2 to 8°C for up to 24 hours, can also be used in case of delay in testing. Do not use hemolyzed or contaminated specimens. Turbid specimens should be centrifuged or allowed to settle and only the clear supernatant should be used for testing.

Materials Provided with the Kit

Reagent

Immutex IM reagent, Positive Control, Negative Control.

Accessories

Glass slide with six reaction circles, sample dispensing pipettes, mixing sticks and rubber teats.

Additional Material Required

A high intensity direct light source, stopwatch.

Test Procedure

Bring all reagents and samples to room temperature before use.

Qualitative Method

1. Gently mix the Immutex IM reagent to resuspend the stabilized horse erythrocyte reagent.
2. Place one drop of the sample to be tested onto one of the reaction circles of the glass slide using a sample dispensing pipette provided with the kit.
3. Place one drop of the positive and negative control onto separate reaction circles of the glass slide.
4. Add one drop of Immutex IM reagent to the test

specimen on the slide. Do not let the dropper tip touch the liquid on the slide.
5. Add one drop of Immutex IM reagent to each of the controls.
6. Mix with separate mixing sticks, spreading the mixture uniformly over the entire reaction circle.
7. Immediately start the stopwatch. Rock the slide gently, back and forth, for 2 minutes.
8. Leave the slide undisturbed on the work table for a further 1 minute.
9. Pick up the slide at the end of 1 minute and observe for agglutination by rocking the slide gently back and forth.

Interpretation of Results

Agglutination is a positive test result and indicates presence of heterophile antibodies in the test specimen. No agglutination is a negative test result and indicates absence of heterophile antibodies in the test specimen.

Remarks

1. Markedly lipemic, hemolyzed and contaminated serum sample could produce nonspecific results.
2. Use of plasma rather than serum can lead to false positive results.
3. Heterophile antibodies may be found in disease other than infectious mononucleosis. Low titers have been detected in cytomegalic inclusion disease and toxoplasmosis.
4. Do not read the results beyond indicated testing time limit.
5. As with all diagnostic tests, the result of the test should be correlated with clinical findings to arrive at the final diagnosis.
6. The reagent performance should be validated by occasionally running the positive and negative controls provided with the kit.

Troubleshooting

Immutex

Problem: False positive results

Possible causes	*Solutions*
1. Plasma is used as a test specimen	Only serum must be used for testing. Should a delay in testing occur, store samples at 2–8°C
2. Markedly lipemic and contaminated serum samples could produce non-specific results	Do not use lipemic and contaminated serum samples for testing
3. Drying of the reagent on slide	Do not read results beyond 3 minutes. The test should not be carried out directly under the fan
4. In other disease conditions, there is a possibility of occurrence of false positives	Heterophile antibodies may be found in disease other than Infectious Mononucleosis. Low titers have been detected in cytomegalic inclusion disease and Toxoplasmosis
5. Incorrect interpretation of results	Ensure that the results are interpreted at 3 minute and as per instructions given in the package insert. Positive and negative controls should be run with each series of tests and results should be compared with these

Problem: False negative results

Possible causes	*Solutions*
1. Prozoning effect.	In serum with very high titers, prozoning may be observed.
2. Hemolyzed samples may have been used.	Avoid using hemolyzed samples for testing.
3. Reagents not brought to room temperature. Cold reagents could give false negative results.	All reagents must be brought to room temperature before use.

RAPID TEST FOR IGM ANTIBODIES TO LEPTOSPIRA: LEPTOSPIROSIS (LEPTOCHEK WB®) (DEVICE)

(*Courtesy:* Tulip Group of Companies)

Introduction

Leptocheck-WB is a rapid, self-performing, qualitative, sandwich immunoassay for the detection of *Leptospira* specific IgM antibodies in human serum/plasma or whole blood specimen. It is useful for the serodiagnosis of current or recent leptospirosis. The broadly reactive genus specific antigen employed in the test allows the detection of *Leptospira* infections caused by a wide range of strains of different serovars.

Summary

Leptospira are actively motile, delicate spirochetes possessing a large number of closely wound spirals and characteristic hooked ends. There are several species of *Leptospira* and they may be saprophytic or parasitic. They can be distinguished only under dark ground illumination in the living state or by electron microscopy. *Leptospirosis* is a zoonotic disease of worldwide prevalence. Humans are infected when the water contaminated by the urine of carrier animals enters the body through cuts or abrasions on the skin or through intact mucosa of the mouth, nose or conjunctiva. Clinical symptoms include fever, chills, headache, conjunctivitis, myalgia and Gl-related symptoms, kidney infection is a common sequelae.

Diagnosis may be made by demonstration of *Leptospira* microscopically in blood or urine, by isolating them in culture or by inoculation of guinea pigs, or by serological tests.

Leptocheck-WB, qualitatively detects the presence of IgM class of *Leptospira* specific antibodies in human serum/plasma or whole blood specimen.

Principle

Leptocheck-WB utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flows through the membrane assembly of the test device, the anti-human IgM-colloidal gold conjugate forms a complex with IgM antibodies in the sample. This complex moves further on the membrane to the test window 'T' where it is immobilized by the broadly reactive *Leptospira* genus specific antigens coated on the membrane, leading to the formation of a red to deep purple colored band at the test region 'T' which confirms a positive test result. Absence of this colored band in test region 'T' indicates a negative test result. The unreacted conjugate and the unbound complex if any move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies coated on the control window 'C' of the membrane assembly, forming a red to deep purple colored band. The control band serves to validate the test results.

Reagents and Material Supplied

Each kit contains:

A. Individual pouches, each containing:
   1. Test Device Membrane test assembly predispensed with Anti Human IgM-colloidal gold conjugate, *Leptospira* genus specific antigens at test window 'T' and anti-rabbit antiserum predispensed at the control window 'C'.
   2. Desiccant pouch
   3. 5 µL sample loops.
B. Sample running buffer
C. Package insert.

Storage and Stability

The sealed pouches in the test kit and the kit components may be stored between 4 and 30°C for the duration of the shelf-life as indicated on the pouch.

Optional Material Required: 10 µL precision micropipettes.

Note

1. For in vitro diagnostic use only. Not for Medicinal Use.
2. Do not use beyond expiry date.
3. Read the instructions carefully before performing the test.
4. Handle all specimens as potentially infectious.
5. Follow standard biosafety guidelines for handling and disposal of potentially infective material.

Specimen Collection and Preparation

1. Blood samples collected with a suitable anticoagulant such as EDTA or Heparin or Oxalate can also be used.
2. No prior preparation of the patient is required before sample collection by approved techniques.
3. Fresh serum/plasma is preferable. Anticoagulated whole blood can also be used as specimen. Serum/plasma may be stored at 2 to 8°C up to 24 hours in case of delay in testing. For long-term storage, freeze the specimen at –20°C for 3 months or –70°C for longer periods. Whole blood should be used immediately and should not be frozen.
4. Repeated freezing and thawing of the specimen should be avoided.

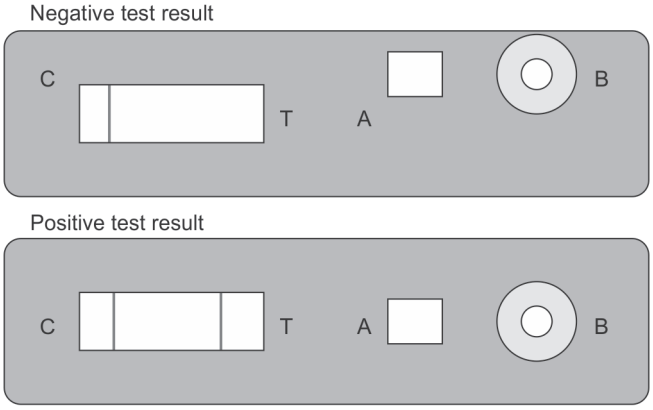
5. Do not use hemolyzed, clotted, contaminated, viscous/turbid specimen.
6. Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only used for testing.
7. For each sample, a new sample loop should be used.

Testing Procedure and Interpretation of Results

1. Bring the kit components to room temperature before testing.
2. Open the pouch and retrieve the test device. Once opened, the device must be used immediately.
3. Label the test device with the patient's identity.
4. Add 10 µL of serum/plasma or whole blood with a micropipette into the sample port "A", OR using the 5 µL sample loop provided with the kit. Dip the loop into the sample and then blot into the sample port 'A'. Repeat this step twice for each sample. Ensure that the loop does not retrieve clots or debris from the sample.
5. Add 5 drops of sample running buffer to the reagent port 'B'.
6. At the end of 15 minutes read the results as follows.

Negative Test Result

Only one colored band appears in the control window 'C'.



In addition to the band in control window 'C', another red/purple band appears in the test window 'T' indicating the presence of specific IgM antibodies to *Leptospira*.

- The test should be considered invalid if neither the control band 'C' nor the test band 'T' appears. Repeat the test with a new device.

Performance Characteristics

Leptocheck-WB was evaluated at the Royal Tropical Institute, Amsterdam in parallel with other licensed tests for the serodiagnosis of *leptospirosis*. The 47 sera evaluated were from diverse serogroups of *Leptospira*.

Leptocheck-WB had a performance comparable to the other tests.

Remarks

1. The intensity of the test line depends upon the stage of the disease and the titres of the antibodies in the test specimen.
2. As specific antibodies reach detectable levels about one week after the onset of disease, a sample collected very early may yield a negative test result.
3. If the test is negative and if leptospirosis is still suspected, the test should be repeated with the second sample collected at a later date in conjunction with clinical reexamination.
4. In endemic areas, faint bands may appear occasionally due to borderline IgM titres present as a result of previous exposures.
5. It is recommended that the positive results obtained must be reconfirmed using a confirmatory test such as the MAT (microscopic agglutination test).
6. High titres of RF and heterophile antibodies may interfere with the test; in such cases, the results must be interpreted with caution.
7. The results must be correlated with clinical findings to arrive at the diagnosis.
8. Do not use the test kit beyond expiration date.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. The flow properties of the nitrocellulose membrane are partially affected leading to the movement of partially aggregated gold-sol particles	Check the pouch for pinholes and also observe the desiccant for any color change. The results of the test should be correlated with clinical findings.

Problem: Faint Lines observed in control and test region

Possible causes	*Solutions*
1. Hemolyzed blood samples were used for testing	Do not use hemolyzed blood samples for testing
2. Reading taken after 15 minutes	Read results exactly at 15 minutes

Problem: delayed results and altered flow

Possible causes	*Solutions*
1. Whole blood samples having microclots or fibrin	Ensure that the whole blood collected directly from fingerprick (without anticoagulant) should be free from microclots to avoid altered flow and delayed reaction time

Problem: false negative results

Possible causes	*Solutions*
1. Inadequate quantity of sample used for performing the test	The exact number of drops of the sample as mentioned in the pack insert should be dispensed for performing the test using the dropper provided with the kit
2. The kit is exposed to very high temperatures leading to deterioration of the antibodies coated on the device	Store at recommended temperature when not in use
3. Turbid or contaminated serum samples were used for testing	Do not use turbid or contaminated serum samples for testing
4. Sample tested early after infection	Specific antibodies reach detectable levels about one week after onset of disease; hence, follow-up testing after day 10 is recommended to allow seroconversion

Problem: Invalid results

Possible causes	*Solutions*
1. Pinholes/defect in the pouch. The nitrocellulose membrane has lost its flow properties due to absorbance of moisture	Check the pouch for pinholes and also check the color of the desiccant (silica gel). A change in color form deep blue to white indicates absorbance of moisture
2. The device is removed from the refrigerator and tested immediately leading to hydration of the sites on the nitrocellulose membrane hence adversely affecting its flow properties	The test pouch should be brought to room temperature before being tested

RAPID TEST FOR MALARIA PAN/PV/PF (PARAMAX-3®) (DEVICE)

(Courtesy: Tulip Group of Companies) (Various other diagnostic combinations are also available)

Introduction

Paramax-3 is a rapid self-performing, qualitative, two-site sandwich immunoassay utilizing whole blood for the detection of *P. falciparum* specific histidine-rich protein-2 (Pf HRP-2), *P.* vivax specific pLDH and pan malaria specific pLDH. The test can be used for the specific detection of *P. falciparum* and *P. vivax* malaria, differentiation of other malarial species and for the follow-up of antimalarial therapy.

Summary

Four species of the *Plasmodium* parasites are responsible for malaria infections in human viz. *P. falciparum, P. vivax, P. ovale* and *P. malariae.* Of these, *P. falciparum* and *P. vivax* are the most prevalent. Early detection and differentiation of malaria is of utmost importance due to incidence of cerebral malaria and drug resistance associated with falciparum malaria and due to the morbidity

associated with the other malarial forms. As the course of treatment is dependent on the species, differentiation between *P. falciparum* and *P. vivax* is of utmost importance for better patient management and speedy recovery.

In Paramax-3 the detection system for *P. falciparum* malaria is based on the detection of *P. falciparum* specific histidine-rich protein-2 (Pf HRP-2) which is a water-soluble protein that is released from parasitised erythrocytes of infected individuals. The detection system of *P. vivax* is based on the presence of *P. vivax* specific pLDH. Further the detection of other malarial infections such as *P. ovale* and *P. malariae* is achieved through the pan malaria specific pLDH. Since pLDH is a product of viable parasites, the pan band may also be used to monitor course of effective antimalarial therapy.

Paramax-3 detects the presence of *P. falciparum* specific Pf HRP-2, *P.vivax* specific pLDH and pan specific pLDH in whole blood specimen and is a sensitive and specific test for the detection of all malaria species, differentiation for *P. falciparum* and *P. vivax* and monitoring successful antimalarial therapy.

Principle

Paramax-3 utilizes the principle of immunochromatography. As the test sample flows through the membrane assembly of the device after addition of the clearing buffer, the colored colloidal gold conjugates of anti-HRP-2 antibody, anti-*P. vivax* specific pLDH antibody and anti-pan specific pLDH antibody complexes the HRP-2/corresponding pLDH in the lyzed sample. This complex moves further on the membrane to the test region where it is immobilized by the monoclonal anti-Pf. HRP-2 antibody and/or monoclonal anti-*P. vivax* specific pLDH antibody and/or monoclonal pan specific pLDH antibody coated on the membrane leading to formation of a pink-purple colored band in the respective regions which confirms a positive test result. Absence of a colored band in the test region indicates a negative test result for the corresponding antigen. The unreacted conjugate along with the rabbit globulin colloidal gold conjugate and unbound complex if any, move further on the membrane and are subsequently immobilized by anti-rabbit antibodies coated on the membrane at the control region, forming a pink-purple band. This control band serves to validate the test performance.

Reagents and Material Supplied

Paramax-3 kit contains:

A. Individual pouches, each containing:
   1. Test Device Membrane assembly predispensed with monoclonal anti-HRP-2 antibody-colloidal gold conjugate, monoclonal anti-*P.vivax* specific pLDH antibody-colloidal gold conjugate, monoclonal anti-pan specific pLDH antibody-colloidal gold conjugate, rabbit globulin colloidal gold conjugate, monoclonal anti-Pf. HRP-2 antibody, monoclonal anti-*P. vivax* specific pLDH antibody, monoclonal anti-pan specific pLDH antibody and anti-rabbit antibody at the respective regions.
   2. Desiccant pouch
   3. 5 μL sample loop.
B. Clearing buffer in a dropper bottle
C. Package insert.

Optional Material Required

Calibrated micropipettes capable of delivering 5 μL sample accurately.

Storage and Stability

The test kit may be stored between 4–30°C till the duration of the shelf-life as indicated on the pouch/carton. Do not freeze.

Note

Read the instructions carefully before performing the test. For in vitro diagnostic use only. Not for Medicinal Use. Do not use beyond expiry date. Do not intermix reagents from different lots. Handle all specimens as potentially infectious. Follow standard biosafety guidelines for handling and disposal of potentially infective material.

Specimen Collection and Preparation

Fresh anticoagulated whole blood should be used as a test sample and EDTA or Heparin or Oxalate can be used as suitable anticoagulant. The specimen should be collected in a clean glass or plastic container. If immediate testing is not possible, then the specimen may be stored at 2 to 8°C for up to 72 hours before testing. Clotted or contaminated blood samples should not be used for performing the test. Fresh blood from finger prick/puncture may also be used as a test specimen.

Test Procedure

1. Bring the Paramax-3 kit components to room temperature before testing.
2. Open the pouch and retrieve the device, sample loop and desiccant. Check the color of the desiccant. It should be blue. If it has turned colorless or pink, discard the device and use another device. *Once opened, the device must be used immediately.*
3. Tighten the vial cap of the clearing buffer provided

with the kit in the clockwise direction to pierce the dropper bottle nozzle.

4. Evenly mix the anticoagulated blood sample by gentle swirling. Dip the sample loop into the sample. Ensuring that a loop full of blood is retrieved, blot the blood so collected onto the sample pad in the sample port 'A' (this delivers approximately 5 µL of the whole blood specimen).

Or

In case finger prick blood is being used, touch the sample loop to the blood on the finger prick. Ensuring that a loop full of blood is retrieved, immediately blot the specimen on to the sample pad in the sample port 'A'. (care should be taken that the blood sample has not clotted and the transfer to the sample pad is immediate).

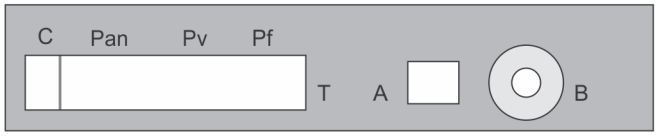
Or

Alternatively, 5 µL of the anti coagulated or finger prick specimen may be delivered to the sample pad in the sample port 'A' using a micro pipette.

Note: Ensure that the blood from the sample loop has been completely taken up by the sample pad.

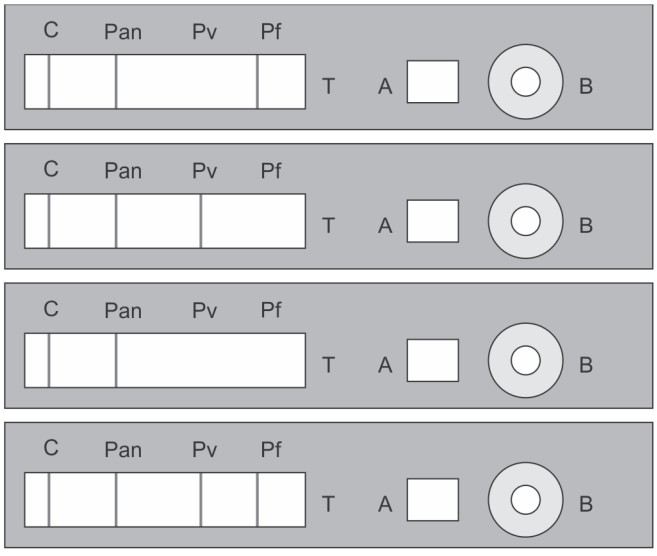
5. Dispense four drops of the clearing buffer into port 'B', by holding the plastic dropper bottle vertically.
6. At the end of 15 minutes, read the results as follows.

Negative for Malaria



- Only one pink-purple band appears at the control region 'C'.

Positive for Malaria



- *P. falciparum* malaria: In addition to the control band, a pink-purple band appears at the 'Pf' and 'Pan' regions respectively.
- *P. vivax* malaria: In addition to the control band, a pink-purple band appears at 'Pv' and 'Pan' regions respectively.
- Other species: In addition to the control band, one pink-purple band appears only at 'Pan' region.
- Mixed infection: In addition to the control band, a pink-purple band appears at 'Pf', 'Pv' and 'Pan' regions respectively.

7. The test should be considered invalid if no bands appear on the device. Repeat the test with a new device ensuring that the test procedure has been followed accurately.

Limitation of the Test

1. As with all diagnostic tests, the test result must always be correlated with clinical findings.
2. The results of test are to be interpreted within the epidemiological, clinical and therapeutic context. When it seems indicated, the parasitological techniques of reference should be considered (microscopic examination of the thick smear and thin blood films).
3. Any modification to the above procedure and/or use of other reagents will invalidate the test procedure.
4. The device and buffer of different lots must not be mixed and used.
5. In case of infection due to *P.vivax* or *P. falciparum,* or due to mixed infection by these species, the 'Pan' malaria band will also be positive. Hence, differentiation of infection due to *P. ovale* or *P.malariae* cannot be done.
6. While monitoring therapy, if the reaction of the test remains positive with the same intensity after 5 to 10 days, post-treatment, the possibility of a resistant strain of malaria has to be considered.
7. Usually, the 'Pv' and 'Pan' bands turn negative after successful anti-malarial therapy. However, since treatment duration and medication used affect the clearance of parasites, the test should be repeated after 5–10 days of start of treatment.
8. In *P. falciparum* malaria infection, HRP-2 is not secreted in gametogony stage. Hence, in "Carriers", the HRP-2 band may be absent.
9. The HRP-2 levels, post-treatment, persist up to 15 days, the 'Pan' band can be used to monitor success of therapy, in *P. falciparum* malaria cases.
10. In a few cases, where the HRP-2 band is positive and the 'Pan' malaria band is negative, it may indicate

a case of post-treatment malaria. However, such a reaction pattern may also be obtained in a few cases of untreated malaria. Retesting after 2 days is advised, in such cases.

SLIDE TEST FOR C-REACTIVE PROTEIN (RHELAX CRP®)

(Courtesy: Tulip Group of Companies)

Summary

The C-reactive protein (CRP) is a serum protein which is synthesized in the liver. Its rate of synthesis and secretion increases within hours of an acute injury or the onset of inflammation and may reach as high as 20 times the normal levels. Elevated serum concentration of CRP is an unequivocal evidence of an active tissue damage process; and CRP measurement, thus provides a simple screening test for organic disorders. Apart from indicating inflammatory disorders, CRP measurement helps in differential diagnosis, in the management of neonatal septicemia and meningitis where standard microbiological investigations are difficult.

Its use in postoperative surveillance is of great importance. CRP levels invariably rise after major surgery but fall to normal within 7–10 days. Absence of this fall is indicative of possible septic or inflammatory postoperative complications. Serum CRP measurement also provides useful information in patients with myocardial infarction there being an excellent correlation between peak levels of CRP and creatine phosphokinase (CPK).

Reagent

1. RHELAX CRP reagent: A uniform suspension of polystyrene latex particles coated with anti-CRP antibodies. The reagent is standardized to detect CRP concentrations greater than 0.6 mg/dl.
2. Positive control, reactive with RHELAX CRP reagent.
3. Negative control, non-reactive with RHELAX CRP reagent.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of reagent is as per the expiry date mentioned on the reagent vial label.

Principle

The RHELAX CRP slide test for detection of CRP is based on the principle of agglutination. The test specimen (serum) is mixed with RHELAX CRP latex reagent and allowed to react. If CRP concentration is greater than 0.6 mg/dl, a visible agglutination is observed. If CRP concentration is less than 0.6 mg/dl, then no agglutination is observed.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the reagents derived from human source have been tested for HBsAg and anti-HIV antibodies and are found to be non-reactive.
3. The Reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagent be verified with the positive and negative controls provided with the kit.
5. Shake the latex reagent well before use to disperse the latex particles uniformly and improve test readability.
6. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry.
7. Accessories provided with the kit only must be used for optimum results.

Specimen Collection and Preparation

No special preparation of the patient is required prior to specimen collection by approved techniques.

Only serum should be used for testing. Should a delay in testing occur, store the sample at 2 to 8°C. Samples can be stored for up to a week. Do not use hemolyzed serum.

Material Provided with the Kit

Reagent

The RHELAX CRP latex reagent, positive control, negative control.

Accessories

Glass slide with six reaction circles, sample dispensing pipettes, mixing sticks, rubber teat.

Additional Material Required

Stopwatch, test tubes, a high intensity direct light source, isotonic saline.

Test Procedure

Bring reagent and samples to room temperature before testing.

Qualitative Method

1. Pipette one drop of the test specimen (serum) on the glass slide using a disposable pipette provided with the kit.
2. Add one drop of RHELAX CRP latex reagent to the drop of test specimen on the slide. Do not let the dropper tip touch the liquid on the slide.
3. Using a mixing stick, mix the test specimen and the RHELAX CRP latex reagent uniformly over the entire circle.
4. Immediately start a stopwatch. Rock the slide gently back and forth, observing for agglutination macroscopically at 2 minutes.

Semiquantitative Method

1. Using isotonic saline, prepare serial dilutions of the test specimen positive in the qualitative method 1:2, 1:4, 1:8, 1:16 and so on.
2. Pipette each dilution of the test specimen onto separate reaction circles.
3. Add one drop of RHELAX CRP latex reagent to the drop of test specimen on the slide. Do not let the dropper tip touch the liquid on the slide.
4. Using a mixing stick, mix the test specimen and the latex reagent uniformly over the entire circle.
5. Immediately start a stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at 2 minutes.

Interpretation of Test Results Qualitative Method

Agglutination is a positive test result and indicates the presence of detectable levels of CRP in the test specimen. No agglutination is a negative test result and indicates the absence of detectable levels of CRP in the test specimen.

Semiquantitative Method

Agglutination in the highest serum dilution corresponds to the amount of CRP in mg/dL present in the test specimen.

Concentration of CRP can be calculated as follows:

CRP (mg/dL) = S × D

where S = Sensitivity of the reagent, i.e. 0.6 mg/dL.

D = Highest dilution of serum showing agglutination.

Remarks

1. Markedly lipemic, hemolyzed and contaminated serum samples could produce non-specific results.
2. Use of plasma rather than serum can lead to false positive results.
3. The CRP is found to be present after the first trimester of pregnancy and persists until delivery.
4. The CRP levels increase in women who are on oral contraceptives.
5. The CRP response is not affected by the commonly used anti-inflammatory or immunosuppresive drugs, including steroids, unless the disease activity is affected and it covers an exceptionally broad incremental range upto 3000 times.
6. Do not read results beyond indicated testing time limits.
7. Since CRP production is a non-specific response to tissue injury, it is recommended that results of the test should be correlated with clinical findings to arrive at the final diagnosis.
8. In cases where an increase in CRP levels is suspected, but the screening test shows a negative result, semiquantitation should be done to rule out prozone effect.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Plasma is used as a test specimen	Only serum must be used for testing
2. Samples are stored for a long period	Should a delay in testing occur, store samples at 2–8°C. Samples can be stored for upto a week at 2–8°C
3. Markedly lipemic, hemolyzed and contaminated serum samples	Lipemic, hemolyzed and contaminated samples produce non-specific results. Avoid such samples
4. Drying of the reagent on slide	Do not read results beyond 2 minutes. The test should not be carried out directly under the fan
5. Presence of dust or debris on the glass slide used	Dust or debris could be misinterpreted as agglutination therefore only clean and dry glass slides must be used for testing

Contd...

Contd...

Possible causes	*Solutions*
6. Latex particles contaminated with positive control/positive sample	Care must be taken to see that the latex reagent dropper tip does not touch the sample or control taken on the slide during dispensing of the reagent
7. CRP levels increase in women who are on oral contraceptives	Check the history of the patient
8. Wrong dropper used for dispensing the sample	Accessories provided with the kit only must be used for optimum results
9. Increase in drop size, thereby leading to excess reagent dispensed	Excess reagent dispensed gives false positive results at borderline concentrations. Ensure that exactly one drop of reagent is dispensed onto the slide
10. Reagent dropper not held vertically while dispensing	To ensure accurate dispensing of the regent, hold the reagent dropper vertically while dispensing the reagent
11. Dried latex particles observed in the latex reagent • During slide test with negative control • In the dropper of the vial (due to freezing of the latex reagent during storage) • Improper dispensing of the entire reagent from dropper	Immediately after performing the test, transfer the contents of the reagent dropper back into the reagent vial Ensure that no reagent is left behind in the dropper. Close the cap of the reagent vial properly and store it back at 2–8°C Do not freeze the reagent vial
12. Cross contamination due to the usage of the same mixing stick	Separate mixing stick should be used for mixing the controls and the sample

Problem: False negative results

Possible causes	*Solutions*
1. The reagent may be damaged due to microbial contamination or exposure to extreme temperatures.	Performance of the reagents can be verified by using positive control/known positive sample.
2. Weak agglutination may be interpreted as negative.	Shake the latex reagent well before use to disperse the latex particles uniformly and improve test readability.
3. Samples stored for a long period of time are used as specimens.	Samples can be stored for upto a week at 2–8°C.
4. Hemolyzed samples may be used for testing.	Do not use hemolyzed samples for testing.
5. Prozoning due to high levels of CRP. CRP is an acute phase reactant; it is normal to have peaks in CRP levels in acute conditions such as trauma, myocardial infarction and ischemic heart diseases.	Dilute the specimen and rerun the test. Carry out a semiquantitative assay to determine the CRP level in the sample.
6. If the conclusion of false negative results has been arrived at by comparision with another kit, other kit could be giving a false positive reaction.	Run the test with a third kit to validate results.
7. Decrease in drop size, thereby leading of less amount of reagent dispensed.	Less amount of reagent dispensed gives false negative results at borderline concentrations. Ensure that exactly one drop of reagent is dispensed onto the slide.
8. The latex reagent might have been frozen.	The latex reagent should never be frozen as freezing leads to the dissociation of human IgG coated on the latex. The free IgG neutralizes the RF present in the sample thereby leading to false negative results.

Problem: Positive control giving negative reaction

Possible causes	*Solutions*
1. The positive control may have deteriorated due to contamination or exposure to extreme temperatures	Check the performance of the latex reagent; using known positive samples, if the latex reagent is working then the positive control may have deteriorated

Problem: Positive result with our kit and negative with another kit or vice versa

Possible causes	*Solutions*
1. The sensitivity of our kit is 0.6 mg/dL hence if another kit has a cutoff of more or less than 0.6 mg/dL then the other kit may produce a false positive/negative result accordingly	Check the sensitivity of the another kit with known true CRP calibrator before confirming the test results

SLIDE TEST FOR ANTISTREPTOLYSIN O (RHELAX ASO®)

(Courtesy: Tulip Group of Companies)

Summary

Streptococcus belongs to the family of Lactobacillaceae and the majority are facultative anerobes. The facultative anerobic streptococci are divided into two categories:
a.Those which produce soluble hemolysin and
b.Those which do not produce soluble hemolysin

The first group of streptococci are called β-hemolytic streptococci which can be further subdivided into group (a), group (b), group (c) and group (d). It includes most of the species associated with primary streptococcal infections in humans. The group (a) β-hemolytic streptococci produce various exotoxins such as streptolysin O and streptolysin S that can act as antigens. The affected individuals produce specific antibodies against streptolysin O, namely anti streptolysin O (ASO). Determination of these antibodies is very useful for the diagnosis of streptococcal infections and their relative effects such as rheumatic fever and acute glomerulonephritis. An elevated ASO titer of more than 200 IU/mL may indicate an acute streptococcal infection.

Reagent

1. RHELAX ASO reagent: A uniform suspension of polystyrene latex particles coated with streptolysin O.
2. Positive control, reactive with RHELAX ASO reagent.
3. Negative control, nonreactive with RHELAX ASO reagent.

The RHELAX ASO reagent system is standardized to detect antibodies to streptolysin O in concentrations ranging from 200 to 4000 IU/mL.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of reagent is as per the expiry date mentioned on the reagent vial labels.

Principle

The RHELAX ASO slide test for detection of antibodies to streptolysin O is based on the principle of agglutination. The test specimen (serum) is mixed with RHELAX ASO latex reagent and allowed to react. If antibodies to streptolysin O are present in concentrations more than 200 IU/mL, but less than 4000 lU/mL, then a visible agglutination is observed. If antibodies to streptolysin O are not present or are in concentrations less than 200 IU/mL, then no agglutination will be observed.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the reagents derived from human source have been tested for HBsAg and anti-HIV antibodies and are found to be non-reactive. However, handle the material as if infectious.
3. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagent be verified with the positive and negative controls provided with the kit.
5. Shake the RHELAX ASO latex reagent well before use to disperse the latex particles uniformly and improve test readability.
6. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry.
7. Accessories provided with the kit only must be used for optimum results.

Specimen Collection and Preparation

No special preparation of the patient is required prior to specimen collection by approved techniques.

Only serum should be used for testing. Should a delay in testing occur, store the sample at 2 to 8°C. Samples can be stored for up to a week. Do not use hemolyzed serum.

Material Provided with the Kit

Reagent

RHELAX ASO latex reagent, positive control, negative control.

Accessories

Glass slide with six reaction circles, sample dispensing pipettes, mixing sticks, rubber teat.

Additional Material Required

Stopwatch, high intensity direct light source, isotonic saline, pipettetes, test tubes.

Test Procedure

Bring reagent and samples to room temperature before testing.

Qualitative Method

1. Pipettete one drop of test sample onto the glass slide using a disposable pipette provided with the kit.
2. Add one drop of RHELAX ASO latex reagent to the drop of test sample on the slide.
3. Using a mixing stick, mix the serum and the RHELAX ASO latex reagent uniformly over the entire circle. Do not let the dropper tip touch the liquid on the slide.
4. Immediately start a stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at 2 minutes.

Semiquantitative Method

1. Using isotonic saline, prepare serial dilutions of the serum sample positive in the qualitative method 1:2, 1:4, 1:8, 1:16 and so on.
2. Pipette the diluted specimens onto the slide. Start with the 1:2 diluted test specimen.
3. Add a drop of RHELAX ASO reagent to it and mix well. Spread the mixture uniformly over the entire circle.
4. Immediately start a stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at 2 minutes. Proceed similarly with each dilution as test specimen.

Interpretation of Test Results

Qualitative Method

Agglutination is a positive test result and indicates the presence of detectable levels of antistreptolysin O in the test specimen.

No agglutination is a negative test result and indicates the absence of detectable levels of antistreptolysin O in the test specimen.

Semiquantitative Method

Agglutination in the highest serum dilution corresponds to the amount of ASO in IU/mL present in the test specimen. The concentration of ASO can be calculated as follows:

$$\text{ASO (IU/mL)} = S \times D$$

where S = Sensitivity of the reagent, i.e. 200 IU/ ml.

D = Highest dilution of serum showing agglutination.

Remarks

1. Markedly lipemic, hemolyzed and contaminated serum samples could produce non-specific results.
2. Serum samples having markedly higher protein content may produce nonspecific reagent aggregation.
3. Use of plasma rather than serum can lead to false positive results.
4. Do not read results beyond 2 minutes.
5. It is recommended that all positive test results should be further tested with methods enabling quantitation of ASO titers.
6. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.

Troubleshooting

Problem: False positive results

Possible causes	Solutions
1. Plasma is used as a test specimen	Only serum must be used for testing
2. Samples are stored for a long period	Should a delay in testing occur, store samples at 2–8°C. Samples can be stored for upto a week at 2–8°C
3. Cross contamination due to the usage of the same mixing stick	Separate mixing stick should be used for mixing the controls and the sample
4. Markedly lipemic, hemolyzed and contaminated serum samples could produce non-specific results	Avoid using lipemic, hemolyzed and contaminated serum samples for testing

Contd...

Contd...

Possible causes	Solutions
5. Drying of the reagent on the slide	Do not read results beyond 2 minutes. The test should not be carried out directly under the fan
6. Presence of dust or debris on the glass slide used	Dust or debris could be misinterpreted as agglutination therefore only clean and dry glass slides must be used for testing
7. Latex particles contaminated with positive control/positive sample	Care must be taken to see that the latex reagent dropper tip does not touch the sample or control taken on the slide during dispensing of the reagent
8. Wrong dropper used for dispensing the sample	Accessories provided with the kit only must be used for optimum results
9. Increase in drop size, thereby leading to excess reagent dispensed	Excess reagent dispensed gives false positive results at borderline concentrations. Ensure that exactly one drop of reagent is dispensed onto the slide
10. Reagent dropper not held vertically while dispensing	To ensure accurate dispensing of the reagent, hold the reagent dropper vertically while dispensing the reagent
11. Cross contamination due to the usage of the same mixing stick	Separate mixing stick should be used for mixing the controls and the sample.
12. Dried latex particles observed in the latex reagent • During slide test with negative control • In the dropper of the vial (due to freezing of the latex reagent during storage) • Improper dispensing of the nature reagent from dropper	Immediately after performing the test, transfer the contents of the reagent dropper back into the reagent vial Ensure that no reagent is left behind in the dropper. Close the cap of the reagent vial properly and store it back at 2–8°C Do not freeze the reagent vial

Problem: Delayed agglutination

Possible causes	*Solutions*
1. Reagents not brought to room temperature before testing.	Bring the regents to room temperature before carrying out the test.

Problem: False negative results

Possible causes	Solutions
1. The reagent may be damaged due to microbial contamination or exposure to extreme temperatures	Performance of the reagents can be verified by using positive control/known positive sample
2. Weak agglutination may be interpreted	Shake the latex reagent well before use to disperse the latex particles uniformly and improve the test readability
3. Prozoning due to antibodies to streptolysin O being in concentrations greater than 4000 IU/mL	Dilute the serum and check for agglutination. If no agglutination is observed with the neat sample but agglutination is observed with the diluted sample, then it may be due to prozoning. Determine the titer of ASO
4. Samples stored for a long period of time are used as specimens	Samples can be stored for upto a week at 2–8°C
5. Hemolyzed samples may be used for testing	Avoid using hemolyzed samples.
6. If the conclusion of false negative results has been arrived at by comparison with another kit, this other kit could be giving a false positive reaction	Run the test with a third kit to validate the results
7. Decrease in drop size, thereby leading to less amount of reagent dispensed	Less amount of reagent dispensed gives false negative results at borderline concentrations. Ensure that exactly one drop of reagent is dispensed onto the slide
8. The latex reagent might have been frozen	The latex reagent should never be frozen as freezing leads to the dissociation of human IgG coated on the latex. The free IgG neutralizes the RF present in the sample thereby leading to false negative results

Problem: Positive control giving negative reaction

Possible causes	*Solutions*
1. The positive control may have deteriorated due to contamination or exposure to extreme temperatures	Check the performance of the latex reagent; using known positive samples, if the latex reagent is working then the positive control may have deteriorated

Problem: Positive result with this kit and negative with other kit or vice versa

Possible causes	*Solutions*
1. The sensitivity of our kit is 200 IU/mL hence, if the other kit has a cutoff of more or less than 200 IU/mL then the other kit may produce a false positive/negative result accordingly	Check the sensitivity of the other kit with known true ASO calibrator

SLIDE TEST FOR RHEUMATOID FACTORS (RHELAX RF®)

(Courtesy: Tulip Group of Companies)

Summary

Sometimes autoantibodies are produced by the human body against self-antigens. The precise role that this aberrant immunity plays in the pathogenesis of certain rheumatic diseases is unknown. However, the presence of these autoantibodies serve as credible marker of the disease.

In rheumatoid arthritis, diagnostically useful autoantibodies termed as "Rheumatoid factors (RF) can be detected which are immunoglobulins of the classes IgM, IgG, IgA and IgE. Practically, IgM class RF with specificity to human IgG (Fc) is the most useful prognostic marker of RF. The clinical significance of RF determinations consists in differentiation between rheumatoid arthritis, in which RFs of modified IgM class have been demonstrated in the serum of approximately 80% of the cases examined and rheumatic fever, in which RFs are almost always absent. The agglutination test is most frequently used because of its greater sensitivity and simplicity. The RHELAX RF is a latex agglutination slide test for detection of rheumatoid factors of the IgM class.

Reagent

1. RHELAX RF reagent: A uniform suspension of polystyrene latex particles coated with suitably modified Fc fraction of IgG. The reagent is standardised to detect 10 IU/mL of RF or more.
2. Positive control, reactive with the RHELAX RF reagent.
3. Negative control, nonreactive with the RHELAX RF reagent.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagents at 2 to 8°C. Do not freeze.
2. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

The RHELAX RF slide test for detection of rheumatoid factors is based on the principle of agglutination. The test specimen is mixed with RHELAX RF latex reagent and allowed to react. If RF is present within detectable levels, then a visible agglutination is observed. If RF is absent below detectable levels, then no agglutination is observed.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the reagents derived from human source have been tested for HBsAg and anti-HIV antibodies and are found to be nonreactive. However, handle the material as if infectious.
3. The reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagent be verified with the positive and negative controls provided with the kit.
5. Shake the RHELAX RF latex reagent well before use to disperse the latex particles uniformly and improve test readability.

6. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry.
7. Accessories provided with the kit only must be used for optimum results.

Specimen Collection and Preparation

No special preparation of the patient is required prior to specimen collection by approved techniques.

Only serum should be used for testing. Should a delay in testing occur, store the sample at 2 to 8°C. Samples can be stored for up to a week. Do not use hemolyzed serum.

Material Provided with the Kit

Reagent

Rhelax RF latex reagent, positive control, negative control.

Accessories

Glass slide with six reaction circles, sample dispensing pipettes, mixing sticks, rubber teat.

Additional Material Required

Stopwatch, test tubes, high intensity direct light source, isotonic saline.

Test Procedure

Bring reagent and samples to room temperature before use.

Qualitative Method

1. Pipette one drop of serum onto the glass slide using the disposable pipette provided with the kit.
2. Add one drop of RHELAX RF latex reagent to the drop of serum on the slide. Do not let the dropper tip touch the liquid on the slide.
3. Using a mixing stick, mix the serum and the RHELAX RF latex reagent uniformly over the entire circle.
4. Immediately start a stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at 2 minutes.

Semiquantitative Method

1. Using isotonic saline, prepare serial dilutions of the serum sample positive in the qualitative method 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and so on.
2. Pipette each dilution of the serum sample onto separate reaction circles.
3. Add one drop of RHELAX RF latex reagent to each drop of the diluted serum sample on the slide. Do not let the dropper tip touch the liquid on the slide.
4. Using a mixing stick, mix the sample and the latex reagent uniformly over the entire circle.
5. Immediately start a stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at 2 minutes.

Interpretation of Test Results

Qualitative Method

Agglutination is a positive test result and indicates the presence of rheumatoid factors in the test specimen. No agglutination is a negative test result and indicates the absence of rheumatoid factors in the test specimen.

Semiquantitative Method

Agglutination in the highest serum dilution corresponds to the approximate amount of rheumatoid factors in IU/mL present in the test specimen.

To calculate the RF in lU/mL, use the following formula:

RF (IU/mL) = S × D

Where S = Sensitivity of the reagent, i.e. 10 IU/ mL.

D = Highest dilution of serum showing agglutination.

Remarks

1. Markedly lipemic, hemolyzed and contaminated serum samples could produce non-specific results.
2. Use of plasma rather than serum can lead to false positive results.
3. Do not read results beyond 2 minutes.
4. Rheumatoid factors are not exclusively found in rheumatoid arthritis but sometimes in syphilis, systemic lupus erythematosus, hepatitis, hypergammaglobulinemia also.
5. It is recommended that results of the test should be correlated with clinical findings to arrive at the final diagnosis.
6. The RHELAX RF reagent is free from prozone effect at RF levels between 10 IU/mL to 2300 IU/mL of RF concentration.
7. The RHELAX RF reagent is sensitive to the presence of IgM RF with heterogeneous specificity.

Troubleshooting

Problem: False positive results

Possible causes	Solutions
1. Plasma is used as a test specimen	Only serum must be used for testing
2. Samples are stored for a long period	Should a delay in testing occur, store samples at 2–8°C. Samples can be stored for upto a week at 2–8°C
3. Markedly lipemic, hemolyzed and contaminated serum samples could produre non-specific results	Avoid using lipemic, hemolyzed and contaminated serum samples for testing
4. Drying of the reagent on the slide	Do not read results beyond 2 minutes. The test should be carried out directly under the fan
5. Presence of dust or debris on the glass slide used	Dust or debris could be misinterpreted as agglutination therefore, only clean and dry glass slides must be used for testing
6. Latex particles contaminated with positive control/positive sample	Care must be taken to see that the latex reagent dropper tip does not touch the sample or control taken on the slide during dispensing of the reagent
7. Wrong dropper used for dispensing the sample	Accessories provided with the kit only must be used for optimum results
8. Increase in drop size, thereby leading to excess reagent dispensed.	Excess reagent dispensed gives false positive results at borderline concentrations. Ensure that exactly one drop of reagent is dispensed onto the slide
9. Reagent dropper not held vertically while dispensing	To ensure accurate dispensing of the reagent, hold the regent dropper vertically while dispensing the reagent
10. Dried latex particles observed in the latex reagent: • During slide test with negative control • In the dropper of the vial (due to freezing of the latex reagent during storage) • Improper dispensing of the entire reagent from dropper	Immediately after performing the test, transfer the contents of the reagent dropper back into the reagent vial Ensure that no reagent is left behind in the dropper. Close the cap of the reagent vial properly and store it back at 2–8°C Do not freeze the reagent vial

Problem: False negative results

Possible causes	Solutions
1. The reagent may be damaged due to microbial contamination or exposure of extreme temperatures	Performance of the reagents can be verified by using positive control/ known positive sample
2. Weak agglutination may be interpreted as a negative result	Shake the latex reagent well before use to disperse the latex particles uniformly and improve test readability
3. Samples stored for a long period of time are used as specimens	Samples can be stored only for a week at 2–8°C
4. Hemolyzed samples may be used for testing	Avoid using hemolyzed samples
5. If the conclusion of false negative result has been arrived by comparison with another kit, this other kit could be giving a false positive reaction	Run the test with a third kit to validate the results
6. Decrease in drop size, therby leading to less amount of reagent dispensed	Less amount of reagent dispensed gives false negative results at borderline concentrations. Ensure that exactly one drop of reagent is dispensed onto the slide
7. The latex reagent might have been frozen	The latex reagent should never be frozen as freezing leads to the dissociation of human IgG coated on the latex. The free IgG neutralizes the RF present in the sample thereby leading to false negative results

Problem: Positive control giving negative reaction

Possible causes	*Solutions*
1. The positive control may have deteriorated due to contamination or exposure to extreme temperatures	Check the performance of the latex reagent using known positive samples. If the latex reagent is working then the positive control may have deteriorated

Problem: Delayed agglutination

Possible causes	*Solutions*
1. Reagents not brought to room temperature before testing	Bring the reagents to room temperature before carrying out the test

SLIDE TEST FOR ANTI DEOXYRIBONUCLEOPROTEIN (RHELAX SLE®)

(Courtesy: Tulip group of companies)

Summary

The presence of autoantibodies to nuclear proteins is a common finding in systemic lupus erythematosus (SLE) and other collagen diseases.

Anti-DNP is present in high titers in the serum of majority of SLE patients with active disease but is present occasionally in remission states. Although anti-DNP is found exclusively in SLE, only low titers may be detected in diseases such as chronic hepatitis, periarteritis nodosa, dermatomyositis, scleroderma and drug hypersensitivity.

Reagent

The Rhelax SLE reagent is a ready-to-use uniform suspension of polystyrene latex particles coated with deoxyribonucleoprotein (DNP). Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

Store the reagent at 2 to 8°C. Do not freeze. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Latex particles coated with DNP will agglutinate when mixed with serum containing anti-DNP. No agglutination indicates absence of anti-DNP in the serum.

Note

1. *In vitro* diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the components derived from human source have been tested for HBsAg and anti-HIV antibody and are found to be non-reactive. However, handle the material as if infectious.
3. The reagents contain sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
4. The reagent can be damaged due to microbial contamination or exposure to extreme temperatures. It is recommended that the performance of the reagent be verified with the positive and negative controls provided with the kit.
5. Shake the latex reagent well before use to disperse the latex particles uniformly and to improve test readability.
6. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Use fresh clear serum samples. In case of delay in testing, store the serum samples at 2 to 8°C for up to 72 hours. For longer storage, freeze the serum. However, repeated freezing and thawing of samples should be avoided.

Material Provided with the Kit

Reagent Pack

Rhelax SLE latex reagent, positive control, negative control.

SEROLOGICAL DIAGNOSIS RHEUMATIC DISORDERS WITH LATEX AGGLUTINATION TESTS RF, CRP, ASL

Spectrum			*Probable diagnosis*	*Further test indicated*
Latex agglutination tests				
CRP	ASL	RF		
–	+	–	Rheumatic fever	Anti-streptolysin titer determination
+	+	–	Acute rheumatic fever	ADNase B, AHy, ANADase
–	–	+	Chronic polyarthritis (CP)	Quantitative RF determination
+	–	+	Inflammation in acute attack	Quantitative RF determination
+	–	–	Acute inflammatory (CP) (early stage)	Quantitative RF determination
–	–	+	Lupus erythematosus (LE), Polyarteritis nodosa, Dermatomyositis, Polymyositis, scleroderma	ANA (antinuclear Ab) Anti-ds-DNA against native double filament
–	–	–	Seronegative chronic polyarthritis (e.g. juvenile arthritis)	ANA/HLA B-27
(+)	–	–	Behçet's disease Collagen/MCTD (mixed connective tissue disease) Reiter's disease Psoriasis LE Pseudo-LE (drug induced)	HLA B 27 ANA, Anti-ds-DNA HLA B 27, *Yersinia* antibody detection - ANA AMA (antimitochondrial antibody)
+	–	–	Inflammatory-degenerative rheumatic disorders Gout (acute attack) Reactive arthritis (after infection)	— Uric acid Gonococcal detection, virus serology (e.g. Rubella infection)
+	–	+	By chronic appearance of age and other inflammatory conditions	Quantitative RF
– = Negative, + = Positive, (+) = Weakly positive				

Accessories Pack

Glass slide with six reaction circles, mixing sticks, rubber teats, sample dispensing pipettes.

Additional Material Required

Test tube (10 × 75 mm), Pipettes, isotonic saline, stopwatch, direct light source.

Test Procedure

Bring all reagents and samples to room temperature before use.

Qualitative Method

1. Place one drop of sample to be tested onto one of the reaction circles of the glass slide using a sample dispensing pipette provided with the kit.
2. Place one drop of positive and negative control onto separate reaction circles of the glass slide.
3. Gently shake the latex reagent and add one drop to each sample and control taken on the slide.
4. Mix with separate mixing sticks, spreading the mixture uniformly over the entire reaction circle.
5. Immediately start a stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at 3 minutes.

Semiquantitative Method

1. Using isotonic saline prepare serial dilutions of the serum sample 1:2, 1:4, 1:8, 1:16, 1:32.
2. Place each dilution of the serum sample onto separate reaction circles of the slide.

3. Add one drop of well-mixed latex reagent to each dilution of the sample on the slide.
4. Mix with separate mixing sticks, spreading the mixture uniformly over the entire reaction circle.
5. Immediately start a stopwatch. Rock the slide gently, back and forth, observing for agglutination macroscopically at 3 minutes.

Interpretation of Results

Qualitative Method

Agglutination is a positive test result and indicates presence of anti-DNP in the test specimen.

No agglutination is a negative test result and indicates absence of anti-DNP in the test specimen.

Semiquantitative Method

The titer of the serum is the reciprocal of the highest dilution which gives agglutination.

Remarks

1. Markedly lipemic, hemolyzed and contaminated serum samples could produce nonspecific results.
2. Use of plasma rather than serum can lead to false positive results.
3. Anti-DNP may be found in diseases other than SLE. Low titers have been detected in rheumatoid arthritis, chronic hepatitis, periarteritis nodosa, dermatomyositis, scleroderma, atypical pneumonia, tuberculosis and lymphoma.

Troubleshooting

Problem: False positive results

Possible causes	*Solutions*
1. Contamination of the latex reagent with positive control or positive sample	Precaution should be taken so that the dropper tip of the reagent does not touch the samples and controls on the glass slide
2. Samples are stored for a long period	Should a delay in testing occur, store samples at 2–8°C. Samples can be stored for upto a week at 2–8°C
3. Cross contamination due to the usage of the same mixing stick	Separate mixing stick should be used for mixing the controls and the sample
4. Markedly lipemic, hemolyzed and contaminated serum samples	Using lipemic, hemolyzed and contaminated samples produce non-specific results. Avoid using such samples
5. Drying of the reagent on the slide	Do not read results beyond 2 minutes. The test should not be carried out directly under the fan
6. Presence of dust or debris on the glass slide used	Dust or debris could be misinterpreted as agglutination therefore only can dry glass slides must be used for testing
7. Latex particles contaminated with positive control/positive sample	Care must be taken to see that the latex reagent dropper tip does not touch the sample or control taken on the slide during dispensing of the reagen
8. Wrong dropper used for dispensing the sample	Accessories provided with the kit only must be used for optimum results
9. Increase in drop size, thereby leading to excess reagent dispensed	Excess reagent dispensed gives false positive results at borderline concentrations. Ensure that exactly one drop of reagent is dispensed onto the slide
10. Reagent dropper not held vertically while dispensing	To ensure accurate dispensing of the reagent, hold the reagent dropper vertically while dispensing the reagent
11. Cross contamination due to the usage of the same mixing stick	Separate mixing stick should be used for mixing the controls and the sample
12. Dried latex particles observed in the latex reagent: • During slide test with negative control • In the dropper of the vial (due to freezing of the latex reagent during storage) • Improper dispensing of the entire reagent from dropper	Immediately after performing the test, transfer the contents of the reagent dropper back into the reagent vial Ensure that no reagent is left behind in the dropper Close the cap of the reagent vial properly and store it back at 2–8°C. Do not freeze the reagent vial
13. Low titers of Anti-DNP may also be found in clinical conditions such as RA, chronic hepatitis, periarteritis nodosa, dermato myosis, Scleroderma, atypical pneumonia, tuberculosis and lymphoma.	The clinical history of the patient should be checked for any of these disorders

Problem: Delayed positive results

Possible causes	*Solutions*
1. Cold reagents have been tested	The reagents should be tested only after attaining room temperature

Problem: False negative results

Possible causes	Solutions
1. The reagent may be damaged due to microbial contamination or exposure to extreme temperatures	Performance of the reagents can be verified by using positive control/ known positive sample
2. Weak agglutination may be interpreted as negative	Shake the latex reagent well before use to disperse the latex particles uniformly and improve test readability
3. Samples stored for a long period of time are used as specimens	Sample can be stored for upto a week at 2–8°C. Only serum must be used for testing
4. Sample may be hemolyzed or contaminated	Avoid using hemolyzed or contaminated samples for testing
5. If the conclusion of false negative results has been arrived at by comparison with another kit, this other kit could be giving a false positive reaction	Run the test with a third kit to validate results
6. The latex reagent might have been frozen	The latex reagent should never be frozen as freezing leads to the dissociation of the DNP coated on the latex. The free IgG neutralizes the RF present in the sample thereby leading to false negative results

Problem: Positive control giving negative reaction

Possible causes	*Solutions*
1. The positive control may have deteriorated due to contamination or exposure to extreme temperatures	Check the performance of the latex reagent; using known positive samples, if the latex reagent is working then the positive control may have deteriorated

Problem: Delayed agglutination

Possible causes	*Solutions*
1. Reagents not brought to room temperature before testinG	Bring the reagents to room temperature before carrying out the test

AUSTRALIA ANTIGEN HBSAG (VIRUTEX HBsAg)

(*Courtesy:* Tulip Group of Companies)

Slide Test for Hepatitis B Surface Antigen

Summary

Blood containing hepatitis B virus (HBV) is potentially infectious. In most cases, detectable levels of hepatitis B surface antigen (HBsAg) circulate in the bloodstream of an infected person, 2 to 3 weeks prior to the appearance of clinical symptoms. These levels are especially elevated in the symptomatic phase, thereafter the levels slowly decline. Detection of HBV using HBsAg as a marker to screen blood donors is essential to reduce the risk of transmission of hepatitis B by blood transfusion.

Reagent

1. Virutex HBsAg reagent A uniform suspension of polystyrene latex particles coated with IgG class of monoclonal Anti-HBsAg antibodies.
2. Positive control, reactive with the Virutex latex reagent.
3. Negative control, nonreactive with the Virutex latex reagent.

Virutex HBsAg reagent conforms to the sensitivity requirements of a "Third generation" test. Each batch of reagent undergoes rigorous quality control at various

stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf life of reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Latex particles coated with anti-HBsAg antibodies will agglutinate when mixed with serum or plasma containing hepatitis B surface antigen within the detectable levels. Agglutination is absent when the hepatitis B surface antigen is absent or not within the detectable levels.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the reagents derived from human source have been tested for HBsAg and anti-HIV antibody and are found to be non-reactive. However, handle the material as if infectious.
3. Reagent contains sodium azide 0.1% as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
4. The reagents can be damaged due to microbial contamination or exposure to elevated temperatures. It is recommended that the performance of the reagents be verified by testing with the negative or positive controls provided with the kit.
5. Shake the latex antigen vial gently before use to disperse the latex particles uniformly and improve the test readability.
6. Use only a thoroughly clean and dry glass slide. Clean the slide with distilled water and wipe dry before use.
7. Accessories provided with the kit only must be used for optimum results.

Sample Collection and Storage

No special preparation of the patient is required prior to sample collection by approved techniques. Do not use hemolyzed samples. Though plasma may be used, fresh serum is preferable. In case of delay in testing, store the samples at 2 to 8°C for up to 24 hours.

Material Provided with the Kit

Reagent Pack

Latex reagent coated with anti-HBsAg antibody, positive control reactive with the latex reagent, negative control nonreactive with the latex reagent.

Accessories Pack

Glass slide with six reaction circles, mixing sticks, rubber teats, sample dispensing pipettes.

Additional Material Required

Test tubes (10 × 75 mm), pipettes, isotonic saline, stopwatch, direct light source.

Procedure

Bring reagent and samples to room temperature before testing.

1. Pipette one drop of sample to be tested onto one of the reaction circles of the glass slide using a sample dispensing pipette, provided with the kit.
2. Prepare a 1:40 dilution (0.05 mL serum + 1.95 mL isotonic saline) of samples to be tested in isotonic saline.
3. Pipette one drop of the diluted sample on the next reaction circle of the glass slide.

3a. In steps 1 and 3 above, carefully aspirate the sample into the dispensing pipette avoiding sample entering the rubber teat and subsequent cross contamination.

4. Place one drop of positive and negative control onto the remaining reaction circles of the slide (do not dilute controls).
5. Shake the latex reagent vial gently to uniformly disperse the reagent suspension. Add one drop of the latex reagent to each of the samples and controls on the slide.
6. Mix with separate mixing sticks, spreading the mixture uniformly over the entire reaction circle.
7. Immediately start a stopwatch. Rock the slide gently back and forth, observing for agglutination macroscopically at 5 minutes.

Interpretation of Results

1. No agglutination with diluted and neat samples is a negative test result: HBsAg absent.
2. Agglutination with neat sample but no agglutination with diluted sample is a positive test result: HBsAg present (weak positive).
3. Agglutination with both neat and diluted samples is a positive test result: HBsAg present (moderate positive).
4. Agglutination with diluted sample but no agglutination with neat sample is a positive test result: HBsAg present (strong positive).

Remarks

1. The positive control has been inactivated at 60°C for 10 hours and is not expected to be infectious.

2. Presence of autoantibodies such as RF and heterophile antibodies may interfere with the test giving a false positive result. Probability of such an occurrence is low (less than 1% of all samples).
3. Since Virutex is only a quick screening test, for confirmation of the results, a confirmatory test should be used.
4. Positive and negative controls should be run with each series of tests and the results compared with unknown specimens to distinguish possible granularity from agglutination.
5. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
6. The reusable glass slide should be first immersed in sodium hypochlorite 5% solution and then rinsed with tap water and then distilled water. Wipe thoroughly dry before use.
7. As the biggest risk to laboratory personnel is from uncharacterized random samples, it is strongly recommended that as a safety measure hand gloves should be worn during the entire test procedure.
8. Samples that are contaminated, hemolyzed, lipemic or highly icteric may give nonspecific reactions.

ONE-STEP TEST FOR HBsAg VIRUCHECK® DEVICE

(*Courtesy:* Tulip Group of Companies) (dipstick method also available)

Principle

Virucheck one-step test for HBsAg utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flows through the membrane assembly within the test device, the colored anti-HBsAg colloidal gold conjugate complexes with HBsAg in the sample. This complex moves further on the membrane to the test region where it is immobilized by the anti-HBsAg antiserum coated on the membrane leading to formation of a pink colored band which confirms a positive test result. Absence of this colored band in the test region indicates a negative test result. The unreacted conjugate and unbound complex if any move further on the membrane and are subsequently immobilized by the antimouse antiserum coated on the membrane at the control region, forming a pink band. This control band serves to validate the test results.

Reagents and Materials Supplied

Each individual pouch contains:

1. Test device: Contains membrane assembly predispensed with anti-HBsAg antiserum-colloidal gold conjugate and anti-HBsAg antiserum and antimouse antiserum coated at the respective regions.
2. Disposable plastic dropper.

Storage and Stability

The sealed pouches in the test kit may be stored between 4–30°C for the duration of the shelf-life as indicated on the pouch.

Note

1. For in vitro diagnostic use only. *Not for medicinal use.*
2. Do not use beyond expiry date.
3. Read the instruction carefully before performing the test.
4. Handle all specimens as potentially infectious.
5. Follow standard biosafety guidelines for handling and disposal of potentially infective material.

Specimen Collection and Preparation

No special preparation of the patient is necessary prior to collection by approved techniques. Though fresh serum/plasma is preferable, serum/plasma specimen may be stored at 2 to 8°C for up to 24 hours, in case of delay in testing. Do not use hemolyzed, turbid or contaminated samples. Turbid samples must be centrifuged and clear supernatant must be used for testing.

Testing Procedure and Interpretation of Results

1. Bring the sealed pouch to room temperature, open the pouch and remove the device. Once opened, the device must be used immediately.
2. Dispense two drops of serum/plasma specimen into the sample well 'S' using the dropper provided. Refrigerated specimens must be brought to room temperature prior to testing.
3. At the end of 15 minutes, read the results as follows (Fig. 22.27):

Negative

Only one colored band appears on the control region 'C'.

Positive

In addition to the control band, a distinct colored band also appears on the test region 'T'.

4. The test should be considered invalid if neither the test band nor the control band appear. Repeat the test with a new device.
5. Although, depending on the concentration of HBsAg in the specimen, positive results may start appearing as early as 2 minutes, negative results must be confirmed only at the end of 15 minutes.
6. In case of doubtful result at 15 minutes, the test may be extended up to 30 minutes to get a clear background.

Limitation of the Test

1. Presence of elevated levels of other antigens such as RF and cross reacting autoantibodies such as antibodies to HLA DR4 may yield false positive results. This may occur in less than 1% of the specimens. For confirmation of results, a confirmatory test must be used.
2. This test detects the presence of HBsAg in the specimen and hence should not be used as the sole criterion for the diagnosis of hepatitis infection.
3. As with all diagnostic test, the results must be correlated with clinical findings.

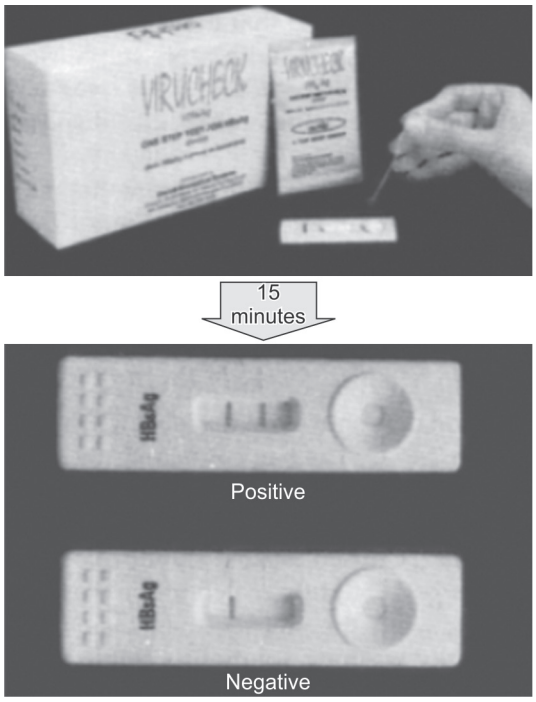


FIG. 22.27: Virucheck result reading

HCV FLAVICHECK® DEVICE

Courtesy: Tulip Group of Companies

One-Step Immunochromatographic Test for HCV Antibodies

Introduction

Flavicheck-HCV is a rapid self-performing, third generation, qualitative one-step, two-site sandwich immunoassay for the detection of antibodies specific to the hepatitis C virus in human serum and plasma. The test employs recombinant proteins derived from the core, NS3, NS4, and NS5 regions of the HCV genome. Combination of these proteins in a double antigen sandwich system not only affords antibody detection to greater number of HCV-encoded epitopes but also earlier detection of seroconversion following HCV infection.

Summary

Hepatitis C virus (HCV) is a single-stranded RNA virus of the Flaviviridae family. The HCV is now known to be the causative agent for most, if not all non-A, non-B hepatitis (NANBH). Antibodies to the hepatitis C encoded antigens are prevalent in the sera of HCV infected individuals. Detection of these antibodies indicates exposure to the hepatitis C virus.

Principle

Flavicheck-HCV utilizes the principle of lateral flow immunochromatography, a unique two site double antigen sandwich immunoassay on a membrane. As the test specimen flows through the membrane assembly of the test device, the colored HCV specific recombinant antigen-colloidal gold conjugate complexes with HCV antibodies in the sample. This complex moves further on the membrane to the test region 'T' where it is immobilized by the HCV specific recombinant antigens coated on the membrane leading to formation of a colored band which confirms a positive test result. Absence of this colored band in the test region indicates a negative test result. The unreacted conjugate and unbound complex, if any, move further on the membrane and are subsequently immobilized by the antirabbit antibodies coated on the membrane at the control region 'C', forming a colored band. This control band serves to validate the reagent and assay performance.

Reagents and Materials Supplied

Kit Components

1. Flavicheck-HCV membrane test assembly (device) comprises of HCV specific recombinant antigen-colloidal gold conjugate co-dispensed with rabbit IgG colloidal gold conjugate; predispensed with HCV specific recombinant antigen at region T', and anti-rabbit antiserum predispensed at the region 'C', along with a plastic sample dropper and desiccant.
2. Package insert.

Storage and Stability

The test kit should be stored between 4–30°C for the duration of the shelf-life of the kit as indicated on the pouch/kit label.

Note

1. In vitro diagnostic test. Not for medicinal use.
2. Do not use beyond expiry date.
3. Read the package insert carefully before performing the test.
4. Handle all specimen as potentially infectious.
5. Follow standard biosafety guidelines for personal protection, handling and disposal of potentially infectious material.

Specimen Collection and Preparation

1. No prior preparation of the patient is required before sample collection by approved techniques.
2. Fresh serum/plasma is preferable. Serum/plasma may be stored at 2 to 8°C up to 24 hours in case of delay in testing. For long-term storage, freeze the specimen at –20°C for 3 months or –70°C for longer periods.
3. Repeated freezing and thawing of the specimen should be avoided.
4. Do not use hemolyzed, clotted, contaminated, viscous/turbid specimen.
5. Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only used foresting.
6. Do not heat inactivate the sample.

Test Procedure and Interpretation of Results

1. Let the sealed pouches attain room temperature (25–30°C).
2. Tear open the sealed pouches and retrieve the appropriate number of test devices as required. Label the test devices appropriately. Once opened, the devices must be used immediately. The addition of sample must be done at the center of the sample port holding the sample dropper in a vertical position. Ensure the drops are free falling. Use a new sample dropper for each specimen to avoid cross contamination.
3. Dispense two drops of specimen in to the sample port (S) using the dropper provided.
4. At the end of 15 minutes, read the results as follows:

Negative Test Result

Appearance of only one colored band at the control region'C'.

Positive Test Result

Appearance of a colored band at the test region 'T'in addition to the band at control region 'C'.

5. The test should be considered invalid if neither the test nor the control bands appear. Repeat the test with a new device.
6. Based on the concentration of antibodies to HCV in the specimen a positive result may start appearing as early as 2 minutes, however, negative results must be confirmed only at the end of 15 minutes.
7. In case of doubtful results at 15 minutes, the test may be extended up to a maximum of 30 minutes if required.

Remarks

1. Though Flavicheck-HCV is a sensitive and reliable screening test, it should not be used as a sole criterion for diagnosis of HCV infection.
2. All positive specimen should be further tested using appropriate supplemental confirmatory tests. Test samples that are positive by a third generation double antigen sandwich-based assays may be reactive with very early seroconversion samples, which are negative/intermediate with blot-based assays. Such samples should be reconfirmed with the RNA-PCR-based method or must be followed up for seroconversion at a later date.
3. As with all diagnostic tests, results must be correlated with clinical findings to arrive at the final diagnosis.

4. Absence of antibodies to HCV does not indicate that an individual is absolutely free of HCV infection as the collection of sample and its timing vis-a-vis seroconversion will influence the test outcome.
5. Do not compare the intensity of test lines and the control lines to judge the concentration of antibodies in the test specimen.
6. Since various tests for HCV differ in their performance characteristics and antigenic composition, their reactivity patterns may differ.
7. Testing of pooled samples is not recommended.

TORCH Infections: Introduction

Toxoplasma Infection

Toxoplasma gondii is an obligate intracellular protozoan parasite that is probably capable of infecting all species of mammals, including man. The detection of IgM antibodies to *T. gondii* is particularly helpful for the diagnosis of acute/primary infections in "risk" individuals in association with AIDS, organ transplantation and pregnancy. As most of Toxoplasma infections are mild or asymptomatic in otherwise healthy individuals, the detection of *T. gondii*-specific IgM, in absence of detectable specific IgG, has become important for the monitoring of primary infections in pregnant women, as the parasite can lead to birth defects. Moreover, as *T. gondii* infections are most severe in immunocompromised patients, where the disease can be fatal, acute infections due to this parasite have to be distinguished from other disorders. Recently developed, IgM capture assays provide the clinician with a helpful and reliable test, not affected by rheumatoid factor.

Rubella Infection

Infection with Rubella virus in children and adults is a self-limited, mild disease characterized by an erythematous rash, mild upper respiratory symptoms and suboccipital lymphadenopathy. After recovery, the individual is immune to subsequent infection with rubella virus. Primary infection of a pregnant woman, however, particularly in the first trimester of pregnancy, may result in a high risk of fetal infection with severe complications. Congenital Rubella is characterized by cataracts, deafness, congenital heart disease and other malformations which may occur singularly or in combination. It is extremely important therefore, to identify those women who are not immune to rubella and to immunize them well before they become pregnant. This can be achieved by screening the serum for the presence of antibodies to Rubella and a positive result is indicative of immunity. The ELISA has been shown to be a sensitive and reliable procedure for detection of antibodies to Rubella.

Cytomegalovirus Infection

Cytomegalovirus (CMV) infections are widespread and approximately half of the adult population have antibodies to CMV. The majority of CMV infections are asymptomatic but CMV infections can cause serious disease in the newborn infant and the immunocompromised individual. About 2% of pregnant women have either a primary or a reactivated CMV infection during pregnancy and it is estimated that 10 to 20% of congenitally infected newborns will show evidence of disease. Clinical symptoms may range from severe disease with jaundice, hepatosplenomegaly, and central nervous system involvement, to asymptomatic infants who will, however, later develop hearing defects. CMV infections are frequent in individuals with deficient cellular immunity such as cancer patients or person with Acquired Immunodeficiency Syndrome, or those receiving immunosuppresive agents. The detection of antibodies against CMV may be of value as an aid in the diagnosis and in determining the immune status of the patient. Various procedures such as complement fixation, indirect hemagglutination, and indirect immunofluorescent assay have been used to detect CMV antibody. More recently, the enzyme-linked immunosorbent assay (ELISA) has been developed and utilized for serological detection of CMV.

Herpes Simplex Virus (1 + 2) Infections

Herpes Simplex Virus types 1 (HSV-1) and 2 (HSV-2) are large complex DNA-containing viruses which have been shown to induce during infection the synthesis of several proteins, possessing an high number of crossreactive determinants and just a few of type-specific sequences. The majority of primary genital herpes infections and recurrent genital herpes infections are caused by HSV-2. Nongenital herpes infections such as common cold sores are caused primarily by HSV-1. The detection of virus-specific IgM antibodies is important in the diagnosis of acute/primary virus infections or reactivation of a latent one, in the absence of evident clinical symptoms. Asymptomatic infections may happen for HSV in apparently healthy individuals and during pregnancy. Severe herpes infections may happen in immuno-suppressed or immunocompromised patients. Recently developed, IgM enzymeimmunoassays provide the

clinician with a helpful and reliable diagnostic test for the monitoring of "risk" population.

TOXOPLASMA INFECTIONS

Slide Test for Antibodies to *Toxoplasma gondii*, Toxogen®

(*Courtesy*: Tulip Group of Companies)

Summary

Toxoplasmosis is an infectious disease caused by the parasite *Toxoplasma gondii* and affects both animals and humans. In humans this infection is usually acquired by ingesting inadequately cooked meat or from feces of infected cats. Approximately 25 to 50% of the adult population are asymptomatically affected with Toxoplasmosis.

Acquired Toxoplasmosis is usually asymptomatic and benign. In pregnant women, however, the infection acquires a special significance as the parasite may enter the fetal circulation through placenta and cause congenital Toxoplasmosis.

The consequences of congenital Toxoplasmosis range from spontaneous abortion and prematurity to generalized and neurological symptoms. Some infants with congenital Toxoplasmosis may also remain asymptomatic at birth and develop the disease during childhood or adolescence.

Reagent

1. Toxogen latex reagent: A uniform suspension of polystyrene latex particles coated with *Toxoplasma gondii* soluble antigens.
2. Positive control, reactive with the Toxogen latex reagent.
3. Negative control, nonreactive with the Toxogen latex reagent.

TOXOGEN latex reagent is standardized to detect 10-15 lU/mL or more of *Toxoplasma* antibodies. Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2 to 8°C. Do not freeze.
2. The shelf-life of the reagent is as per the expiry date mentioned on the reagent vial label.

Principle

Latex particles coated with *Toxoplasma gondii* antigens will agglutinate when mixed with serum containing antibodies to *Toxoplasma gondii*. Agglutination is absent when antibodies to *Toxoplasma gondii* are absent.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the reagents derived from human source have been tested for HBsAg and antibody to HIV and found to be non-reactive. However, handle the material as if infectious.
3. Reagent contains 0.1% Sodium Azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. Shake the latex/reagent vial gently before use to disperse the latex particles uniformly and improve the test readability.
5. Recap the reagent vials immediately after performing the test.
6. Use only a clean and dry glass slide. Clean the slide with distilled water and wipe dry before use.
7. Accessories provided with the kit only must be used for optimum results.
8. The positive control is prediluted and ready to use.

Specimen Collection and Storage

No special preparation of the patient is required prior to sample collection by approved techniques. Fresh serum should be used for testing. In case of delay in testing, store the sample at 2 to 8°C for upto 48 hours.

Material Provided with the Kit

Reagent Pack

Toxoplasma gondii latex reagent, positive control, negative control.

Accessories Pack

Glass slide with six reaction circles, mixing sticks, rubber teats, sample dispensing pipettes.

Additional Material Required

Test tubes (10 × 75 mm), pipettes, isotonic saline, stop watch, direct light source, 5% 2-mercaptoethanol solution.

Test Procedure

Bring reagent and samples to room temperature before use. Dilute sample to be tested 1: 16 with 0.9% saline (0.1 mL of serum+1.5 mL of 0.9% saline).

Qualitative Method

1. Place one drop of diluted serum on the reaction circle of the glass slide using a disposable pipette provided with the kit.

2. Add one drop of well mixed latex reagent to the drop of diluted serum sample.
3. Using a mixing stick, mix the sample and the latex reagent uniformly over the entire circle.
4. Immediately start a stopwatch. Rock the slide gently back and forth. Observe for agglutination macroscopically at 5 minutes.

Semiquantitative Method

1. Using isotonic saline, prepare serial dilutions of the serum samples positive in the qualitative method starting frorn 1:32, 1:64, 1:128, 1:256 and so on.
2. Pipette each dilution of the serum sample onto separate reaction circles of the slide.
3. Add one drop of well mixed latex reagent to each dilution of the serum sample.
4. Using a mixing stick, mix the sample and the latex reagent uniformly over the entire circle.
5. Immediately, start a stopwatch. Rock the slide gently back and forth. Observe for agglutination macroscopically at 5 minutes.

Interpretations of Results

Qualitative Method

Agglutination is a positive test result and indicates presence of diagnostically significant level of antibodies to *Toxoplasma gondii*. No agglutination is a negative test result and indicates absence of diagnostically significant level of antibodies to *Toxoplasma gondii*.

Semi Quantitative Method

The highest dilution of serum showing agglutination corresponds to the titer of antibodies to *Toxoplasma gondii*.

Differentiation IgG - IgM

By previous treatment of the sera with reducing agents, such as 2-mercaptoethanol, it is possible to observe the type of immunoglobulins responsible for the reaction.

Add 50 µL of the 2-mercaptoethanol solution to 1 mL of 1:16 diluted serum under test. Incubate for 60 minutes at 37°C.

At the end of the incubation period, proceed using the semiquantitative test procedure as outlined above.

When antibody titer drops 2 or more dilutions after mercaptoethanol treatment, it can be considered IgM positive.

Significance of Test Results

a. Serum samples that test negative in 1:16 dilution indicate absence of diagnostically significant anti-toxoplasma titer.
b. Serum samples positive at 1:16 dilution indicate residual titer due to past exposure.
c. Positive titers from 1:32 -1:128 dilution should be suspect of incipient toxoplasmosis. Evolution of titer 3 weeks later should be determined. Increase of at least two dilutions should be considered indicative of acute toxoplasmosis.
d. Titer of 1:256 or more suggest possible active infection.
e. Determination of IgM antibodies is also advisable in (c) and (d) cases.

Remarks

1. Markedly lipemic, hemolyzed and contaminated serum samples could give rise to non-specific result.
2. Use of plasma rather than serum can lead to false positive results.
3. Positive and negative controls should be run with each series of tests to validate the results.
4. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.

RAPID IMMUNOCONCENTRATION TEST FOR HIV-1 AND HIV-2 ANTIBODIES FLOW THROUGH METHOD RETROQUICK-HIV®

(*Courtesy:* Tulip Group of Companies)

Introduction

Retroquick-HIV is a membrane based flow through immunoassay for the detection of antibodies to HIV-1 and HIV-2 in human serum and plasma. Highly purified synthetic peptides of gp 120 and gp 41 (HIV-1) and gp 36 (HIV-2) corresponding to the immunodominant regions of the HIV 1 and HIV 2 utilized in the test system assist in visual, qualitative, simultaneous detection and differentiation of antibodies to HIV-1 and 2.

Summary

Acquired Immunodeficiency syndrome (AIDS) is caused by at least two retroviruses, the HIV-1 and the HIV-2, collectively referred to as HIV-1/2. Antibodies to HIV-1 envelope protein (gp 120), transmembrane protein (gp 41) and HIV-2 transmembrane protein (gp 36) are prevalent in sera of individuals with AIDS or ARC or who are at high risk of contracting AIDS. Detection of these antibodies indicates exposure to the HIV 1/2 virus.

Principle

Retroquick-HIV test comprises of a test device striped with distinct bands of purified gp 120 and gp 41 synthetic peptide specific to HIV-1 at test region '1' and gp 36 synthetic peptide specific to HIV-2 at test region '2'. The third band striped at region 'C' corresponds to the assay performance control. First the membrane assembly is hydrated with wash buffer and then the specimen is added. Antibodies to HIV-1 and/or 2 if present, are captured by the respective antigens. After washing with wash buffer, protein A conjugated gold sol reagent is added to reveal the presence/absence of bound antibodies. Post final wash a positive reaction is visualized by the appearance of purple colored bands at the test region '1' and/or '2'. The absence of bands at test region '1' and '2' is a negative test result. The appearance of control band serves to validate sample addition, reagent and assay performance.

Reagents and Materials Supplied

Kit Components

Retroquick-HIV immunoconcentration test kit for HIV-1 and HIV-2 antibodies comprises of the following components:

1. Ready to use individually pouched, flow through test devices striped with HIV-1 specific purified synthetic peptides at test region '1' and HIV-2 specific purified synthetic peptides at test region '2' and a blue dyed protein A based control band at region 'C' along with a specimen dropper and dessicant.
2. Dropper bottle with ready to use wash buffer solution.
3. Dropper bottle with ready to use protein A conjugated gold sol solution.
4. Package insert.

Storage and Stability

The unopened Retroquick-HIV kit, as well as kit components upon opening, must be stored at 2 to 8°C, till the duration of the shelf-life as indicated on the kit/kit component labels.

1. In vitro diagnostic test. Not for medicinal use.
2. Read package insert carefully before performing the test.
3. Do not use beyond expiry date.
4. Flow through device, wash buffer and protein A conjugate of the same lot are optimized as a system. It is important that the kit components of the same lot are used for achieving accurate and reproducible results. Do not intermix reagents from different lots.
5. The sequence of addition of reagents should be followed meticulously for achieving accurate results.
6. Handle all specimens as potentially infectious.
7. Follow standard biosafety guidelines for personal protection, handling and disposal of potentially infectious material.
8. After use, the kit components must be returned to the recommended storage temperature immediately.

Specimen Collection and Preparations

1. No prior preparation of the patient is required before sample collection by approved techniques.
2. Fresh serum/plasma is preferable. Serum/plasma may be stored at 2 to 8° C upto 24 hours in case of delay in testing. For long-term storage, freeze the specimen at –20°C.
3. Repeated freezing and thawing of the specimen should be avoided.
4. Do not use hemolyzed, clotted, contaminated, viscous/turbid specimen.
5. Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only used for testing.
6. Do not heat -inactivate the specimen.
7. Frozen samples for retrospective studies must be centrifuged at 3000 rpm for 15 minutes and the clear supernatant must be used for tests.

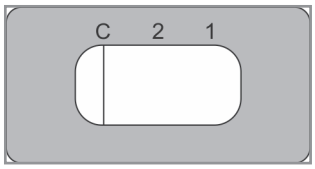
Test Procedure

1. Bring all reagents and specimen to room temperature (25–30°C) before use. Tighten the Wash Buffer solution and Protein A Gold Conjugate dropper bottle caps in a clockwise direction to pierce the respective dropper bottle nozzles. The addition of specimen/ reagents must be done at the center of the reaction port, holding the sample dropper/dropper bottles in a vertical position. Ensure the drops are free falling. Use a new sample dropper for each specimen to avoid cross contamination.
2. Tear open the foil pouches and retrieve the required number of Retroquick-HIV membrane test devices and label appropriately.
3. Add two drops of wash buffer into the reaction port of the device and allow to soak through completely.
4. Using the sample dropper provided, add one drop of the serum/plasma specimen into the reaction port. Allow to soak through completely.
5. Add three drops of wash buffer to the reaction port and allow to soak through completely.

6. Add two drops of protein A gold conjugate to the reaction port and allow to soak through completely.
7. Add two drops of wash buffer and allow the wash buffer to soak through completely.
8. Read and record the results immediately.

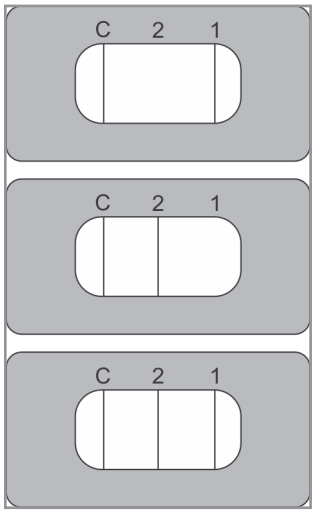
Interpretation of Results

Negative Test Result



Appearance of only one control band corresponding to control region 'C'.

Positive Test Results



In addition to the control band 'C', appearance of reactive band at test region '1': Specimen positive for Antibodies to HIV 1.

In addition to the control band 'C', appearance of reactive band at test region '2': Specimen positive for Antibodies to HIV 2.

In addition to the control band 'C', appearance of reactive bands at test region '1' and test region '2' Specimen positive for Antibodies to HIV 1 and HIV 2.

Invalid Test Result

The test should be considered invalid if neither the test band nor the control band appears. In case of invalid results, the test should be repeated using a fresh device.

Remarks

1. The addition of reagents must be accomplished without interruptions.
2. After addition of the wash buffer, in step 7 of the procedure, if the background in the reaction port is high, the samples must be recentrifuged appropriately so as to pellet invisible particulate matter. Test should be rerun with the clear supernatant.
3. The presence of antibodies to HIV 1/2 indicates previous exposure to HIV 1 and/or HIV 2 virus but does not constitute a diagnosis of AIDS.
4. Absence of antibodies to HIV 1/2 does not indicate that an individual is absolutely free of HIV 1 or HIV 2 as the collection of sample and its timing vis-a-vis seroconversion will influence the test outcome.
5. Since HIV 1 and HIV 2 viruses are similar in genomic structure and morphology and antibodies to them have (30–70%) cross reactivity, reactive test bands for HIV 1 and HIV 2 do not necessarily imply mixed infection with HIV 1 and HIV 2.
6. Though Retroquick-HIV is a reliable and sensitive screening test, it should not be used as a sole criterion for diagnosis of HIV infection.
7. All positive specimen should be further tested using appropriate supplemental confirmatory tests.
8. As in all tests the results must be correlated with clinical findings before arriving at the final diagnosis.
9. Since various tests for HIV 1/2 differ in their performance characteristics and antigenic composition, the reactivity patterns may differ.
10. The results of Retroquic-HIV must be read within 30 minutes of test completion.
11. Do not compare the intensity of the test lines and the control lines to judge the concentration of the antibodies in the test sample.
12. Testing of pooled specimen is not recommended.
13. The control band in fresh unused membrane test devices is blue colored and changes to blackish purple color after test performance.
14. The control band would not develop if the sample addition has not been done.

RAPID TEST FOR SIMULTANEOUS/DIFFERENTIAL DETECTION OF TOTAL ANTIBODIES TO HIV-1 AND HIV-2 IN HUMAN SERUM/PLASMA RETROSCREEN®

(*Courtesy:* Tulip Group of Companies)

Retroscreen-HIV, is a rapid, self-performing, qualitative, sandwich immunoassay for simultaneous and differential detection of total antibodies, i.e. IgG, IgM, IgA etc to HIV-1 and HIV-2 virus in human serum/plasma.

Summary and Explanation

Retroscreen-HIV is an immunochromatographic test for simultaneous and differential detection of total antibodies to HIV-1 and HIV-2 virus in human serum/plasma. Highly purified recombinant antigens – gp41 and p24-O fusion polypeptide, representing HIV-1 and HIV-1 group "O" and synthetic peptide gp36 represeting HIV-2 are stripped on the membrance as two separate test bands. An assay control forms the third band. The same antigens are also coated on colloidal gold. Synthetic gp36 is chosen instead of recombinant gp36 to reduce cross-reactivity and enable better discrimination between HIV-1 and HIV-2 samples.

Principle of the Assay

Retroscreen-HIV utilizes the principle of Immuno-chromatography, a unique two-site immunoassay on a nitrocellulose membrane. The conjugate pad contains four components – recombinant gp41 conjugated to colloidal gold, recombinant p24-O conjugated to colloidal gold, synthetic peptide gp36 conjugated to colloidal gold and rabbit IgG conjugated to colloidal gold. As the test specimen flows through the membrane test assembly, the highly specific HIV-1/2 antigens-colloidal gold conjugate complexes with the HIV-1/2 specific antibodies in the specimen and travels on the membrane due to capillary action along with the rabbit IgG-colloidal gold conjugate. This complex moves further on the membrane to the test region where it is immobilized by the HIV-1/2 antigens coated on the membrane at two separate test regions for HIV-1 and HIV-2. This leads to the formation of colored band(s). The absence of colored band(s) in the test regions indicated the presence of antibodies to HIV-1/2 in the specimen.

The unreacted conjugate and unbound complex, if any, move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies coated on the membrane at the control region (C), forming a colored band. This control band acts as a procedural control and serves to validate the results.

Kit Components

Retroscreen-HIV kit has following components:

1. Device: Stripped with HIV-1 and HIV-2 specific antigens and anti-rabbit IgG along with HIV specific antigen and rabbit IgG gold conjugate. Each device is individually pouched along with single-use sample dropper and desiccant.
2. Sample running buffer: Buffer containing surfactant and preservatives.
3. Instructions for use.

Storage and Stability

Retroscreen-HIV is stable up to the expiry date mentioned on the label when stored at 4–30°C. Once the pouch is opened, the device must be used immediately.

Material Required but not Provided

1. Disinfectant
2. Disposable gloves
3. Biohazard waste container.

Sample Collection

1. Retroscreen-HIV uses human serum/plasma as specimen.
2. No special preparation of the patient is necessary prior to specimen collection by approved techniques.
3. Preferably use fresh sample. However, specimen may be stored refrigerated (2–8°C) for short duration. For long storage, freeze at –20°C or below.
4. If serum is to be used as speciemen, allow blood to clot completely. Centrifuge to obtain clear serum.
5. Repeated freezing and thawing of the specimen should be avoided.
6. Do not heat inactivate before use.
7. Do not use turbid, lipemic and hemolyzed serum/plasma.
8. Do not use hemolyzed, clotted or contaminated specimens.
9. Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only used for testing.
10. Refrigerated specimens must be brought to room temperature prior to testing.

Precautions

1. For in vitro diagnostic use only. Not for medicinal use.
2. Bring all reagents and specimen to room temperature before use.
3. Do not use beyond expiration date.
4. Read the instructions carefully before performing the test.

5. Handle all specimens as if potentially infectious.
6. Do not pipette any material by mouth.
7. Do not eat, drink or smoke in the area where testing is done.
8. Use protective clothing and wear gloves when handling samples.
9. Use absorbent sheet to cover the working area.
10. Immediately clean up any spills with sodium hypochlorite.
11. Dispose off all the reagents and material used as if they contain infectious agent.
12. Do not mix components of one lot with another.
13. If desiccant color at the point of opening the pouch has turned from blue to pink, another test device must be run.

Test Procedure

1. Bring the sealed aluminium foil pouch of Retroscreen-HIV device to room temperature.
2. Open a foil pouch by tearing along the "notch".
3. Remove the testing device and the specimen dropper. Once opened, the device must be used immediately.
4. Label the device with specimen identity.
5. Place the testing device on a flat horizontal surface.
6. Carefully dispense one drop (25 μL) of serum/plasma into the specimen well "S" using the sample dropper provided.
7. Add two drops of sample running buffer into the same well "S".
8. Observe the development of visible colored band at test regions (HIV-1 and HIV-2).
9. Positive results may be onserved within 15 minutes.
10. The test should be considered invalid if the control band (CTNL) does not appear. The test is also invalid if neither the control nor the test bands appear. Repeat the test with a new Retroscreen-HIV device.

Limitations

1. Retroscreen-HIV alone cannot be used to diagnose HIV infection even if the sample is repeatedly or has high intensity of bands.
2. A negative result with Retroscreen-HIV does not preclude the possibility of exposure to or infection with HIV.
3. The membrane is laminated with an adhesive tape to prevent surface evaporation. Air pockets or patches may appear, which do not interfere with the test results. Presence of a band at the test region(s) even if low in intensity or formation is a positive result.
4. The deliberate slow reaction kinetics of Retroscreen-HIV is designed to maximize and enhance reaction time between sample capture and tracer elements to improve test sensitivity.
5. Most positive results develop within 15 minutes. However, certain sera sample may take a longer time to flow. Therefore, negatives should be confirmed only at 30 minutes. Do not read results after 30 minutes.
6. Since HIV-1 and HIV-2 viruses are similar in genomic structure and morphology, antibodies to them may cross react. Reactive test bands for both HIV-1 and HIV-2 do not necessarily imply mixed infection. However, to reduce cross-reactivity and better discrimination, Retroscreen-HIV uses a synthetic peptide gp36 with highly conserved epitopes for HIV-2 detection instead of recombinant gp36 antigen.
7. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the result of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.
8. Retroscreen-HIV should only be used as a screening test and its results should be confirmed by other supplemental methods before taking clinical decisions.

TUBERCULOSIS

Mycobacterium Tuberculosis

Tuberculosis is the leading cause of death in the world from a single infectious disease. The World Health Organization (WHO) has declared it a global emergency. The resurgence of the disease with Multi Drug Resistant TB (MDRTB) is a major concern.

Microbiology

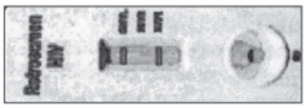
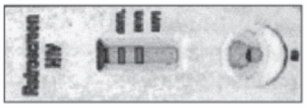
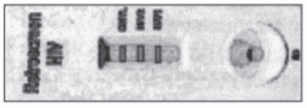
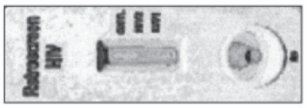
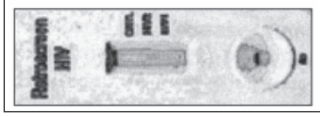
Mycobacterium is straight or curved rod shaped, non-motile bacteria. They are obligate aerobes, growing well in well aerated regions (lungs is the primary organ) and intracellular in nature.

It exhibits **"acid fastness"** – due to the impermeability of their cell walls to certain dyes and stains. Despite this once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. One such method is the **"Ziehl-Neelson stain".** Acid-fast bacilli appear pink in a contrasting background.

They can be cultured in solid and liquid media.

Solid media: Lowenstein-Jenson, Middle Brook medium.

Liquid medium: Dubos' medium.

	**Negative** A colored band appears only in the control area marked "CNTL"
	**HIV-1 positive** A colored band appears in control area as well as in the area marked "HIV-1". The sample is reactive for HIV-1
	**HIV-2 positive** A colored band appears in control area as well as in the area marked "HIV-2". The sample is reactive for HIV-2
	**HIV-1 and HIV-2 dual positive** A colored band appears in control area as well as in the area marked "HIV-1" and "HIV-2". This indicates a mixed infection.
 	**Invild** The test should be considered invalid if the control band (CTNL) does not appear. The test is also invalid if only the test band and no control band appear. Repeat the test with a new **Retroscreen-HIV** device.

Classification

Mycobacteria can be classified as:
Tubercle bacilli – causing tuberculosis
Human – *M. tuberculosis*
Bovine – *M. bovis*
Murine – *M. microti*
Avian – *M. avium*
Cold blooded – *M. marinum.*
Lepra bacilli – causing leprosy
Human–*M. leprae*
Murine–*M. leprae* murium.
Mycobacteria causing skin ulcers
M. ulcerans
M. bathei.
Atypical mycobacteria
Photochromogens
Scotochromogens
Rapid growers
Saprophytic mycobacteria
M. butyricum, M. phlei, M. stercosis.

Pathogenesis

The source of infection is usually an open case of pulmonary tuberculosis. The disease progression can be divided into 5 stages:

Stage 1: Inhalation of droplet nuclei containing the bacteria that are non-specifically taken up by alveolar macrophages. They are not activated.

Stage 2: After 7–21 days of infection. The bacilli multiply in the macrophages until it bursts.

Stag 3: Lymphocyte infiltration and macrophage activation at the site of infection occurs. Activated lymphocytes releases cytokines and gamma interferon. At this stage the tuberculin skin becomes positive, indicating that the host is developing an immune response. But this response is also responsible for much of the immune pathology.

In this stage, the **"tubercle"** formation begins—They are vascular granuloma composed of central zone of giant cells with or without caseation and peripheral zone of lymphocytes and fibroblasts.

Stage 4: In this stage, the growing tubercle invades the bronchus and also invades an artery or other blood supply line. The hematogenous spread of the bacilli may result in extrapulmonary tuberculosis, also known as **"Miliary Tuberculosis"**. They may affect any part of the body – bones, joints, lymph nodes, etc. The lesions that are formed can be either exudative (soft tubercle) or productive lesions (hard tubercle).

Stage 5: The tubercle liquefies, the liquid is very conducive to the growth of bacilli and the bacteria begin to multiply rapidly extracellularly. The large antigen load causes the wall of bronchi to become necrotic and rupture resulting in cavity formation. When these lesion heals, it becomes fibrous and calcifies forming **"Ghon complex"**.

Immune Response

Much of the immune response of the host is through cell mediated immunity (CMI). It is also responsible for much of the pathology associated with tuberculosis. The activated lymphocytes and macrophages release cytokines and gamma interferon.

Serological Response

There are many mycobacterial antigens against which serological response is produced by the host.

List of mycobacterial antigens

- A60
- MTB 48
- 38KDa
- LAM
- 16KDa
- ESAT –1
- L4 – PIM
- 19KDa
- 14KDa
- Antigen 85 complex.

Virulence Factors

The bacteria do not possess the typical bacterial virulence factors such as toxins, capsules and fimbriae. Many structural and physiological properties contribute to the virulence.

Slow intracellular growth: It is an effective means of evading the immune system. Once phagocytosed, it can inhibit phagosome-lysosome fusion.

Slow generation time: Because of the slow generation time, the immune system may not readily recognize the bacteria or may not be triggered to eliminate them.

High lipid concentrations in cell wall: This accounts for impermeability and resistance to ant microbial agents, resistance to killing by acidic and alkaline compounds.

Cord factor: It is primarily associated with virulent strains of bacteria. It is known to be toxic to mammalian cells.

Clinical Manifestations

Tuberculosis is usually classified as pulmonary or extrapulmonary. Before the recognition of HIV, more than 80% of all cases were limited to the lungs. However, up to two-third of HIV infected patients with tuberculosis may have both pulmonary and extrapulmonary disease or extrapulmonary disease alone.

Pulmonary	*Extrapulmonary*
Pulmonary	Pleural TB
tuberculosis	TB lymphadenitis TB pericarditis TB meningitis Skeletal TB

Clinical Manifestation of Pulmonary TB

- Cough: One of the earliest and most common symptoms, present in 40–80% cases
- Sputum: Initially not productive, but becomes productive indicating tissue necrosis
- Fever: Present in 65–80% of patients. In patients with advanced stage of disease, fever persists even after initiation of therapy
- Pleuritic chest pain
- Dyspnea
- Hemoptysis
- Chills/sweats
- Fatigue/malaise
- Anorexia/weight loss.

Not all infected persons show the clinical symptom. 10–20% are asymptomatic. It is important to differentiate between TB infection and TB disease to understand this.

TB Infection and Disease: Differentiation

	TB infection	*TB disease*
Bacteria	Present	Present
Tuberculin skin test	Positive	Positive

Chest X-ray	Normal	Reveals lesion
Sputum: Smear/culture	Negative	Positive for bacilli
Symptoms	No symptoms	Cough, fever, weight loss
Infectious	Not infectious	Infectious before treatment

Diagnosis

The diagnosis of TB infection and disease can be made clinically with symptoms. The laboratory diagnosis of tuberculosis may be established by demonstrating the bacillus in the lesion by microscopy, isolating it in culture or by transmitting the infection to experimental animals. Immunological response to the disease can be made by demonstrating of hypersensitivity to tuberculoprotein. Serological response to mycobacterial antigens in serum can be demonstrated.

Tuberculous Skin Testing

Many methods have been described for tuberculin testing. The method used routinely is the technique of Mantoux. In this 0.1 mL of PPD containing 5TU is injected intradermally. A positive test indicates hypersensitivity to tuberculoprotein denoting infection with TB bacilli or BCG vaccination. Persons who have never had contact with tubercle bacilli are tuberculin negative.

Pitfalls of Laborartory Diagnosis

Even there are many laboratory methods, there are many pitfalls in accurate diagnosis.

Non-specificity: Microscopy, in spite being a rapid test suffers from specificity due to poor staining and also lack of species identification. Saprophytic mycobacteria may give false results. In fluorescent microscopy, background fluorescence can give false positive results.

Time: Culture, being one of the confirmatory methods for diagnosis is time consuming. It takes 6–8 weeks for a culture report. Species identification will take more time. The clinician must start empirical treatment and wait for laboratory confirmation. Particularly drug resistant mycobacteria will take time for identification. Once therapy is started, the patient may not respond. This will lead to waste of time and money from the patient and clinician point of view.

False negativity: Skin testing may be false negative (anergy) due to impaired immune response. Also infection by atypical mycobacteria may interfere with the results.

Importance of Serological Diagnosis

- One of the most extensively researched areas
- Very important tool for diagnosis in smear negative cases
- IgG, IgM, IgG antibodies have been found to be useful in diagnosis
- It is sensitive and specific
- It is rapid and cost effective
- Antibody patterns correlates with the clinical condition.

Treatment

Chemotherapy has revolutionized the management of tuberculosis. Antituberculosis drugs are of two types, bactericidal and bacterostatic

Bactericidal: Rifampicin, pyrazinamide, isoniazid and streptomycin.

Bacteriostatic: Ethambutol.

They can also be classified as first line and second line drugs.

The major problem in chemotherapy is drug resistance, which in tubercle bacilli is due to mutation, with an approximate rate of once in 10^8 cell divisions.

Drug resistance may be "primary" (pretreatment, initial), when the patient is infected with a strain of tubercle bacillus which is already drug resistant or "acquired" (secondary, post-treatment), when the infecting strain initially sensitive becomes resistant, usually as a result of improper or inadequate treatment. This is the more common type of resistance.

A very serious consequence of unchecked drug resistance is the emergence and spread of "Multi Drug Resistant TB (MDRTB)". It is a global problem and its presence in those with concomitant HIV infection makes it more dangerous.

RAPID TEST FOR DETECTION OF ANTIBODIES TO *MYCOBACTERIUM TUBERCULOSIS* (DEVICE) SEROCHECK-MTB®

(*Courtesy*: Tulip Group of Companies)

Serocheck-MTB is a rapid, self performing, qualitative, two site sandwich immunoassay for the detection of antibodies to *Mycobacterium tuberculosis* in human serum/plasma or whole blood.

Summary

Lack of specificity of AFB smear, delayed reporting of mycobacteria by culture and requisite of expertise and expensive newer automated techniques, has led to the

development of rapid and relatively simple serological tests based on the detection of serum antibodies to selected mycobacterial antigens, 38 kDa and LAM.

Principle

Serocheck-MTB utilizes the principle of immunochromatography. As the test sample flows through the membrane assembly of the device, after addition of the sample running buffer, the colored recombinant tuberculosis antigens (38 kDa/ LAM) –colloidal gold conjugate complexes with *Mycobacterium tuberculosis* specific antibodies in the sample. This complex moves further on the membrane to the test region where it is immobilized by the recombinant tuberculosis antigens (38 kDa/LAM) coated on the membrane leading to formation of a purple colored band which confirms a positive test result for tuberculosis.

The unreacted conjugate and rabbit immunoglobulin conjugated to colloidal gold move further on the membrane and are subsequently immobilized by the antirabbit antibodies coated on the membrane at the control region, forming a purple colored band. This control band serves to validate the test results.

Reagents and Materials Supplied

Each kit contains

A. Individual pouches, each containing:

1. Test device: Membrane assembly pre-dispensed with recombinant tuberculosis antigens (38 kDa/LAM) – colloidal gold conjugate, rabbit immunoglobulin-colloidal gold conjugate, recombinant tuberculosis antigens (38 kDa/LAM), and anti-rabbit antibody at the respective regions.
2. Disposable plastic sample dropper
3. Desiccant pouch.

B. Sample running buffer in a dropper bottle

C. Package insert.

Storage and Stability

The test kit may be stored between 4–30°C till the duration of the shelf-life as indicated on the pouch/corton. Do not freeze.

Note

1. For in vitro diagnostic use only. Not for medicinal use.
2. Do not use beyond expiry date.
3. Do not intermix reagents from different lots.
4. Read the instructions carefully before performing the test.
5. Handle all specimens as potentially infectious.
6. Follow standard biosafety guidelines for handling and disposal of potentially infective material.

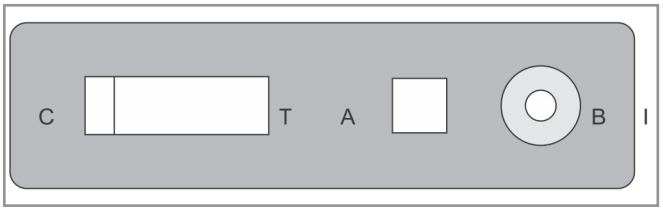
Specimen Collection and Preparation

No special preparation of the patient is necessary prior to specimen collection by approved techniques. Though fresh serum/plasma is preferable, specimens may be stored at 2–8°C for upto 24 hours, in case of delay in testing. Blood samples collected with a suitable anticoagulant such as EDTA or Herparin or Oxalate can also be used. Do not freeze whole blood samples. Do not use turbid, lipamic and hemolyzed serum/plasma specimens. Do not use hemolyzed, clotted or contaminated whole blood samples.

Testing Procedure and Interpretation of Results

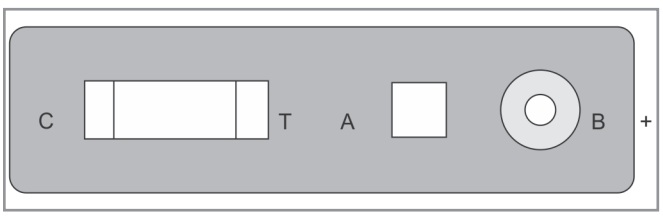
1. Bring the Serocheck-MTB kit components to room temperature before testing.
2. Open the pouch and remove the device, sample dropper and desiccant. Check the color of the desiccant. It should be blue. If it is turned colorless or pink, discard and use another device. Once opened, the device must be used immediately.
3. Label the test device with patients identity.
4. Tighten the vial cap of the sample running buffer provided with the kit in the clockwise direction to pierce the dropper bottle nozzle.
5. Add one drop of serum/plasma or whole blood with the sample dropper provided in the sample port 'A'.
6. Dispense 5 drops of sample running buffer into port 'B', by holding the plastic dropper bottle vertically.
7. At the end of 15 minutes read the results as follows.

Negative for antibodies to *Mycobacterium tuberculosis*



Only one purple band appears in the control window 'C'.

Positive for antibodies to *Mycobacterium tuberculosis*



In addition to the control band, a purple band appears in the test window 'T'.

8. The test should be considered invalid if no bands appear on the device. Repeat the test with a new device ensuring that the test procedure has been followed accurately.

Limitations of the Test

1. As with all diagnostic tests, the test results must always be correlated with clinical findings.
2. The results of the test are to be interpreted within the epidemiological, clinical and therapeutic context.
3. Any modifications to the above procedure and/or use of other reagents will invalidate the test procedure.
4. Do not compare the intensity of the test line and control line to determine the concentration of the antibodies in the test specimen.
5. Testing of pooled samples is not recommended.
6. In immunocompromised TB patients, such as in patients with HIV, since antibodies to *Mycobacterium tuberculosis* may not be present at levels indicative of active disease, and the test may give a negative results.
7. Patients with recent case of active tuberculosis infection may continue to have antibody titer within the detectable limits of the test and such samples may give a positive test results, after such patients have been cured.
8. Positive test results may be obtained in Leprosy patients. However, the clinical presentation of leprosy cannot be confused with that of tuberculosis.

TB IgG, IgA, IgM AB, MFD. ANDA

(*Courtesy:* Lilac Medicare)

In vitro diagnostic test: Enzyme immunoassay for the Determination of IgA, IgG or IgM antibodies against mycobacteria in human serum.

Serodiagnosis of Mycobacterial Infections

The A60 antigen complex is an inter-specific antigen found in the cytosol of typical and atypical mycobacteria. It reacts with antibodies created during mycobacterial infections (tuberculosis, leprae, etc.), and also reacts with the antibodies produced during Nocardia infections.

Antimycobacterial antibodies are absent in healthy individuals. However, inapparent or abortive infections due to mycobacteria are much more frequent than usually suspected. In particular, IgM antibodies are frequently observed after a contact inherent to professional occupations (e.g. hospital personnel and social workers) or to adverse social conditions.

In the latter case, the positive IgM reaction is observed most readily among babies and infants growing in unhealthy conditions. A positive IgM test observed in the serum and CSF is most useful in establishing the diagnosis of tuberculous meningitis for the serodiagnosis of latent pulmonary or extrapulmonary tuberculous primary infection and for the prognosis of relapses.

The large amount of work that has been carried out to establish the clinical validity of the Anda-Tb IgG test allowed the following conclusions to be drawn:

- Healthy people are negative, even if they have a positive intradermal reaction and even if they live in a country with severe endemy.
- The prevalence of inapparent subclinical infections is largely under-evaluated in the third-world, but also in developed countries among certain social and professional groups: people in regular contact with individuals belonging to the third or fourth-world (e.g. food store employees, hospital personnel and jailed people), non-tuberculous diseased people, positive HIV patients in hospitals and others. All show a percentage of A60 seropositives, sometimes well superior to that seen in the population at large which presents a frequency of positives fluctuating between 1.5 and 3%.
- In patients suffering from a tuberculous infection, the test shows the presence of IgG antibodies if the patient has undergone an antigenic booster stimulus. The test will be positive mostly in cases of patent active infection. It will also be positive in case of a booster vaccination in healthy people.
- In patients affected by extrapulmonary tuberculosis, the test will be effective according to the organ infected.
- In 10 to 20% of the patients, the humoral immunologic activity is weak. Patients showing such an anergy may appear negative.
- Tuberculous meningitis provokes the formation of antibodies in the cerebrospinal fluid (CSF), detectable at a 1:10 dilution.

The presence of IgG antibodies indicates a good immunological response of the patient to the infection. An anergy affecting some patients before or at the beginning of the treatment concerns as well the cellular immunity (PPD) as the IgG output. The production of IgA antibodies is largely independent from the production of IgG antibodies and may occur while the patient is in an IgG anergic state. IgA antibodies easily form complexes with antigen and are at the origin of inflammatory processes in various organs. IgA antibodies are readily detected in the serum of some apparently healthy individuals at risk, in the sputum of some patients suffering from a pulmonary tuberculous infection and in biological fluids of patients suffering from extra-pulmonary infections. In particular, specific IgA antibodies

are detected in about 30% of the patients suffering from Crohn's disease. Interestingly, people in contact with these patients only show elevated IgG antibodies. Note that the presence of anti-A 60 antibodies in those patients is probably due to secondary mycobacterial infections, as other studies do not report their presence and as nucleic acids specific to mycobacteria have never been discovered in that type of patient.

Principle of the Method

Anda-Tb is an immunoenzymatic test with dosage on a solid phase. Samples of diluted human sera, sputum (IgA) or cerebrospinal fluid (CSF) are distributed in the wells of the microtitration plates coated with the A60 mycobacterial complexes. Their incubation allows for the formation of antigen-antibodies complexes. The unbound components of the sera are eliminated by washing. The wells are thereafter incubated with peroxidase-labelled anti-human IgA, IgG or IgM antibodies that bind to the antibody complexes present. The unbound antibodies are eliminated by washing. A solution of tetra-methyl-benzidine (TMB) containing hydrogen peroxide, is thereafter introduced in the wells. A color develops during the reaction of peroxidase with TMB, whose quantity is proportional to that of specific antibodies present in the sample.

For IgA and IgG tests, a reference curve is constructed by plotting the optical densities of the references. The concentration of the unknown sera analyzed at the same time as the references is then determined from the reference curve and transformed into relative sero-units, which allows the user to take into account the inevitable daily variations which occur during the determinations.

For IgM tests, a threshold value is determined from the optical density obtained with a positive reference. The sample whose optical density is equal to or greater than this value is considered positive.

The controls included in the IgG tests are intended to verify if the test was properly carried out. They are not to be used as references.

The method employed is a standard ELISA technique.

TUMOR MARKERS

Definitions of Terms

Tumor

A swelling or enlargement occurring in inflammatory conditions.

Neoplasm

A new growth of tissue characterized by uncontrolled proliferation of cells.

Cancer

An abnormal growth or swelling.

Metastasis

Spread of cancer from the site of origin to another site through blood or lymph.

Benign Tumor

A tumor that does not harm the body.

Malignant Tumor

A tumor that can threaten a person's life.

Tumor Marker

A substance present in or produced by a host in response to the presence of tumor.

Features of Tumor Marker

- Should be identified by biochemical, immunological or molecular biological methods
- Measured easily, reliably and cost effectively using an assay with high analytical sensitivity and specificity.
- Quantitative level of tumor marker reflects tumor burden with diagnostic sensitivity (few false negatives) and specificity (few false positives).
- Test result influence patient care and outcome.

What are the Clinical Applications of Tumor Markers?

Monitoring Treatment

One of the most important applications of tumor markers lies in supervising the course of the disease, especially during treatment. Most other clinical procedures lack the sensitivity and convenience for such frequent examinations. The levels of the tumor marker will inform whether the patient is experiencing remission or relapse and will also determine the effectiveness of the treatment.

During the course of chemotherapy, the level of the tumor marker may indicate when there is a need for a redesign of medication, because many a times tumor cells develop drug resistance.

Detection of Recurrence

Monitoring tumor marker for the detection of recurrence following surgical removal of the tumor is an important clinical application. It is desirable to monitor the patient using highly sensitive onco Immunoassays tests in order to detect recurrence as early as possible.

While monitoring for recurrence, the slope (the rate of increase of tumor marker concentrations with time)

of tumor marker is important. The slope is a major factor guiding therapeutic strategies.

Prognosis

In patients with cancer, tumor markers help in assessing the tumor aggressiveness, which in turn determines how a patient should be treated. Because serum concentration of tumor marker increases with tumor progression and usually reaches the highest levels when tumor becomes metastasized, the serum level of tumor markers at diagnosis are likely to reflect the aggressiveness of the tumor and help predict the outcome for the patient. A low serum level indicates that the tumor is at an early stage or still organ confined.

Diagnosis

Diagnosis is a procedure that determines definitively whether a person has cancer. The frequency of raised levels of an isolated tumor marker in nonmalignant diseases and the overlap between normal concentrations and the concentrations of tumor markers in patients with proven cancer discourages their isolated usage for diagnosis.

The use of multiple markers simultaneously to observe specific patterns of tumor marker is widely accepted as a reliable tool for diagnosis.

Diagnostically Important Tumor Markers

Tumor marker	*Condition*
PSA	Prostate cancer
CEA	Colorectal cancer, breast cancer
AFP	Testicular cancer, liver cell cancer
HCG	Germ cell tumors, trophoblast cancer
CA 125	Ovarian cancer
CA 15-3	Breast cancer
CA 19-9	Pancreatic cancer, biliary tract cancer

What are the Factors that Affect Tumor Marker Diagnosis?

Specificity

Most markers are not specific for a tumor.

Single tumor (breast cancer)	Multiple marker (CA 15-3, CEA)
Single marker (CA 125)	Multiple tumors (ovarian, lung, uterine caner)

Multiple Epitopes: Epitopes are Antibody Binding Sites on the Antigen

Tumor cells have many tumor antigens. Each antigen has multiple epitopes. Use of Polyclonal antibodies as capture and tracer may lead to non-specific binding (Fig. 22.28).

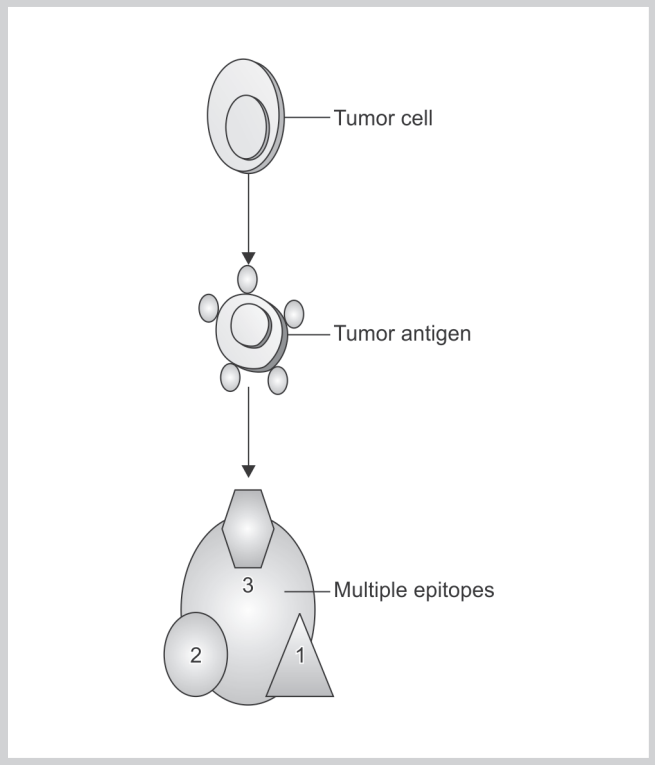


FIG. 22.28: Schematic representation of tumor cell

Hook Effect

By definition, *"a falsely low value produced by the immunoassay when the actual concentration of the sample is highly elevated is termed as Hook effect".* It occurs most commonly in sandwich type immunoassay when only one incubation is performed.

Serum Half-life of Tumor Marker

It refers to the time for the serum concentration of the tumor marker to drop to half of its original concentration.

Use of Polyclonal Antibodies

Polyclonal antibodies have poor reproducibility and can cross react. Also the lot-to-lot variation is more in polyclonal antibodies.

Sensitivity

Many immunoassay techniques suffer from poor sensitivity. Sensitivity of an immunoassay is very important in case of tumor recurrence after surgery, where the levels of the tumor marker should be very low. Increase in values after surgery indicates tumor recurrence. Also detection of very low values may have a prognostic effect.

Malignant disease	*Multiple markers*	*Comment*
Metastatic breast cancer	CA 15-3 and MCA	Differentiate from adenocarcinoma of other primary site
Pancreatic cancer	CEA and CA 19-9	Elevation of both specific for pancreatic cancer
Ovarian and colorectal adenocarcinoma	CA 125 to CEA ratio	Discrimination between ovarian and colorectal adenocarcinomas
Testicular cancer	HCG and AFP	Together used are most useful in staging and monitoring of testicular cancer

New Concepts in Tumor Marker Diagnosis

Multiple Marker Testing (MMT)

Due to lack of specificity, use of more than one marker increases the chances of detecting tumors. Using multiple tumor markers increases the possibility of detecting an elevation of markers in an increasing number of benign and nonmalignant diseases.

Use of Monoclonal Antibodies

The presence of multiple epitopes on tumor antigen together with non- specificity of tumor markers necessitates the use of Monoclonal antibodies (MAb) for detection. Both the tracer and capture antibodies should be monoclonal in nature. MAb are very expensive and only few have them. MAb have the following advantages:

- High specificity
- High reproducibility
- No lot-to-lot variation
- No cross reaction
- Ideal for tumor marker immunoassays.

Ultra Sensitive Assay Technology

Use of third generation Streptavidin–Biotin based immunoassays will enhance the sensitivity and specificity of the assay.

Short Notes on Different Tumor Markers

PSA (Prostate Specific Antigen)

- It is a 33 KDa single chain glycopeptide produced only in the prostatic secretory epithelium
- It is a major protein in the seminal plasma
- Because it is a serine protease, it forms complex in the serum with various protease inhibitors
- The major PSA complex detected in the serum is the PSA –α1 –antichymotrypsin (PSA –ACT) complex. It is also bound with α 2-chymotrypsin (PSA – A2M)
- The tissue specificity makes it the most useful marker for the diagnosis and treatment of prostate cancer
- A small portion of psa remains free in the blood unbound to any carrier protein. This portion is "Free PSA".

Issues of PSA Diagnosis

Determination of PSA value in the serum is a good indicator for prostate cancer. But like any laboratory test, there is a significant overlap between PSA levels found in cancer and benign prostatic hyperplasia. Thus, it is important to obtain sequential levels in low or borderline elevated values. A rise in the level as compared to an earlier measurement is an ominous sign.

What are the Limitations of PSA Testing?

Detection does not always mean saving lives

- False positive tests
- False negative tests
- Elevation in different conditions.

Role of Free PSA

The introduction of free PSA (f-PSA) testing has introduced a greater level of specificity in identifying early prostate cancer. In 1998, the FDA between 4.0-10.0. This has often been the diagnostic gray zone for total approved f-PSA testing as a diagnostic aid for men with

Criteria for Ideal Immunoassay

Application	*Specificity*	*Sensitivity*	*Precision*	*Conc. Range*
Screening	Less critical	Critical	Less critical	Not critical
Diagnosis	Highly desirable	Highly desirable	Less critical	Not critical
Monitoring	Less critical	Desirable	Critical	Wide range desirable
Recurrence	Important	Highly desirable	Less critical	Not critical
Prognosis	Not critical	Critical	Not critical	Not critical

total PSA values PSA testing and f-PSA may aid in the stratification.

New Concepts in Prostate Cancer Diagnosis

The value of total PSA, even though an indicator for prostate cancer, does not differentiate from BPH (Benign Prostatic Hyperplasia).

In this circumstance it is advisable to perform free PSA and PSA ratio.

What is PSA Ratio?

It is the ratio of free and bound PSA in the body. It is also known as FREE PSA%.

$$\text{Free PSA \%} = \frac{\text{Free PSA in sample}}{\text{Total PSA in sample}} \times 100$$

Advantages of PSA Ratio Testing

- It enhances the specificity of PSA testing in prostate cancer.
- Combined with total PSA, DRE and biopsy findings, helps to predict the postoperative pathological stage and grade, and may assist the patient and physician in making more informed treatment decisions.
- Can help differentiate CaP (carcinoma of prostate) from BPH and reduce unnecessary biopsies.

Cancer Antigens

These are epitopes recognized on the surface of high molecular weight glycoproteins on the epithelial cells lining respiratory, gastrointestinal and many other secretory tissues.

CA 125

- An epitope associated with a high molecular weight glycoprotein
- Originally identified by MAb OC 125
- A marker for ovarian cancer
- Elevated in ovarian, lung, pancreatic and uterine cancer
- Used in differential diagnosis between ovarian cancer and benign disease.

CA 15-3

- An epitope on mucin type glycoprotein antigen
- The antigen is present in normal and malignant epithelial cells of certain organs — breast, lung, ovary, etc.
- Elevated levels are observed in patients with metastatic breast cancer

Also elevated in chronic hepatitis, liver cirrhosis, tuberculosis and SLE.

CA 242

- An epitope on sialylated carbohydrate antigen
- Present on mucinous type of glycoprotein
- Elevated in patients with gastrointestinal cancer
- Discrimination between benign and malignant pancreatic disease
- Complement to CEA in colorectal cancer.

hCG

A glycoprotein secreted by the syncytiotrophoblasts of placenta contains α and β subunits.

Forms of hCG

In maternal serum found in many forms

- Intact hCG
- Free subunits (free β)
- Partially degraded form.

Objectives of hCG Estimation

- Pregnancy test
- Ectopic pregnancies
- Molar pregnancies
- Diagnosing trophoblastic disorders
- Germ cell neoplasms
- Testicular carcinoma.

Strategies of hCG Assays

The hCG assays employ different strategies for estimating hCG from urine (qualitative) and serum (quantitative).

The serum hCG assay measures the intact (whole) molecule when an antibody or the α-subunit and an antibody for β-subunit are used in the immunometric format. This type of assay does not measure free α/ β–subunit because free subunits cannot form sandwich with both antibodies. The total β-hCG assay measures both the intact hCG and free β-subunits. As a tumor marker, a total β-hCG assay may be preferred.

Before Selecting an hCG Assay

It is of utmost importance to check:

- Calibrators—calibrated against 3rd IS (WHO)
- Calibrator matrix—human serum matrix
- Cross reactivity with LH—very little or no cross reactivity.

TUMOR MARKERS STANDARD METHODOLOGIES AVAILABLE ON ELISA AND CLIA, AS ON RIA TOO.

Alpha-Fetoprotein (AFP) ELISA

Summary and Explanation of the Test

Alpha-Fetoprotein (AFP) is a glycoprotein with a molecular weight of 70 kDA. AFP is normally produced during fetal development by the hepatocytes, yolk sac and to a lesser extent by the gastrointestinal tract. Serum concentrations reach a peak level of up to 10 mg/mL at 12 weeks of gestation. This peak level gradually decreases to less than 25 ng/mL after 1 year of postpartum. Thereafter, the levels reduce further to less than 10 ng/mL.

Elevated levels of AFP are found in patients with primary heptatoma and yolk sac-derived germ tumors. AFP is the most useful marker for the diagnosis and management of hepatocellular carcinoma.

AFP is also elevated in pregnant women. Presence of abnormally high AFP concentrations in pregnant women provides a risk marker for Down syndrome.

Interpretation

AFP has a low clinical sensitivity and specificity as a tumor marker. Clinically an elevated AFP value alone is not of diagnostic value as a test for cancer and should only be used in conjunction with other clinical manifestations (observations) and diagnostic parameters. AFP levels are known to be elevated in a number of benign diseases and conditions including pregnancy and non-malignant liver diseases such as hepatitis and cirrhosis.

Expected Ranges of Values

Approximately 97 to 98% of the normal healthy population has AFP levels less than 8.5 ng/mL. In high risk patients, AFP values between 100 and 350 ng/mL suggest hepatocellular carcinoma. Concentrations over 350 ng/mL usually are indication of the disease.

Expected values for the AFP ELISA Test System male and female < 8.5 ng/mL (97–98%).

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal'-persons is dependent upon a multiplicity of factors the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigeneous to the area in which the laboratory is located.

CA 15-3 (CARCINOGENIC ANTIGEN 15-3)

(*Courtesy:* Lilac Medicare)

Summary and Explanation of the Assay

The MUC-1 antigen is a membrane-anchored mucin-type glycoprotein present in malignant and normal epithelial cells of certain organs, e.g. breast, lung, ovary and pancreas. The apo-protein of the MUC-1 mucin contains a transmembrane domain, a cytoplasmic domain, and an extracellular carbohydrate rich domain. The extracellular domain is characterized by polymorphism with respect to the number of a 20 amino acid tandem repeat (VNTR polymorphism). The MUC-1 breast cancer mucin < (CA15-3 antigen) is secreted from tumor cells and can be used as a serological marker of breast cancer.

The expiry date of the complete kit is stated on the label on the outside of the kit box.

Do not use the kit components beyond the expiry date.

Do not mix reagents from different lots of kits.

Store the kit at + 2–8°C.

Expected Values

Normal range < 30 Units.

It is recommended that each laboratory establishes its own normal range to account for such local environment factors as diet, climate, living conditions, patient selection, etc.

Increased

Adenoma of salivary gland; breast cancer; benign breast disease; breast cancer metastasis; lung cancer; ovarian benign disease; recurrence after remission in breast cancer with bone metastasis.

Decreased

Positive response to therapy.

CA 19-9 (CARBOHYDRATE AG 19-9, GICAM GASTROINTESTINAL CANCER ANTIGEN) BLOOD MFD: CAN AG,

(*Courtesy:* Lilac Medicare)

Expected Values

Normal range < 37 Units.

It is recommended that each laboratory establishes its own normal range.

Reference Values

		SI units
Norm	< 37 AU/mL	< 37 kU/L
Metastasis	> 1000 AU/mL	> 1000 kU/L

Usage

Tumor marker antigen that is helpful in post-therapeutic monitoring to determine the success of therapy or the presence of cancer recurrence. Useful for monitoring gastrointestinal cancers, head and neck tumors, and gynecologic tumors. Predicts the recurrence of stomach, pancreatic, liver, and colorectal malignancies. Is used in combination with other tumor markers to measure the effectiveness of treatment or earlier detection of recurrence and development of metastases. Most effective for monitoring pancreatic cancer.

Increased in

Intra-abdominal carcinoma, pancreatic carcinoma (most frequently elevated marker; elevated levels found in 80% of patients with pancreatic cancer), and possibly with other adenocarcinomas such as lung, gastric, biliary, and colonic. Also cholangitis, cirrhosis and pancreatitis (acute).

Description: A carbohydrate antigen, related to the Lewis blood group antigen. CA 19-9 is a carbohydrate antigen that has been shown to be elevated in the sera or some patients with gastrointestinal tumors. Elevated levels can indicate recurrence of cancer before radiographic or clinical findings by 1 to 7 months.

CA242 MFD: CAN AG,

(*Courtesy:* Lilac Medicare)

During malignant transformation of cells, substances which are not present or present in very low amounts in normal cells, may be expressed and secreted into body fluids. Tumor-associated substances may be determined with immunological methods using reagents specific for the tumor-associated antigen, and used for detection of malignant cells or presence of tumor in the body, to study the status of the disease and/or follow the effect of therapy.

The CA242 epitope, identified by the C242 monoclonal antibody (MAb), is a sialylated carbohydrate antigen present on mucinous type of glycoprotein (s) (named CanAg) in carcinomas of many organs. The CA242 antigen is shedded from the tumor and the CA242 epitope can be detected in serum from patients with carcinomas using the C242 MAb.

The CA242 levels are low in healthy subjects and subjects with benign diseases, while elevated levels are commonly found in patients with gastrointestinal cancer.

The CA242 test may be used as an aid in the diagnosis and management of patients with known or suspected gastrointestinal carcinomas. The test can be used for diagnostic studies of carcinomas in different organs, to monitor the effect of different treatments of cancer, detection of recurrent cancer disease, and for studies of the prognostic significance of pre-and post treatment levels of CA242.

The CA242 test should not be used as a substitute for any established clinical examination of malignancy, but may be used as a complement to existing clinical and laboratory methods.

Reference Value

Normal value: In healthy subjects < 20 Units/mL.

CA 50 (Carbohydrate Antigen 50)

Normal value < 17 U/ mL.

Increased

Colorectal adenocarcinomas, digestive tract carcinoma, esophageal squamous cell carcinoma, non-small cell lung carcinoma, pancreatic cancer, transitional cell bladder carcinoma.

Decreased

Positive response to therapy.

Description: A tumor marker that increases with many malignancies, particularly those the digestive tract. This test is not specific enough for screening and correlates more with tumor progression than with tumor regression.

CA 125 (CANCER ANTIGEN 125) MFD: MONOBIND

(*Courtesy:* Lilac Medicare)

Summary and Explanation of the Test

Cancer Antigen 125 (CA-125) is a glycoprotein that occurs in blood as high molecular weight (M > 200,000). High concentrations of this antigen are associated with ovarian cancer and a range of benign and malignant diseases. Although the specificity and sensitivity of CA-125 assays are somewhat limited, especially in early diagnosis of ovarian cancer, the assay has found widespread use in the differential diagnosis of adnexal masses, in monitoring disease progression and response to therapy in ovarian cancer, and in the early detection of recurrence after

surgery or chemotherapy for ovarian cancer. Published literature has shown that elevated serum CA-125 levels can be observed in patients with serious endometroid, clear cell and undifferentiated ovarian carcinoma. The serum CA-125 is elevated in 1% of normal healthy women, 3% of normal healthy women with benign ovarian diseases, 6% of patients with non-neoplastic conditions (including but not limited to first trimester pregnancy, menstruation, endometriosis, uterine fibrosis, acute salpingitis, hepatic diseases and inflammation of peritoneum or pericardium).

Expected Ranges of Values

The serum CA-125 is elevated in 1% of normal healthy women, 3% of normal healthy women with benign ovarian diseases, 6% of patients with non-neoplastic conditions (including but not limited to first trimester pregnancy, menstruation, endometriosis uterine fibrosis, acute salphingitis, hepatic diseases and inflammation of peritoneum or pericardium).

Expected values for the CA-125 ELISA Test System Healthy and non-pregnant subjects ≤ 35 U/mL.

CARCINOEMBRYONIC ANTIGEN (CEA) MFD: MONOBIND

(*Courtesy:* Lilac Medicare)

Carcinoembryonic antigen (CEA) is a glycoprotein with a molecular weight of 180 kDa. CEA is the first of the so-called carcinoembryonic proteins that was discovered in 1965 by Gold and Freeman. CEA is the most widely used marker for gastrointestinal cancer.

Although CEA is primarily associated with colorectal cancers, other malignancies that can cause elevated levels of CEA include breast, lung, stomach, pancreas, ovary and other organs. Benign conditions that cause significantly higher than normal levels include inflammation of lung and gastrointestinal (GI) tract and benign liver cancer. Heavy Smokers, as a group, have higher than normal baseline concentration of CEA.

Expected Ranges of Values

Nearly 99% of non-smokers have CEA concentrations less than 5 ng/mL. Similarly 99% of smokers have concentrations less than 10 ng/mL.

Expected Values for the CEA ELISA Test System	
Non-smokers	< 5 ng/mL
Smokers	< 10 ng/mL

Increased

Cancer (breast, esophageal, gastrointestinal, ovarian, pancreatic, prostate, pulmonary), chronic ischemic heart disease, cirrhosis, hypothyroidism, inflammatory bowel disease, inflammatory processes, leukemia, neuroblastoma, pancreatitis (acute), pneumonia (bacterial), pulmonary emphysema, radiation therapy (recent), renal failure (acute), tobacco smokers (chronic), and trauma. Drugs include antineoplastics and hepatotoxic drugs.

Decreased

Not clinically significant.

PROSTATE-SPECIFIC ANTIGEN (PSA) TOTAL PROSTATE SPECIFIC ANTIGEN (TPSA) ELISA, MFD: MONOBIND

(*Courtesy:* Lilac Medicare)

Prostate Specific antigen (PSA) is a serine protease with chymotrypsin-like activity. The protein is a single chain glycoprotein with a molecular weight of 28.4 kDa. PSA derives its name from the observation that it is a normal antigen of the prostrate but is not found in any other normal or malignant tissue.

PSA is found in benign, malignant and metastatic prostrate cancer. Since prostate cancer is the second most prevalent form of male malignancy, the detection of elevated PSA levels plays an important role in the early diagnosis. Serum PSA levels have been found to more useful than prostatic acid phosphatase (PAP) in the diagnosis and management of patients due to increased sensitivity.

Interpretation

PSA is elevated in benign prostrate hypertrophy (BPH). Clinically, an elevated PSA value alone is not of diagnostic value as a specific test for cancer and should only be used in conjunction with other clinical manifestations (observations) and diagnostic procedures (prostate biopsy). Free PSA determinations may be helpful in regard to the discrimination of BPH and prostrate cancer conditions.

Expected Ranges of Values

Healthy males are expected to have values below 4 ng/mL.

Expected values for the PSA ELISA Test System Healthy Males < 4 ng/mL.

Usage

Assists in the identification, differentiation, clarification, staging, and localization of tumor; monitoring preoperatively, postoperatively, and for recurrent tumor; assists in the selection of therapeutic interventions or cytotoxic drug therapy; and assists in assessment of tumor response to treatment protocols.

Increased

Benign prostatic hypertrophy, cirrhosis, impotence, osteoprosis, prostate cancer or infarct, prostatic needle biopsy, prostatitis, transurethral resection (TUR), urethral instrumentation, and urinary retention.

PROSTATIC ACID PHOSPHATES (PAP), BLOOD METHOD: BIOCHEMICAL ANALYSIS

Normal: Values are dependent upon laboratory method

		SI unit
Fishman-Lerner	0-0.7 U/dL	
Bessey, Lowry, and Brock (BLB)		
Female	0.02–0.55 U at 37°C	0.3–9.2 U/L
Male	0.15–0.65 U at 37°C	2.5–10.8 U/L
Bodansky	0–3 U/dL	0–16.1 U/L
King-Armstrong	0–3 U/dL	0–5.3 U/L
RIA		2.5–3.7 ng/mL

Increased

Bone cancer (metastatic), hyperparathyroidism, metastatic prostatic carcinoma, multiple myeloma, osteogenesis imperfecta, Paget's disease, prostatic carcinoma (10–25%), and prostatic infarct.

Decreased

Down syndrome. Drugs include estrogen therapy for prostatic carcinoma, and ethanol.

Description

Prostatic acid phosphatase, an isoenzyme of acid phosphatase, is a lysosomal enzyme that hydrolyzes phosphate esters. It is found mainly in the prostate, but is also present in erythrocytes and the kidneys, liver, and spleen. Prostatic tissue has a concentration of acid phosphatase 100 times greater than other tissues. Serum activity of the prostatic isoenzyme is greatly increased in metastatic cancer of the prostate in which the tumor has extended beyond the capsule surrounding the prostate gland. Therefore, this test is used as both a marker for and a monitor of the disease course. For Method refer to Enzymology section.

ELISA TROUBLESHOOTING ASPECTS

Introduction

ELISAs have emerged as the mainstay for diagnosis of various human diseases in a modern clinical laboratory. Despite being an extremely sensitive and specific assay format, several pre-analytical and analytical factors may affect the performance of ELISAs. Thus, it is imperative that laboratory professionals be aware of the problems so that he/she has more control over the final assay results. Many errors can be avoided if the protocol is read and fully understood before starting the assay.

- On identifying assay failure, check the expiration dates of the individual reagents and ensure that all the reagents have been stored as indicated on the product label
- Once this has been established, check for signs of instability or deterioration in reagent solutions, (e.g. precipitation or discoloration)
- All substrate solutions should be colorless
- Use clean plastic disposable pipettes, tips, and containers for reagent preparation and storage
- Avoid cross-contamination of kit reagents by changing pipette tips between addition of each calibrator, sample and reagent
- Ensure that specified incubation times and temperatures have been adhered to and that no substitution of kit reagents has occurred
- To improve accuracy, it is recommended that samples and standards be run in duplicate.

In this technical series, we present various problems commonly encountered in ELISAs and the necessary corrective action.

Problem: High negative control value or high background

Possible causes	*Corrective action*
• Contamination of negative control wells by positive control	• When washing, do not allow wells to overflow
• Contamination of negative control vial	• Check pipette barrel for residual fluid of dried material. Remove if present • Always format negative control wells before positive control • Use new pipette tip for each sample • Check the pipette tips are long enough to provide air space between top of tip and the barrel • Rerun with fresh reagents
• Insufficient washing or contamination of negative control by conjugate	• Make sure wells are completely filled. While washing ensure residual conjugate is removed from well • Pipette all specimen and reagents in the center bottom of the microwell Avoid contact with inner wall and rim • Rewash
• Non-specific attachment of antibodies	• Unsuitable blocking buffer or omission of blocking buffer • Wells no preprocessed to prevent non specific attachment of antibodies
• Antispecies conjugate reacts with reagent coated on plate	• Set-up controls to assess weather any reagent binds unexpectedly to any reagent

Problem: Low positive control value or low absorbance

Possible causes	*Corrective action*
• Reagent not at room temperature	• Make certain all kit components are at RT (22–28°C).
• Test volume low	• Ensure pipette tips are fitted correctly/tightly • Check pipette barrels for obstructions • Check calibration of pipettes
• Substrate A and B not freshly combined or incorrectly prepared (in case of 2-reagent substrate system)	• Prepare substrate immediately before use • Follow working reagent preparation
• Contamination of substrate with or bacterial contamination of positive control	• Rerun the assay with fresh reagents
• Incubation time too short	• Check calibration of timers • Record time of incubation
• Moisture in pouches	• Check whether desiccant in pouch is in working condition • Seal unused wells in pouches • Date pouches when first opened
• Improper incubation temperature	• Check incubator temperature/room temperature (22–28°C)
• Room temperature too low for substrate incubation	• Check temperature of the working area
• Washing step too vigorous	• Reduce pressure in wash system
• Reagent not mixed before using	• Mix the reagents before use
• Wells allowed to dry after assay has started	• Complete all assay steps without interruption
• Failure to add stop solution	• Addition of stop solution increases intensity of color reaction and stabilizes final color reaction
• Insufficient conjugate concentrate added in preparing working stock	• Prepare conjugate accurately • Follow working reagent preparation as described by the manufacture

Problem: Entire plate gives positive OD or color all over plate

Possible causes	*Corrective action*
• Inadequate was volume or contamination of substrate by residual conjugate left in well	• When washing, fill the wells to the rim and ensure no overflow
• Too strong conjugate	• Check dilution
• Antispecies antibodies react with absorbed antigen	• Check suitable controls
• Serum factors in heated sera	• Do not heat sera
• Substrate solution contaminated by conjugate	• Check pipette barrels for residual fluids or dried material, remove if present • Pipette tips should be long enough to provide air space between top of tip and pipette barrel • For automated system, make sure reagent lines are in proper position. Do not switch lines
• Substrate solution is not fresh	• Do not hold substrate solution longer than manufacturer claims
• Failure to stop reaction	• Check bottle before use
• Acid not added	• Check assay procedure
• Plate sat idle too long before reading	• Read within 30 minutes of adding stop solution
• Chromogen may not be working	• Use fresh chromogen
• Substrate solution container is dirty	• Do not add fresh substrate to reagent bottle containing old substrate • Clean old solution bottle with acid and thoroughly rinse with distilled water
• Plate exposed to light during substrate incubation	• Place plates in dark immediately after addition of substrate solution (Check Product Insert)

Problem: False positive reactions

Possible causes	*Corrective action*
• Inadequate washing	• Check washer before use to determine they are working properly
• Clogged cannulas in washer	• Perform routine maintenance
• Contamination of wells by conjugate	• Carefully add conjugate to wells. Pipette reagent to center bottom of microwell
• Splashing of conjugate on rims of wells during conjugate addition	• Avoid contact with sides and rims of wells • Check alignment and delivery of automated systems
• Contamination of substrate solution by conjugate	• Check pipette barrels for residual fluid or dried material. Remove if present • Check pipette tips are long enough to provide air space between top of tip and pipette barrel
• RBCs in test sample	• Centrifuge before use
• Evaporation of sample of conjugate during the 37°C incubation (if 37°C is a must, not applicable in RT incubation)	• Place the covered test plate in a prewarmed (37°C) moist incubation box inside the incubator (dry or humidified)
• Mold in incubation box and/or wash buffer bottle	• Visually check incubation boxes and wash buffer bottles • Clean any moldy containers • Be sure all containers are free of cleaning agents before using • Set-up routine cleaning schedule
• Too much conjugate concentrate used in preparing working stock	• Prepare fresh working conjugate • Follow conjugate preparation
• Incubation temperature too high	• Check room temperature, whether at 22–28°C/Check AC

Problem: Poor reproducibility or bad duplication

Possible causes	*Corrective action*
• Bubbles in wells	• Use pin or needle to burst. Use separate pin for each well
• Dispensing error	• Check dispensing instrument
• Finger tips on plates	• Clean bottom surface of plate with wash buffer, blot to dry.
• Misaligned wells in plate	• Realign wells
• Improper washing technique	• Be certain to wash the specific no. of times. Fill each well to the rim with wash buffer. Do not allow well to overflow. Blot plate dry at end of wash

Problem: Poor sensitivity

Possible causes	*Corrective action*
• Usage of non-human serum based calibrators (for quantitative assays)	• Rerun with human serum based calibrators (primary standards)
• Insufficient conjugate concentration added in preparing working stock	• Prepare fresh working conjugate, follow working procedure
• Error in pipetting working conjugate	• Check calibration of pipettes
• First incubation time insufficient	• Repeat run using proper incubation time
• Temperature more than suggested by manufacturer	• Process plate continuously throughout entire assay procedure
• Plates being held too long after first incubation before further processing	

Problem: High absorbance of calibrator

Possible causes	*Corrective action*
• Plates being held too long after first incubation at a higher temperature than what is recommended by the manufacturer before further processing	• Process plate continuously throughout entire assay procedure
• Insufficient sample volume added	• Check calibration of pipettes

Problem: Specimen absorbance out of range of calibrators

Possible causes	*Corrective action*
• Concentration in specimen is too high	• Dilute with '0' calibrator and reassay

Problem: Overall low absorbance

Possible causes	*Corrective action*
• Temperature of room < 20°C	• Increase time of reaction between enzyme/substrate (check with manufacturer) • It is recommended to maintain 22–28°C ambient temperature in the laboratory

Problem: Controls out of range

Possible causes	*Corrective action*
• Contamination of controls	• Rerun assay with new controls
• Contamination of calibrators	• Rerun assay with new calibrators

Problem: strips slip from holder

Possible causes	*Corrective action*
• Improper handling	• Grasp holder on grip marks when tapping

Problem: Strips do not fit in holder

Possible causes	*Corrective action*
• Improper alignment or incorrect holder	• Rotate strip 180 and reinsert or use correct holder

Problem: Substrate A is blue

Possible causes	*Corrective action*
• Contaminated	• Obtain fresh substrate A

Problem: Substrates A and B turn blue when mixed

Possible causes	*Corrective action*
• Contaminated	• Obtain fresh substrate A and B

Problem: Stop solution yellow

Possible causes	*Corrective action*
• Contamination	• Obtain fresh stop solution

Problem: Waited over 30 minutes before measuring plate

Possible causes	*Corrective action*
• End product of enzyme reaction may precipitate and cause error	• Rerun the essay

Problem: No color even after 30 minutes incubation with substrate

Possible causes	*Corrective action*
• Improper mixing of substrate A and B	• Remix the substrates
• Substrate not working	• Contact manufacturer

Problem: Color develops very quickly

Possible causes	*Corrective action*
• Contaminated enzymes	• Common in wells, pretreatment may be necessary • Make sure all reservoirs are clean

Problem: Color develops too slowly

Possible causes	*Corrective action*
• Sample not at room temperature	• Bring samples to room temperature before assay run
• Conjugate too weak	• Check dilutions and time when diluted
• Contamination inhibits activity of enzyme, e.g. sodium azide on peroxidase	• Avoid wrong preservatives
• Low temperature of laboratory of substrate solution	• Makes sure temperature of substrate is correct

TECHNICAL TIPS

Washing

The purpose of washing is to separate bound and unbound (free/unwanted) reagents/serum components. This involves the emptying of microwells of reagents followed by the addition of liquid into the wells. Such a process is performed at least 3–6 times for every well. The liquid used to wash wells is usually buffered (PBS) in order to maintain isotonicity, since most Ag-Ab reactions are optimal under such conditions. Tap water is not recommended, since tap water varies greatly in composition (pH, molarity, and so on). Generally, the mechanical action of flooding wells with a solution is enough to wash wells of unbound

reagents. Some workers leave washing solution for a short time (soak time) after each addition (1–5 minutes). Sometimes detergents, notably Tween-20 (0.05%) are added to washing buffers. This can cause problems where excessive frothing takes place producing poor washing conditions, since air is trapped and prevents the washing solution from contacting the well surface. For most cases, this addition does not contribute significantly to the washing procedure. When using detergents, care has to be taken that they do not affect reagents adversely (denature Ag), and greater care is needed to prevent frothing in the wells.

Normal Washing

In washing plate manually, the most important factor is that each well receives the washing solution so that, no air bubbles are trapped in the well or a thumb is not placed over corner wells.

Strip/Plate Washers

- Various washing cycles can be programed
- Careful maintenance is essential, since they are prone to machine errors, such as having a particular nozzle being blocked.

Washing Tips

- Follow procedure for preparation of wash buffer
- Check washer alignment daily as part of routine instrument start-up procedures
- Ensure that the plate is leveled
- Make certain will is completely filled, when washing, to ensure residual conjugate is removed
- Examine the fill volume (a slight dome should be observed at the top of the well)
- When washing do not allow wells to overflow
- Reduce pressure in wash system
- Check washers before use to determine they are working properly. Perform routine maintenance
- Be certain to wash the specified number of times
- Allow approximately 20 seconds soak-time between the addition of wash solution and subsequent aspiration (if soak-time is not indicated in the assay pack insert)
- Examine the wells for complete aspiration of contents
- Upon completion of wash cycle, blot to remove residual fluid.

Pipetting Tips

- Calibrate pipettes regularly according to manufacture's instructions
- Avoid touching side wall of well with tips
- Avoid splashing of sample and reagents
- Avoid blowing out tip contents
- Use a new tip for each sample/control/reagent addition
- New tips should be used on the multichannel pipettes for each reagent to be added
- Reverse pipette when using the multichannel pipettes to add conjugate and substrate solution
- Forward pipette when using the multichannel pipettes to add stop solution
- Check pipette tips are long enough to provide air space between top of tip and pipette barrel
- Check pipette barrel for residual fluid of dried material, remove if present
- Ensure pipettes tips are fitted tightly.

Microplates

- Bring microplate pouches to room temperature before opening
- Level microwells evenly in microplate frame as the individual breakaway wells have very flexible plate frames leading to bowing off wells and yield poor washes
- Place plates in dark immediately after addition of substrate solution, provided the substrate is sensitive to light
- Grasp holder on grip marks when tapping to avoid strips slipping from holder
- Rotate strips 180° and reinsert or use correct holder if strips do not fit in holder
- Seal unused wells in purchase along with the desiccant
- Date the pouches when first opened
- Clean bottom surface of plates with wash buffer to remove fingerprints
- Make sure microwells are at level during washing, reagent addition and plate/strip reading
- Wipe the bottom the plate with a lint-free cloth/towel before reading
- Do not allow microwells to become dry once the assay has begun.

Substrate Preparation

- Use freshly prepared substrate A and substrate B (in 2-reagent substrate systems)
- Do not hold substrate solution longer then 1 hour
- Follow procedure of working substrate solution
- The temperature of solution is important because it effects rate of color reaction
- Do not add fresh substrate to reagent bottle containing old substrate

- Clean old substrate solution bottle with H_2SO_4 and thoroughly rinse with distilled water.

Conjugates

- Store at recommended temperature
- Never store excessively diluted conjugate for use at some later time
- Always make up the working dilution of conjugate just before you need it
- Never leave conjugate on the bench for excessive time.

Addition of Samples

Problems caused by:

- Failure to put sample into buffer in well, leaving it on the side of the plate.

Stopping Reagents

Stopping reagents are added to prevent further enzyme reaction in ELISA. The stopping is usually made at a time when the relationship among the enzyme-substrate product is in the linear phase. Molar concentration of strong acids or strong bases stops enzyme activity by quickly denaturing enzymes. Some stopping reagents are enzyme-specific. Sodium azide is a potent inhibitor of HRPO, whereas EDTA inhibits alkaline phosphatase by the chelation of metal ion cofactors. Since addition of stopping agents may alter the absorption spectrum of the product, the absorption peak must be known. Thus $H_2SO_4^-$ stopped ELISAs are read at 492 nm (450 nm before stop).

Temperature

- Bring test reagents to room temperature (22–28°C) approximately 30 minutes prior to use
- Maintain proper incubation temperature:
  - Lower temperature can decrease OD values
  - Higher temperatures can increase OD values
  - Evaporation in wells can cause edging effect.
- The optimal temperature for incubation is 22–28°C
- Check temperature against calibrated thermometer
- Strict adherence to time must be maintained:
- Check calibration of timers
- Record time of incubation
- Read plate with specified time limits of adding stop solution.

Rotation of Plates While Incubating Reagents

In certain ELISA systems, the plates are rotated during incubation for better antigen-antibody reaction. The effect of rotating plates is to mix the reactant completely during the incubation step. Since the solid-phase limits the surface area of the absorbed reactant, the mixing ensures that, potentially reactive molecules are continuously coming into contact with the solid-phase.

During stationary incubation, mixing only takes place because of diffusion of reagents. Thus, to allow maximum reaction from reagents in stationary conditions, greater times of incubation may be required, than if they are rotated.

Rotation also allow ELISA to be performed independent of temperature conditions. The interaction of antigen and antibodies relies on their closeness, and the kinect energy provided to the system, which is encourage with the mixing during rotation. Stationary incubation relies on the diffusion of molecules and thus is dependent on temperature.

Laboratory Conditions

The laboratory should be devoid of any acid fumes.

CHAPTER 23

Diagnostic Immunology

Nonenzymatic, Quantitative Techniques (Immunodiffusion, Electrophoresis and Turbidimetry)

QUALITATIVE DETERMINATION OF PLASMA PROTEINS BY IMMUNOPRECIPITATION

The principle of immunoprecipitation was first described by Kraus. Originally, immunoprecipitation reactions carried out in test tubes were detected by the fact that turbidity can be observed following the mixing of antigen (Ag) and antibody (Ab) solutions. Centrifugation of such samples results in an insoluble sediment. Layering the antigen solution on top of the antibody solution in a narrow test tube, without mixing, results in a ring-like turbidity at the contact area within a short time. This so-called "ring test" reveals the presence of Ag-Ab precipitation in the solution studied. A distinction of characteristics between the various antigens and antibodies cannot be made.

Immunodiffusion Method of Oudin

With this method, the Ag-Ab precipitation takes place in a gel medium. Due to the different diffusion rates of heterogeneous antigens (e.g. the proteins of blood serum) the differentiation of the various antigens is rendered possible. The linear (one dimensional) double diffusion technique, i.e. diffusion of antigens and antibodies from opposite directions, results in the formation of multilayered precipitations if heterologous Ag solutions and multivalent antisera are used.

Agar is most frequently employed for the gel because of its transparency and solidification characteristics.

Double Diffusion Method of Ouchterlony

Agar gel is poured uniformly onto glass plates or into Petri dishes and allowed to solidify. Holes (wells) are cut into the agar gel which are filled with Ag and Ab solutions, respectively. Both the antigens and antibodies diffuse radially, and form, upon confluence, immunoprecipitates. These immunoprecipitates often take the form of a curved line. Because of its numerous possible modifications, the versatile arrangement of Ag-Ab containing wells, etc. this technique is widely applied in immunological, biochemical and medical laboratories.

Grabar and Williams' Method of Immunoelectrophoresis

This represents a combination of physicochemical and immunochemical techniques. The material to be examined (e.g. tissue exudates, serum, etc.) is separated electrophoretically on agar gel and, subsequently, subjected to the effect of a precipitating antiserum. This is done by placing antiserum in a trough in the agar parallel to the electrophoretically separated proteins. Both the antibodies and antigens diffuse toward one another, forming, upon confluence, well-defined precipitin lines.

To date, approximately 30 different precipitin lines in human serum can be detected by immunoelectrophoresis (IEP), indicating an equal number of individuals serum proteins. Of late, many of these serum proteins have been characterized.

The immunoelectrophoretic technique is a valuable addition to available methods for characterization of proteins. This technique is usefully employed in all areas of protein chemistry and in connection with the investigation of a large number of clinical problems connected with protein metabolism. In particular, the microtechnique of IEP has found widespread acceptance.

Methodology

Double Diffusion of Ouchterlony

Macrotechnique

Reagents

1. Phosphate buffer solution 0.15 mol/L, pH 7.1. This solution is obtained by mixing 67 mL of solution I with 33 mL of solution II.
   Solution I: Distilled water is added to 26.70 g of $Na_2HPO_4.2H_2O$ to total 1,000 mL.
   Solution II: Distilled water is added to 20.41 g of KH_2PO_4 to total 1,000 mL.
2. *Agar*
   Behringwerke agar purum or agar of equal quality which possesses satisfactory transparency.
3. *Antisera*
   Antisera to plasma proteins.

Preparation of Agar Gel Slides

Four grams of purified agar is mixed with 100 mL of phosphate buffer solution 0.15 mol/L, pH 7.1, and 300 mL of distilled water. Approximately 40 mg of Thimerosal are added as a preservative. This mixture is heated at 100°C until the solution is clear. Undissolved particles can be removed by centrifugation of the hot solution at 3,000 rpm for 2–3 minutes.

Twenty two milliliters of the hot agar solution is then pipetted into level Petri dishes of 8 cm diameter. Upon standing for a short time, the agar solution will solidify, forming a gel. Holes (wells) are cut into the agar gel by means of a glass or metal punch. For routine examinations, it is best to cut holes of 0.6 to 0.7 cm in diameter approximately 2 cm apart (as measured from the center of the holes). Punch sets of various patterns are available commercially. After cutting the holes, a small amount of agar gel is heated with several drops of water in a test tube. A few drops of this diluted agar solution are added to each hole to seal the gaps between the agar gel and the bottom of the Petri dish.

Gel Diffusion Procedure

Petri dishes prepared in this manner can be used for many types of immunoprecipitation reactions. Multivalent antisera (e.g. antiserum to human serum), heterogeneous antigen solution (e.g. human serum), purified proteins (human IgG, human IgA, or human albumin), or specific antisera (e.g. antisera to plasma proteins) can be analyzed by this method.

The wells in the agar plate are filled completely with approximately 0.1 mL of antigen solution or antiserum. Highly precipitating antisera react optimally with antigen solutions of concentrations between 1 and 10 mg/mL. The Petri dish is covered to prevent drying of the agar, then allowed to stand at room temperature for 2–3 days.

During this period, immunoprecipitates form. Each individual antigen produces a single precipitin line, the location of which depends upon the rate of diffusion and the concentration of the antigen. The diffusion rate of protein depends primarily upon the molecular weight. High molecular globulins (e.g. macroglobulins) are precipitated in the proximity of the point of application, in contrast to antigens of lower molecular weight (e.g. albumin) which are precipitated nearer to antibody well. Immunologically, identical proteins in adjacent wells form precipitin lines which merge completely ("reaction of identity"). Partial immunological identity (partially shared antigenic determinants) is reflected by formation of precipitin arcs, which partially fuse and partially intersect forming so-called "spurs" (e.g. γ G-globulin) and γ A-globulin). Immunological non-identity is reflected by intersection of the precipitin lines without fusion (e.g. albumin and γ G-globulin).

Microtechnique

A microtechnique, using small agar gel plates, with smaller Ag wells and shorter distances between them, is applicable, under certain circumstances. Examples include the need to examine small amounts of test material, or if test results are needed in a short time period. Plates for this microtechnique may be prepared with a 2% agar solution as used for immunoelectrophoresis. Three mL quantities of the hot agar solution are pipetted onto precleaned glass slides (2" × 2") and the agar is then allowed to solidify. The agar wells are cut either individually or by means of a template.

Recording of Results

Photographic recording of results of gel diffusion test is the most satisfactory method of documentation. Precipitin lines can be copied directly onto photographic paper. For this purpose, the surface of the agar gel is carefully rinsed with tap water to remove dust or other undissolved particles in the antisera or antigen solutions. To obtain an optically uniform surface, the agar is covered by tap water or physiological saline solution. The Petri dish is then placed on photographic paper (extra hard) in a dark room and illuminated from above with a lamp of approximately 200 watts. When optimally exposed and developed, the photographic paper will exhibit even fine precipitin lines with great sharpness.

Staining of the precipitin patterns of gel diffusion preparations can be achieved by the methods described under "immunoelectrophoresis" if this should prove necessary.

Possible Sources of Error

1. Specific antisera to plasma proteins are obtained by immunizing rabbits or goats with highly purified plasma proteins. Frequently, traces of contaminating antibodies in such antisera must be removed by absorption. Absorbed antisera may contain minute amounts of antigens (human plasma proteins) used for this purpose. This possibility must be considered when precipitin lines occur between the antisera wells, where two or more types of antisera are used.
2. Occasionally, circular precipitates develop around the antigen wells. These "hallos" occur particularly when markedly lipemic or aged samples of sera or plasma are used. These nonspecific precipitations are easily distinguished from specific immunoprecipitates by their circular arrangements. Strongly hemolytic sera may mimic nonspecific precipitates on photographic records.

Immunoelectrophoresis

Reagents and Equipment

Agar

Agar used for IEP should have a low calcium concentration and should yield a transparent gel with a low solidification point. Agar of good quality can be prepared by washing commercially available agar. It is important to wash agar thoroughly with distilled water since electrophoretic separation of serum proteins depends partly upon the purity of agar. For example, in unwashed agar the protein migration to the anode in the electrical field is limited; the majority of proteins migrate to the cathode due to the marked electro-osmotic potential. Agar specifically prepared for IEP is available from Behring diagnostics.

Particularly suitable as a carrier medium for IEP is the low-ion agarose, which is obtained by the removal of the agaropectin moiety of agar.

Buffer

Michaelis diethylbarbiturate acetate buffer solution pH = 8.2, $\mu = 0.1$.

A total of 13.38 g of sodium 5, 5-diethylbarbiturate and 8.83 g of sodium acetate trihydrate are dissolved in distilled water to yield 1.5 liters. The pH is adjusted to 8.2 by adding approximately 180 mL 0.1 N hydrochloric acid. This solution possesses an ionic strength of $\mu = 0.1$.

Antisera

Depending upon the specific problem to which the technique is being applied (e.g. IEP analysis of human serum) either the multivalent antisera (i.e. antisera containing numerous antibodies, such as those directed against the various proteins of human serum) or the specific antisera (i.e. antisera directed against specific antigens, such as albumin, IgG, etc.) can be employed. Such antisera can be prepared in horses, rabbits, goats chickens, or other suitable animals. The higher the immunoprecipitation titer of the antiserum (as shown by the ring test), the stronger are the precipitin lines on IEP.

With rabbit antiserum to human serum, approximately 30 different human serum proteins can be demonstrated by IEP. A somewhat different immunoelectrophoretic pattern is obtained with horse antihuman antiserum. This difference is due to an altered Ag-Ab ratio which is dependent upon the species specific differences of antibody structures. Rabbit antisera form strong precipitin lines in a wide range of Ag-Ab ratios, whereas horse antisera produce finer and sharper precipitin lines in a rather narrow range of Ag-Ab ratios. For this reason, it may be advisable to use various dilutions when using horse antisera.

Preparation of Agar Gel Slides

Two grams of pure agar is dissolved in 50 mL of diethylbarbiturate acetate buffer, pH 8.2 ($\mu = 0.1$) (can check by pH meter), and 50 mL of distilled water. This solution is heated for 15 minutes in a water bath of 100°C. Any undissolved particles can be removed from the heated agar by centrifugation at 3,000 rpm for 2–3 minutes. Ten mg of Thimerosal is added as a preservative. The hot agar solution is applied by pipette to alcohol cleansed, level glass slides. 3 mL of agar are placed onto each glass slide. After a few minutes the agar solidifies and the glass slides thus prepared are put into moisture chamber. A simple plastic container with Petri dish of water could serve as a moisture chamber.

It is advisable to prepare just sufficient agar solution to cover the glass slides since repeated heating of the gel leads to alterations which interfere with electrophoretic process. Holes and troughs are punched through the agar layer with a die or suitable modification. On both sides of the trough, a hole is made. Agar remaining in the punch is aspirated by a syringe connected to the barrel of the punch by rubber tubing.

Application of Antigens

The antigen mixture, e.g. serum, to be electrophoresed is introduced into the wells by a 26-gauge needle attached to a tuberculin syringe or by means of an appropriate micropipette. Complete fillings of the wells require about 0.002 mL of serum or protein solution. Each slide provides

two Ag wells, permitting antigen analysis in duplicates or comparison of two different antigens. For the analysis of enzymatically cleaved agents or substances which diffuse readily, it is recommended that only one antigen be electrophoresed, since migration to the other side of the Ab trough is a possibility.

Electrophoresis

In principle, the apparatus used for IEP is similar to that used for paper electrophoresis. The ends of the antigen-containing agar covered microscope slides are placed on two parallel bars. Filter paper strips establish the contact between the agar on the slides and the buffer in the troughs. The slides must be horizontal in position. The voltage between the ends of the slides is 45 V (6 volts/cm). Adequate electrophoretic separation is achieved within 45 minutes. Under these conditions the agar slides warm to approximately 25–28°C. Therefore, the application of a lid is necessary to prevent desiccation.

Application of Antiserum

Subsequent to electrophoretic separation, the agar strip from the precut center trough is removed with a 19-gauge injection needle attached to tuberculin syringe. The amount of antiserum applied is approximately 0.04 to 0.06 mL. It is advantageous to vary the antigen and antiserum concentrations. A shorter antibody trough can be used to examine only one serum fraction (e.g. IgG) in order to economize on antisera. After application of antiserum, the slides are placed in a moisture chamber for diffusion. The electophoretically separated proteins (i.e. antigens) and the antiserum diffuse toward one another and the homologous agents undergo an Ag-Ab reaction by forming precipitin lines at the points of confluence. Depending upon the concentration of antigens and antisera, optimal immunoprecipitates are formed between 20 hours and longer.

Possible Sources of Errors

1. Slides must be cleaned with chromic sulfuric acid.
2. The electrophoretic separation of the serum protein must be within an optimal range. Too short or too long a separation distance may be caused by faulty buffer composition (pH, μ), by incomplete contact between agar slides and filter paper strips, or by improper voltage. It is important to maintain uniform distribution of voltage (not of current) through the agar.
3. Wells of proper diameters and the correct distance between the Ag well and Ab trough are decisive for satisfactory IEP. Deviation from these specifications may result in marked dislocations and/or distortion of precipitin patterns.
4. The agar gel slides should be used within 2 or 3 days after preparation and must be stored in moisture containing sealed containers, preferably at 4°C.
5. The sera to be examined should be fairly fresh or stored by freezing at –20°C. Bacterial contamination or autocatalytic processes may result in marked alterations of serum samples, leading to changes in electrophoretic mobility, solubility, etc.
6. Normal control sera should be examined simultaneously with abnormal sera.

Fixation of Precipitin Patterns

Immunoelectrophoretic patterns can be recorded by photographing the unstained or stained precipitin lines. For most purposes, direct photography of the slide is sufficient.

Staining of the precipitin patterns is occasionally of value for chemical characterization of the precipitin lines. The agar slides are washed with physiological saline for one or two days, in order to remove the nonprecipitated protein from the agar. They are then covered by the filter paper and dried completely either in the incubator at 37°C (or in air at room temperature). After removal of the filter paper, the agar slides are placed in 2% acetic acid solution for approximately 5 minutes. The various staining techniques performed are as follows.

Protein Stains

Amidoschwarz (*Amido black*): 0.5% amidoschwarz 10B in methanol-glacial acetic acid (9:1). Stain for 5–10 minutes, then wash with methanol-glacial acetic acid (9:1), for approximately 15 minutes.

Azocarmine: 0.5% azocarmine B in methanol-glacial acetic acid (9:1). Stain for 15 minutes; wash with methanol-glacial acetic acid solution (9:1).

Light green: 0.5% green SF in 5% trichloroacetic acid. Stain for 1 hour; wash with 5% trichloroacetic acid solution.

Bromophenol blue: 0.1% bromophenol blue in $HgCl_2$-saturated methanol. Wash with methanol. Green stain results from acid solution, blue stain from alkaline solution.

Lipoprotein Stains

Oil red: 0.5% oil red O in 50% ethanol (filtered). Stain for approximately two hours. Wash with 50% ethanol.

Sudanblack: Sudanblack (0.1%) is dissolved in 60% ethanol at 37°C, with occasional stirring, during a 24 hours time period. This solution is then filtered at 25°C and stored in dark containers. Before use, 0.1 mL of a 30% sodium hydroxide solution is added to 160 mL of the sudanblack solution. After staining for 2 hours, the slides are then washed using 50% ethanol.

Peroxidase Reaction (for haptoglobin and hemopexin)

Benzidine: Dissolve 0.2 g benzidine in 100 mL of distilled water, then add 0.5 mL glacial acetic acid and 0.2 mL hydrogen peroxide 30%. When stained for 10–20 minutes, the specific precipitates become dark greenish-blue in color.

Fixation of the color: Wash briefly with distilled water, stain for 10 minutes in a 0.1% solution of $Ni(NH_4)_2 (SO_4)_2$, thereafter for 12 hours in 0.2% $Ni(NH_4)_2(SO_4)_2$. The specific color is bluish black.

Ceruloplasmin Stain

p-Phenylenediamine: Solution must be prepared immediately before use. Dissolve 21.6 mg of p-phenylenediamine in 100 mL of sodium acetate buffer solution (pH 5.7, $\mu = 0.1$); add 10 mL of a solution consisting of 0.65 g sodium azide in 1,000 mL of distilled water, and warm to 37°C. Stain for 2 hours at 37°C. Wash twice for 2 hours in sodium acetate buffer (pH 5.7). This technique is suitable for fresh samples only.

Cholinesterase Stain

Indoxyl acetate: 5 mg indoxyl acetate is dissolved in 0.5 mL acetone, to which are added immediately thereafter 22 mL of a diethylbarbiturate acetate buffer solution pH 8.2, $\mu = 0.05$ (1 volume of buffer $\mu = 0.1$ and one volume of distilled water) and 2.5 mL of copper acetate solution 0.1 µmol/L (0.018) g copper acetate in 100 mL distilled water). This solution must be used within 8 hours after preparation. After staining for 2 hours, and subsequent washing in tap water for 1 hour, cholinesterase is indicated by blue color.

Lipid-protein Double Staining

A volume of 0.5% oil red solution in 50% ethanol is suitable as a lipid stain. Place the slide in the dye mixture for approximately 2 hours, then wash 50% ethyl alcohol. A volume of 0.5% light green solution in 5% trichloroacetic acid is then immediately applied to achieve the specific protein stain. The staining time for proteins is approximately one hour. Wash with trichloroacetic acid.

The slides must be photographed after for keeping a permanent record.

Identification and Interpretation of Precipitin Lines

The analysis of immunoelectrophoretic patterns requires a certain degree of experience and training. It is imperative to be able to recognize the various specific precipitin lines. Various techniques are available to achieve identification of given precipitin line by experimental means:

1. Employment of specific antisera
2. Specific stains
3. Selective absorption
4. Employment of purified proteins.

Practical Application of Immunoelectrophoresis

Increased immunoglobulin concentrations are indicated by elongated and thickened precipitin lines of IgG, IgA and IgM immunoglobulins and antigen excess result in positions of these precipitin lines closer to the antibody trough in comparison with those obtained with normal sera. Although IEP does not lend itself to precise quantitation of serum proteins, marked deviations from normal concentrations may be recognized.

Increased IgG, IgA and IgM are frequently associated with acute and chronic liver diseases. This is the case in acute hepatitis, in which a particularly impressive increase in IgM occurs. In infectious mononucleosis, chronic hepatitis and Laennec's cirrhosis all three major immunoglobulins are frequently, but not invariably, increased. In lipoid hepatitis the IgG-globulin precipitin line may be markedly accentuated. Collagen diseases, including Sjögren's syndrome and systemic lupus erythematosus (SLE), as well as certain infections, may be associated with hyperimmunoglobulinemia as indicated by IEP. In trypanosomiasis, the IgM may be excessively increased. Certain virus diseases, such as Coxsackie infections, are often associated with increased immunoglobulins.

In most cases of sarcoidosis IEP has not revealed abnormal serum protein changes, though definite IgA increases have been observed by some. IEP revealed increased immunoglobulin contents in a variety of additional diseases such as dermatitis herpetiformis and dermatitis gestationis and pernicious anemia in which IgA, and mongolism in which the IgA and IgG were increased. Besides all the causes mentioned above, the disorders mentioned under hypoalbunemia, monoclonal and polyclonal gammopathies discussed under serum protein in clinical chemistry chapter can also be assessed by using IEP techniques.

Quantitative Determination of Plasma Proteins by Immunoprecipitation

The reagents used for quantitative determination are specific antisera which react stoichiometrically with the proteins to be determined and form precipitates with them. These immune reactions may occur either in solutions or in gels which contain the antiserum in even distribution. In the first case, the quantity of immune precipitate, measurable at appropriate dilutions as turbidity or by other means, gives a quantitative measure of the antigen concentration. By using, as the reaction medium, a gel containing antiserum, the reagent is arranged in the form of a stationary phase into which the antigen can penetrate either by diffusion or under the influence of an electrical field. The resulting precipitates, which assume different

configurations depending on the method used, can be quantitatively evaluated.

In the four most widely used methods to be described, reaction conditions have been selected which ensure that the quantity of specific antiserum is constant and exceeds the quantity of antigen, while the concentrations of the antigens to be determined can vary within wide ranges.

FUNDAMENTAL QUANTITATIVE CONSIDERATIONS

Photometric Method

Principle: This method is a modification based on the determination of the nitrogen content of isolated immunoprecipitates as described by Heidelberger. In contrast to the tedious technique, the antigen and antibody are allowed to react together at high dilution. Under these conditions, the reaction product of the immune reaction does not result in the formation of the precipitate which sediments, but merely in the appearance of tubidity. The extinction (e.g. at 450 nm) can be used as a measure of the turbidity. If the quantity of antiserum is kept constant and the antigen concentration is varied, a curve can be obtained. When the antigen is present in excess, soluble reaction products are formed. For this reason, only the ascending limb of the curve can be used for quantitative determinations. By reference to this part of the curve, the concentration of antigen in an unknown solution can be determined.

Single Linear Immunodiffusion of Oudin

Principle: Gel containing the antiserum is allowed to solidify in a test tube, and the antigen solution is poured on top of it. The precipitate formed at the boundary layer migrates into gel zone. The distance of the front of the precipitate from the boundary surface is proportional to the square root of the diffusion time, t.

$$h = k \times \sqrt{t}$$

As the migration velocity of the antigen

$$k = \frac{h}{\sqrt{t}}$$

is proportional to the logarithm of its concentration CAg and the distance h through which the precipitate migrates may be expressed by the following equation:

$$\log CAg = a \times k + b = a \times \frac{h}{\sqrt{t}} + b$$

Accordingly, if CAg as a function of $\frac{h}{\sqrt{t}}$, is plotted in a semilogarithmic system, the result is a straight line. Its slope is represented by *a* and it intersects the ordinate at *b*. The slopes *a* and *b* are thus constants determined by the Ag-Ab system under consideration. The length of the cylinder of precipitate is proportional to the negative logarithm of antibody concentration.

The fundamental difference between the techniques of single linear immunodiffusion and single radial immunodiffusion and electroimmunodiffusion (EID) described below is simply that in the former the antigen concentration is determined from the speed of migration of an immunoprecipitate; while in the latter methods, it is determined by measuring the precipitin area on the length of a precipitate which remains constant after a certain time period.

Since the diffusion velocity of any molecule is dependent on temperature, it is essential in using Oudin's technique that the temperature be kept strictly constant during the reaction period.

The results of EID by Laurell's method and single radial immunodiffusion as described by Mancini, Carbonara and Heremans are not significantly affected by temperature fluctuations. One advantage of Oudin's method is that the solutions require less exact volume measurements, and pipetting errors which may affect other methods are of minor importance.

Single Radial Immunodiffusion

Principle: Dissolved antigen molecules diffuse radially from a cylindrical well into an agar gel layer of uniform thickness containing the corresponding antiserum. The resulting precipitate assumes the form of a cylinder or ring. When diffusion ceases, the surface area of the base of the cylinder is directly proportional to the quantity of antigen, at a predetermined concentration of antiserum in the reaction gel.

If the circular area πr^2 (i.e. area of central well + area of precipitate) is plotted on a graph as a function of the quantity of antigen QAg (i.e. antigen concentration × volume of antigen solution), the result is a straight line relationship expressed by the equation $\pi r^2 = k \times QAg + S$.

The point S at which the straight line intercepts the ordinate is a function of the size of the central well. And the slope *k* is inversely proportional to the antiserum concentration in the gel and the gel thickness. By standardizing the technical conditions, it is possible to keep constant the variables contained in *k* and *S* so that the measured radius or diameter of the precipitate ring is a function solely of the quantity of antigen introduced. With the aid of standardized protein preparations, it is

possible to construct a reference curve which can be used to determine the antigen concentrations of unknown solutions. It is essential, however, to deliver identical volumes of the antigen solutions, standard and test solutions, into central wells.

Differences in temperature do not affect the results of this reaction. The only effect of a rise in temperature is to accelerate diffusion and thereby the appearance of a measurable precipitin ring. However, the temperature should not be allowed to rise above 37°C because the gel may melt and because of the risk of irreversible damage to the thermolabile proteins.

Electroimmunodiffusion

Principle: In the course of the electrophoretic migration of an antigen through agarose gel containing the corresponding antiserum, it produces an extended trail of an immunoprecipitate. The length *'I'* of the precipitate is a measure of the antigen concentration provided that the latter is contained in a fixed volume of solvent.

Depending on the electrophoretic migration rate of an antigen, the precipitin peaks may appear more or less rapidly. Fast moving proteins usually produce long narrow precipitin bands ending in a point, while proteins of lower eletrophoretic mobility produce broader precipitates with rounded ends. Because of their slow migration rates, elctrophoresis takes longer; diffusion of the antigen at right angles to the direction of electrophoresis may be responsible for the broadening of the peaks. The migration velocity of the antigens is affected by the field strength and the pH of the buffer solution employed. Careful standardization of these factors is essential for reproducible results. This makes the method somewhat more elaborate than comparable methods based on diffusion alone. On the other hand, the results are usually available in 2 or 3 hours, while the methods of Oudin and Mancini require at least 20–30 hours.

Single Radial Immunodiffusion

Equipment

Antiserum-agar gel plates of single radial immunodiffusion by the method of Mancini, Carbonara and Heremans as modified by Augener can be prepared in polystyrene Petri dishes (8 cm diameter) having perfectly flat bottoms.

Application of exact amounts of antigen samples is best achieved by the use of a microliter syringe with which exact amounts of 1 μL can be delivered.

The immunoprecipitin rings can be measured with a measuring microscope or a magnifying lens with 0.1 mm scale. A simple way of measuring is by using a measuring template (with *Tripartigen* plates from Behring Diagnostics, these are given as part of the kit for immunoglobulin, etc. quantitations).

Procedure

A microliter syringe is used to fill holes in the agar gel layer with 2 μL of antigen solution. At least three different dilutions of each standard solution are required to plot a curve. The remaining holes are used for the solutions being analyzed. Each is filled with 2 μL of the test specimen in suitable dilution; the diluent is physiological saline. After introducing the reagents, the plates are left in a moisture chamber at room temperature. The results are preferably read after 2 days, although an approximate reading can be obtained in one day. After 2 days, the circular immunoprecipitates in the gel layer are distinct and can easily be measured. For *Tripartigen* (from Behring Diagnostics) a Tripartigen ruler (scale) is provided to read the ring diameter and the quantitation is done by noting the corresponding value given in appropriate units from the reference chart provided. With every kit, a standard serum/specific protein solution can also be had for comparing results.

TURBIDIMETRY

Introduction

Diagnosis is a decision point. The decision is the intention to treat. It is the point at which sufficient evidence has been accumulated to state, beyond reasonable doubt, that the patient is or is not suffering from a particular disease.

Laboratory tests remain one of the mainstays on which the clinicians rely for diagnosis and management of the patient. Laboratory tests are indicated for:

1. Detection

The presence or absence of a particular substance, e.g. testing for infectious diseases like Venereal Diseases Research Laboratory (VDRL), hepatitis B surface antigen (HBsAg).

2. Quantification

Accurately determining the concentration of a particular substance as an aid to diagnosis or differential diagnosis (e.g. concentration of CRP in differential diagnosis of viral and bacterial infections) and for establishing the extent

of the clinical condition (e.g. IgA in measuring disease severity).

3. Monitoring

The course of clinical condition or response to therapy.

4. Prognosis

For predicting the probability of occurrence of a disease/disorder (e.g. microalbuminuria for predicting diabetic nephropathy) or predicting the outcome of a disease/disorder.

Microscopy, biochemical assays, microbiology procedures, and immunoassays are various techniques that fulfil the requirements of routine laboratory tests to meet the needs of the clinicians.

Certain clinical analytes can be measured by specific techniques only, whereas for the measurement of certain analytes options exist for selecting the techniques of measurement. For example, urinary albumin can be measured by biochemical methods such as pyrogallol red or coomassie blue. But for the diagnosis of microalbuminuria, a condition where urinary excretion of albumin is in the range of 30–300 mg/L, the accuracy of the measurements by biochemical methods is questionable because these methods also react with other proteins in addition to albumin which are frequently found to be present in urine. Immunochemical methods (immunoassays) which are more sensitive and specific have a distinct advantage and, hence, are preferred.

Immunoassays

Immunoassays are assays that detect the presence of an antigen in the human body with the help of an antibody or detect the presence of an antibody with the help of an antigen. In this text for simplicity, all further information provided is based on considering antibody as a reagent to detect antigen in the human body fluids.

The first reported immunoassays were homogeneous. They are attributed to Kraus (1897), who coined the term **'precipitin'** for the precipitate formed upon mixing an antigen and an antibody.

Meyer in 1922, employed sheep erythrocytes to serve as a label and conjugated human immunoglobulin to them. Anti-immunoglobulin antibodies appearing in rheumatoid arthritis patients were shown to cause visible clumping of these erythrocytes. This method was known as **hemagglutination.**

Singer and Plotz replaced the erythrocytes with latex particles, which were easier to standardize, and these assays are popularly known as **latex agglutination tests.** High degree of sensitivity for a wide variety of antigens/antibodies, which can be detected by these latex agglutination assays, has promoted their usage worldwide for screening since, 1956 in clinical laboratories. The simplicity of performance and obviating the need for equipments, have made these assays extremely popular.

The need for quantitative estimation, and higher sensitivity led to the development of **radioimmunoassays** (RIA) first in 1959 by Berson and Rosalyn Yalow. The first RIA developed was used to detect and quantify insulin.

Since then immunoassays have been used to detect and quantify a variety of molecules native to humans such as proteins, hormones as well as foreign molecules such as bacteria, viruses and parasites.

Qualitative Immunoassays

Qualitative immunoassay techniques provide test results, which only help to identify or indicate the presence of analytes. Various techniques for qualitative detection of antigens have been in use, which include latex agglutination, passive gel diffusion, IEP and Western blotting. These techniques at the best can give a semiquantitative or comparative information about analytes under assay.

Single immunodiffusion technique uses the diffusion of an antigen into agar impregnated with antibody. **Double immunodiffusion technique** allows the direct comparison of two or more test materials providing a simple and direct method for determining whether the antigens in the test specimens are identical, cross-reactive, or non-identical. **Immunoelectrophoresis** has been used over the years for detection of several different antigens present in a common solution. The latex agglutination assays though simple to use are subject to variations in results as the interpretation pattern between negative and weakly reactive samples may vary between laboratory to laboratory and person to person.

Lower sensitivity for many analytes and the need for correct quantification of analytes for:

- Effective monitoring of disease
- For differential diagnosis to aid correct therapy, have created the need for more sensitive and precise quantitative immunoassays.

Quantitative Immunoassays

Quantitative results of immunoassays are extremely useful in:

- Establishing the extent of severity of a disease
- Assessing the course and stage of clinical condition
- Differential diagnosis of many diseases
- Monitoring response to therapy
- Accurate prognosis of disease.

Various techniques have been used to develop quantitative methods that include radial immunodiffusion (RID) and electroimmunoassays, turbidimetric and nephelometric assays and labeled immunochemical assays.

The RID and electroimmunoassay (rocket electrophoresis) though reliable, are slow, relatively involvement intensive, and expensive. This limits their usage in routine laboratories. In many laboratories, the gel-based techniques are restricted to qualitative studies or are used as reference methods.

During the last decade, the gel techniques are increasingly being replaced by optical detection methods.

The various techniques by which quantitative immunoassays are performed can be broadly grouped as follows:

Heterogeneous Immunoassays

These assay systems employ an antibody immobilized on a solid phase, which captures the corresponding antigen from the sample. A second labeled antibody specific to a different epitope of the antigen is used as a basis for signal generation. After the immunochemical reaction has taken place, the bound and unbound-labeled antibodies are separated. The concentration of antigen is then estimated by measuring bound or unbound-labeled antibodies through an appropriate signal generation and measurement system.

Heterogeneous immunoassays can be performed by various techniques such as:

- Radioimmunoassays (RIA)
- Enzyme immunoassays (EIA)
- Fluorescent enzyme immunoassay
- Chemiluminescent enzyme immunoassay.

The difficulties associated with separation of bound and unbound-labeled antibodies, the need for dedicated instrumentation and labor intensive procedures has prompted the usage of heterogeneous assays in speciality laboratories mainly through use of expensive automation.

The need for simpler, affordable, user-friendly assay techniques for detection of routinely encountered clinical analytes still remained to be explored. With the tremendous progress made in instrumentation technology, optics, and software, the face of quantitative estimation for routine parameters has changed dramatically in the recent years. Simultaneous development in purification techniques for polyclonal antibodies, emergence of monoclonal antibodies with high specificity and avidity have been instrumental in the development of homogeneous assay techniques which are simple to perform and easily adaptable for routine laboratory analysis.

Homogeneous Immunoassays

These assays require only the mixing of a sample (antigen) and the immunochemical reagents (antibody) followed by detection of signal. These assays do not require separation of free or bound-labeled materials in the test system for the detection or measurement of the antigen. In homogeneous immunoassays the immunochemical binding produces a detectable signal (agglutination, absorbance, fluorescence, etc.). The simplicity and flexibility associated with the performance of homogeneous assays has made their usage popular with laboratorians worldwide.

The homogeneous assays can be performed by different techniques such as:

- Turbidimetry
- Nephelometry
- Homogeneous enzyme immunoassays
- Enzyme-multiplied immunoassay technique (EMIT)
- Enzyme inhibitor immunoassay
- Enzyme complementation immunoassay
- Substrate linked fluorescence immunoassay (SLFIA)
- Scintillation proximity assay (SPA)
- Electrochemiluminescence (ECL)
- Luminescent oxygen channeling immunoassay (LOCI).

The clinical chemistry analyzers (photometers) were originally developed for colorimetric estimation of chemical or enzymatic reactions. Subsequently, it was shown that the visible scattered light in Kraus's precipitin reaction could be measured by turbidimetry and nephelometry on photometers, to quantitate the immune complex formation. These systems utilize the fast reaction between an antigen with their corresponding antibodies in a liquid phase.

The technique of quantitation by turbidimetry and nephelometry is apparently similar to the popular absorption spectrophotometry used in routine clinical laboratories and hence, adaptable by high throughput as well as small and medium laboratories easily.

Spectrophotometry

Spectrophotometers work on the basis of the Beer's and Lambert's law.

Beer's Law

When a colored solution is illuminated with a monochromatic light (light of a single wavelength), its absorbance is proportional to the concentration of the colored solution when the light path is constant, i.e. $A \alpha C$ where A is the absorbance of light, C is the concentration of solution.

Lambert's Law

When a colored solution is illuminated with a monochromatic light, its absorption is proportional to the length of the light path, when the concentration of the

solution is constant, i.e. A α L, Where A is the absorbance of light, L is the length of the light path.

Beer-Lambert Law

Combining the two laws together, we have the Beer-Lambert law, which states that when a colored solution is illuminated by a monochromatic light, its absorbance is proportional to the concentration of the solution and the length of the light path, i.e. A α C α L.

$$A = K \times C \times L \text{ .....Equation (1)}$$

where K is a constant

In all photometric estimations a reference standard whose concentration is known is used and its color intensity is compared with the color intensity of the test sample, i.e. $A_t = K \times C_t \times L$

$$A_s = K \times C_s \times L$$

where A_t—Absorbance of test, C_t—Concentration of test, A_s—Absorbance of standard, C_s—*Concentration of the standard.*

Since the pathlength is constant (1 cm) in the spectrophotometer, L is constant, concentration of the standard C_s is known, therefore,

$$C_t = \frac{A_t}{A_s} \times C_s \text{ .....Equation (2)}$$

It has been observed with most biochemistry analytes that as the concentration of analyte increases linearly, the absorbance also increases linearly within the pathophysiological concentration. When a graph of concentration vs absorbance is plotted, a straight-line graph is obtained (Fig. 23.1A). A single standard method using a standard of known concentration or a factor method can be employed for calculating the concentration of the unknown.

Certain reactions, however, may not follow the Beer-Lambert law within the pathophysiological concentration for an analyte and, hence, do not provide a straight-line graph. For such analytes, the unknown cannot be determined using a single standard. A graph using different concentration of standards vs absorbance has to be plotted on a graph paper. The plotted curve is known as the standard curve (Fig. 23.1B).

The concentration of the unknown can be interpolated from this standard curve.

Measuring Principles in Biochemistry

Criteria for Wavelength Selection

It has been established that when the wavelength of light used is complementary to the color of the chemical complex to be measured, peak absorbance is obtained. Thus, selection of the wavelength depends on the color of the complex to be measured.

Complementary Filters for Measuring Color Complexes

Color of the complex	*Wavelength/Color*
Yellow	405/Violet
Red	505/Green
Blue-violet	546/Green
Green	630/Red

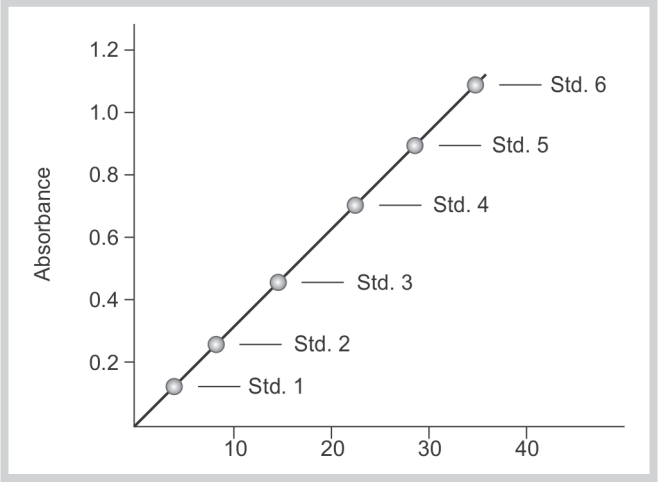


FIG. 23.1A: Illustration of a straight-line graph obtained by plotting absorbance vs concentration of analyte for reactions which obey Beer-Lambert law

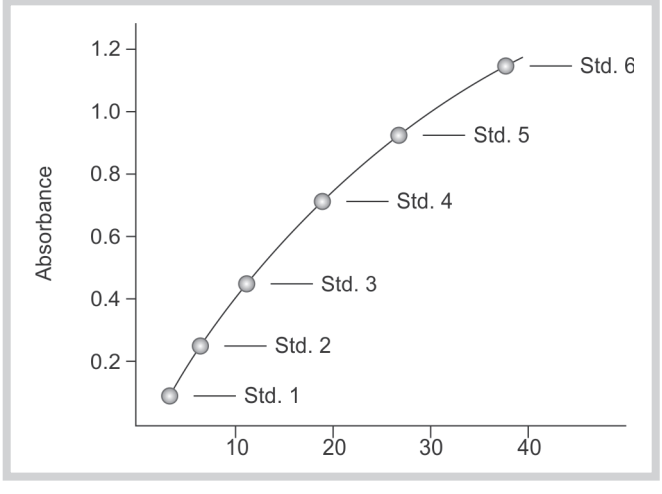


FIG. 23.1B: Illustration of a standard curve obtained by plotting absorbance vs concentration of analyte for reactions which do not obey Beer-Lambert law

For example, for the estimation of hemoglobin using cyanmethemoglobin method, a red colored complex, which is formed during the reaction, is measured using a green filter.

Reading Methods

The measurements of biochemical reactions using enzymes, substrates or specific chemicals are read by methods mentioned below.

1. Equilibrium Methods

Also known as end point methods. Here, the absorbance of end product is measured when the reaction between the reagent and sample has virtually come to equilibrium (end) and the substrate has been converted into a stable end product. The reaction ceases when equilibrium is reached. The concentration of the test specimen can be calculated by using the equation 2 as described earlier.

2. Kinetic Methods

Also known as rate methods where the rate of change of absorbance (ΔA) produced in a fixed time interval is measured.

The kinetic measurements are of two types:

- Fixed time analysis

Where the ΔA produced by the reaction between the reagent and the substrate is measured by stopping the reaction at a fixed time interval.

- Continuous monitoring

Where the ΔA produced is monitored continuously as the reaction proceeds.

Results of the unknown are derived using a factor (K) in the kinetic methods, which is usually provided by the manufacturer or can be calculated as:

$$K = \frac{V_{total} \times 1000}{V_{sample} \times t \times \in \times d}$$

V_{tolal}= total volume of the reaction mixture, V_{sample}= Volume of sample, t = time, ∈ = molar extinction coefficient of the chromogen, d = length of the light path

Standardization of Time Interval for Rate Reactions

Determination of the reaction rate involves the measurement of the amount of change in absorbance (ΔA) produced in a defined time interval. Depending on the reaction, kinetics between a specific reagent and the substrate, the time interval for reading the ΔA can be selected to measure the reaction rate. The different types of reaction curves, which can be obtained as the reaction progresses, typically follow the following patterns (Fig. 23.2).

Curve A

If a graph similar to 'curve A' is obtained then any time interval can be selected for reading reactions, as the rate of change is constant during the entire reaction run.

Curve B

Correct results can be obtained only if the rate is measured along segment II. Incorrect results are obtained if the ΔA is measured during the lag phase (I) or during the phase III.

Curve C

Deviates from linearity over its entire course and ΔA fall off with time. At no time does it give rate of constant changes. Such reaction curves are not suitable for measurements and the reagent systems have to be optimized to obtain correct reaction curves.

Measurement of Immune Complexes by Spectrophotometry

Unlike in classical biochemistry, where the reactants are clear and endpoints are expressed as absorbances, the behavior of light differs for solutions containing suspensions or particulates. Such particles (insoluble immune complexes) are formed as the reaction between antigens and antibodies takes place.

When light of suitable wavelength is allowed to pass through a reaction solution containing antigens (analytes) and the initial absorbance is measured, the absorbance is minimum at this point (Fig. 23.3).

Subsequently, the reagent containing corresponding antibody solution is then added to the antigen in the cuvette and allowed to react. An agglutination reaction begins when a single molecule of antibody binds to at least two corresponding binding sites on different antigen particles. As the reaction proceeds, the agglutinating particles aggregate and form immune complexes. Immune complexes increase in size, become larger, resulting in an increase in turbidity and the scattering of the incident light. Thus, a decreasing part of the incident light (I_o) is transmitted as the reaction proceeds. Spectrophotometers read this decrease in the intensity of the transmitted light as absorbance (Fig. 23.4).

This measurement of reduction in the intensity of the transmitted light at 180° is defined as *turbidimetry*. The turbidity is proportional to the analyte concentration, which in turn is proportional to the amount of agglutination. Based on this proportional relationship, the amount of analyte in the sample causing the turbidity can be easily determined. It should be noted that the nature of immunochemical reaction is exactly the same

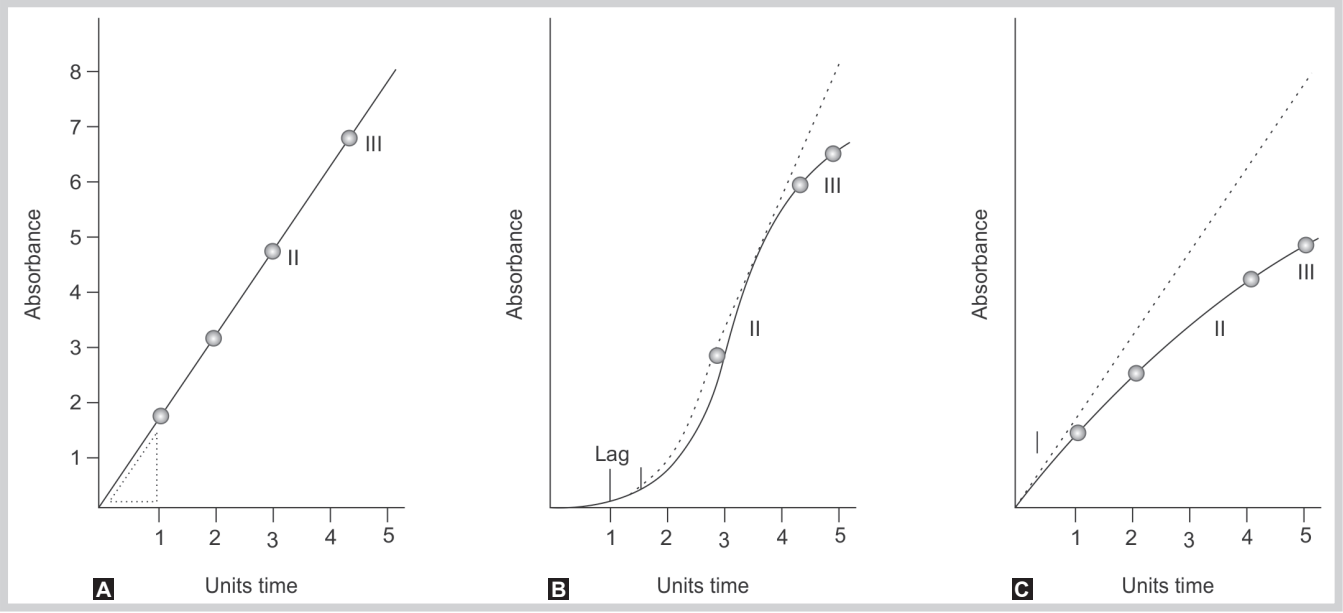


FIG. 23.2: Forms of graphs showing change in reaction rate as a function of time. In A, the rate is constant in all the segments I, II and III. In B, a measurement at segment II will be representative of constant rate, but at segment I (lag phase) and segment III it will be less. In C, the rate falls of continuously

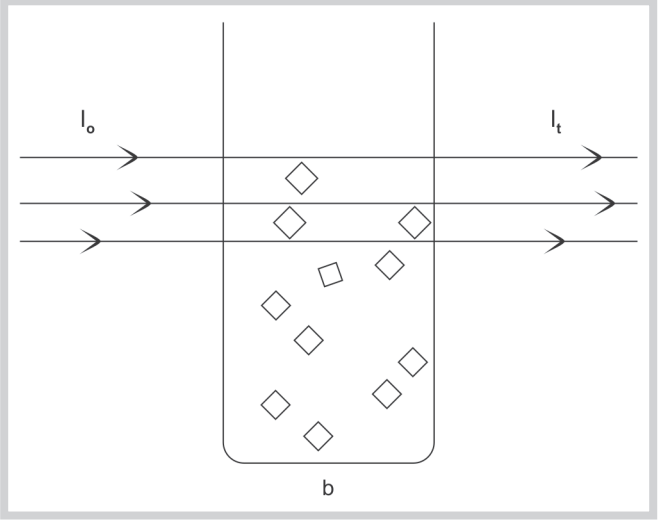


FIG. 23.3: Behavior of light in solution containing antigens where I_o is the intensity of incident light, It is the intensity of transmitted light and 'b' is the cuvette containing antigens in the reaction solution

in turbidimetry and nephelometry. However, it is the detection principle applied for measurement, which differentiates turbidimetry from nephelometry (Fig. 23.5):

Nephelometry measures light scattered or reflected towards the detector, which is away from direct path of the transmitted light. Routine spectrophotometers cannot be used for nephelometry and hence, nephelometers are required. Most nephelometers measure light scattering at a 90° angle. However, in order to measure the forward scatter intensity caused by light scattering from large particles, some nephelometers are designed to measure scattered light at an angle other than 90°.

Selection of Wavelength for Measuring Immune Complexes

The optimum wavelength for optical measurement of immune complexes increases with the size of immune complex to be measured. In general, if the size of the immune complex formed is less than 1/10th the size of the wavelength of incident light, then the light scattering is relatively symmetrical (Fig. 23.6). This uniform scattering of light is known as **Rayleigh scattering.**

On the other hand, when the size of the immune complex to be measured is more than 1/10th the size the wavelength of the incident light of there is concentration of scattered light in forward direction at an angle of 45° or less, away from the axis of the incident light beam (Fig. 23.7). This type of scattering is referred as **Rayleigh-Debye scattering.**

Careful examination of both the figures (Figs 23.6 and 23.7) show that the intensity of scattered light for forward and back scatter (0° and 180°) from small particles is equal but less at 90° (Rayleigh scattering). As the size of the

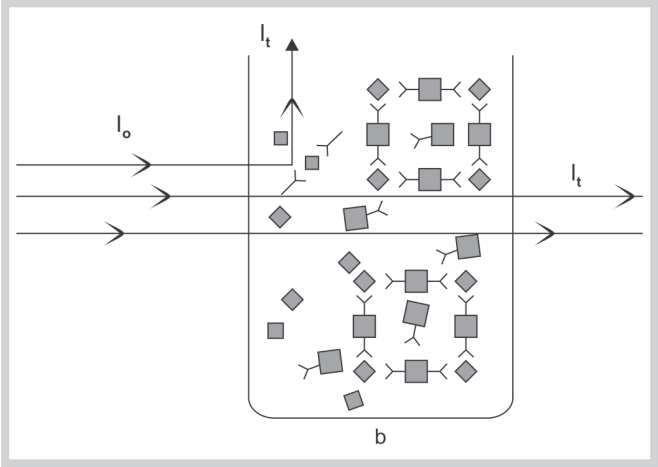


FIG. 23.4: Behavior of light in solution containing immune-complexes (Ag-Ab), where I_o is the intensity of incident light, I_t is the intensity of transmitted light, I_s is the intensity of scattered light and 'b' is the cuvette containing immune-♥complexes in the reaction solution

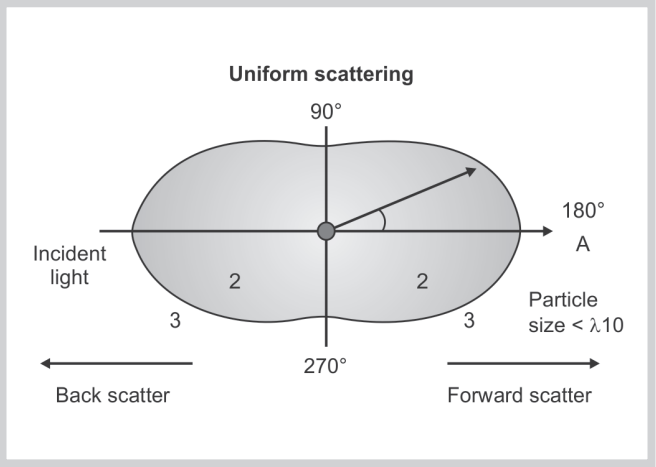


FIG. 23.6: Illustrating Rayleigh scattering for a immune complex with particle size < λ10 of the wavelength of incident light

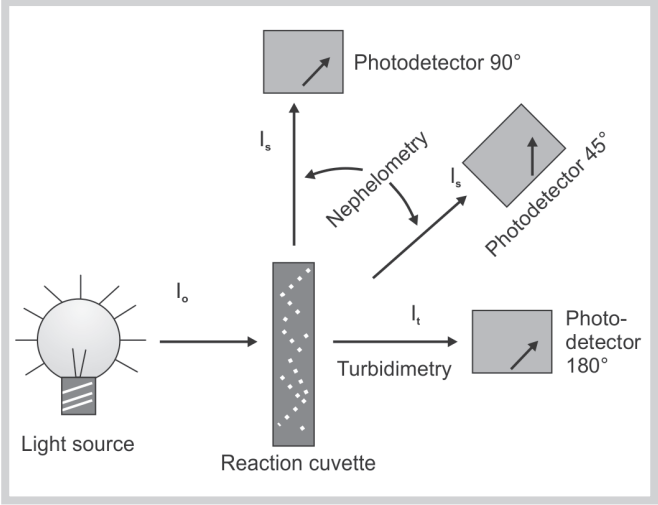


FIG. 23.5: Detection principles in turbidimetry and nephelometry where I_o is the intensity of incident light, 'b' is the reaction cuvette, I_t is the intensity of transmitted light measured by detector at 180°, I_s is the intensity of scattered light measured by detectors placed at 90° and 45°

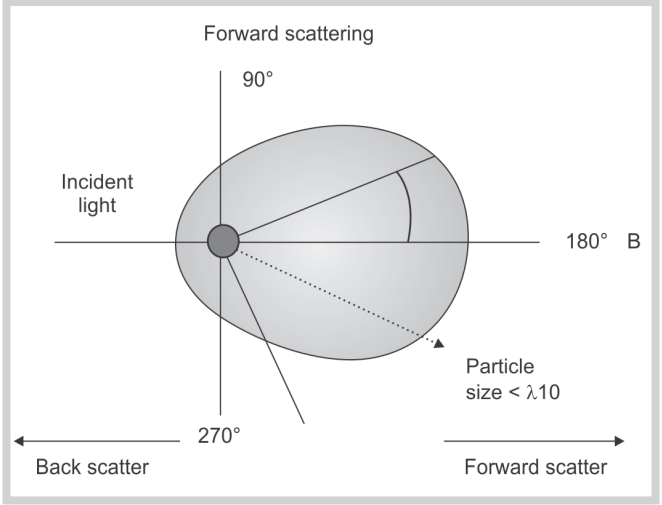


FIG. 23.7: Illustrating Rayleigh-Debye scattering for a immune complex with particle size > λ10 of the wavelength of incident light

particle becomes larger, the angular dependence of light scattering becomes dissymmetrical, increasing in forward scattering and decreasing in backward scattering.

The Rayleigh and Rayleigh-Debye expressions provide useful information about scattering of light by small and intermediate size particles and are important for the optimization of analytical instrumentation for measuring light using turbidimetric and nephelometric assays.

The upper limit on size of immune complexes exhibiting Rayleigh scattering is about 40 nm when a visible light at 400 nm is used. Many of the plasma proteins such as immunoglobulins, albumin, etc. fall below this limit. As the immune complexes, become larger in size from 40 to 400 nm, the angular dependence of scattered light at 400 nm looses the symmetry around the 90° axis, and shows an increase in forward scattering. Some plasma proteins of the IgM class, aggregating immunoglobulin/antigen

complexes fall into this size category. For measuring such complexes a bigger wavelength of light depending on the size of the immune complexes formed, should be used. For latex based assays using a latex particle of approximately 200–300 nm, light of wavelength between 500–600 nm would be ideal for measuring the immune complexes formed.

Biochemical measurements	*Nephelometric measurements*	*Turbidimetric measurements*
Measures absorbance scattering at an by colored complexes formed as a result of biochemical reactions	Measures light reduction in angle away from the incident light due to formation of immune complexes	Measures light at 180°, intensity of light transmitted (as absorbance) at 180°due to the formation of immune complexes
Selection of	*Selection of*	*Selection of*
wavelength of light is complementary to the color of the chemical complex to be measured	wavelength of light depends on the size of the immune complex formed	wavelength of light depends on the size of the immune complex formed

The choice between turbidimetry and nephelometry will depend on application and the available instrumentation. Until recently, it was assumed that for relatively clear solutions in which the transmission of light in the forward direction is greater than 95% small changes in absorption due to turbidity were difficult to measure with precision. The stability and resolution of modern microprocessor driven spectrophotometers and automated clinical chemistry analyzers have greatly improved their ability to measure turbidity with dependable accuracy and precision. Turbidimetric methods have today become competitive in sensitivity with nephelometric methods for immunological quantitation of such solutions.

For some analytes, the signal amplification and assay sensitivity requires the usage of conjugation chemistry to attach antibodies to inert and uniform latex particles. Such reagent systems are referred to as particle enhanced turbidimetry (PET).

PET reagents usually use latex of approximately 200–300 nm for conjugating antibodies to facilitate formation of larger immunecomplexes and thereby generate detectable signals. This leads to decrease in scattering of light at 90° and increase in forward scattering. Turbidimetric assays, therefore, have better precision for measuring larger immune complexes.

In the context of contemporary technology, turbidimetric assays are gaining popularity over nephelometric determination due to their simplicity and overall consistency.

Turbidimetry vs Nephelometry

Turbidimetry	*Nephelometry*
Measures reduction in intensity of transmitted light at 180° due to the formation of immune complexes	Measures scattering of light at an angle (usually 90°) away from the incident light, due to the formation of immune complexes
Can be performed on most spectrophotometers Sensitivity competitive with nephelometric for small immune complexes such as serum proteins More precise for measuring large immune complexes	Requires dedicated nephelometers Sensitive for measuring small immune complexes such as serum proteins Less precise for measuring large immune complexes due to forward scattering of light
Blanking and reading reaction can be performed in the same measuring cuvette	Blanking has to be performed in separate measuring cuvette
Provides better precision due to slower reaction kinetics as blanking of immunochemical reaction can be monitored in a single cuvette	Because of the fast reaction kinetics it is difficult to obtain a sample and a reagent, sample and reagent blank in case of nephelometry

Considerations for Measurements of Turbidimetric Immunoassays (TIA)

As far back in the year 1929, Heidelberger and Kendall have quantitatively described the formation of a precipitate when reacting an antigen with an antibody. They demonstrated that when an increasing amount of an antigen is added to a constant amount of corresponding antibody, the resulting degree of precipitate formed follows a bell-shaped curve as shown in Figure 23.8. To obtain the Heidelberger curve, the antigen concentration is plotted against the absorbances obtained from measuring the Ag-Ab reaction.

The Heidelberger-Kendall curve can be divided in three zones as follows:

The Antibody Excess Zone

In the first stage of the reaction, there is a large excess of binding sites in the reaction mixture available for the antigen to bind. First the antigen binding sites are quickly saturated by antibody before cross-linking begins to occur. This results in formation of small Ag-Ab complexes. In this zone, the absorbances increase proportionally to the analyte concentration.

Zone of Equivalence

In the second stage of reaction binding sites available for the antigen are proportionate to the antigen concentration.

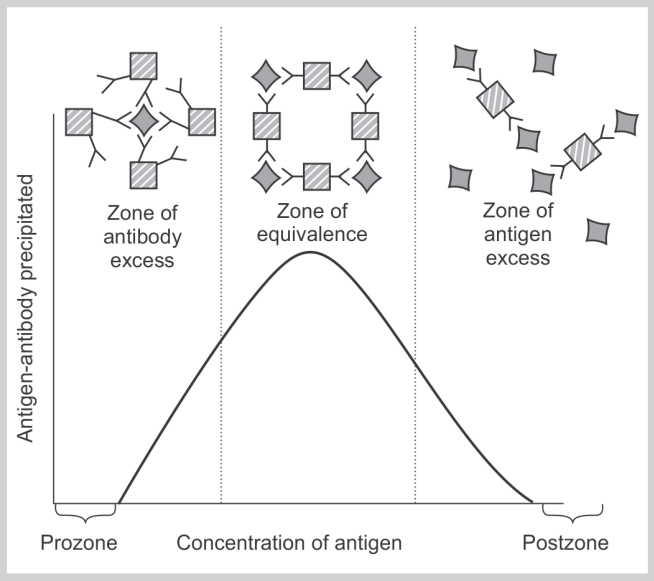


FIG. 23.8: The quantitative immunoprecipitin curve (Heidelberger-Kendall curve)

Here, the probability of cross-linking is more likely resulting in formation of large immune complexes. As the saturation point is reached, there is neither free antigen nor free antibody in the reaction mixture. To this zone, the absorbances increase with the increasing analyte concentration, but does not increase proportionally.

The Antigen Excess Zone

In the third stage of the reaction, the relative concentration of the antigen is so high that most of the binding sites are overcrowded, hindering the formation of real precipitate and favoring the formation of small immune complexes. This is called the prozone effect or the hook effect. The term "prozone" is inappropriately used to describe "postzone" or "antigen excess" in day-to-day parlance. The existence of prozone effect causes very high concentrations of antigen to produce signals, which are similar to the signals generated, by moderate concentrations of the same antigen. It is imperative to know for the assay design as to what concentration of analyte will cause a prozone effect in a turbidimetric immunoassay for a given antibody reagent system.

When the Ag-Ab reaction takes place and the formation of the immune complex is measured optically by turbidimetry, then the absorbance and reaction kinetics in the three zones will follow the following pattern:

Heidelberger-Kendall	*Absorbance curve*
Antibody excess zone	Increases towards maximum
Equilibrium	Reaches maximum
Antigen excess zone	Decreases below maximum

The Heidelberger-Kendall immunoprecipitin curve forms the fundamental basis for all homogeneous Ag-Ab assays including turbidimetry and is usually referred to as the dose response curve.

For many analytes of diagnostic importance, the Ag-Ab reactions neither follow the Beer-Lambert law nor provide a linear relationship between concentration and turbidity. Estimating the concentrations of analytes using a single standard, as in biochemical analysis therefore, results in inaccurate results near the zone of equivalence. The ΔA is directly proportional to the concentration of analyte only in the initial region of the antibody excess zone. Use of single standard for calculating concentration of analyte may be acceptable only for lower analyte concentrations. As the analyte concentrations increase, the error in measurement will start to magnify. Therefore, for having a larger measuring range, the turbidimetric assays use that part of the dose response curve, which covers the maximum portion of the antibody excess region and demonstrates a linear reaction as the standard curve.

The standard curve is plotted using a number of standards containing different concentrations of analyte being measured (usually 5–6). The highest concentration of the standard is chosen in such a way that the analyte absorbance at that concentration will lie on the linear extreme of the standard curve. The lowest concentration of the analyte is usually selected below the reference values of the analyte of interest.

The linear range between the highest and lowest standards used for the preparation of standard curve is referred to as the measuring range of the assay (Fig. 23.9).

Optimization, Standardization and Quality Control of Turbidimetric Assays

To measure the Ag-Ab reactions, reliably all the factors that affect the reaction rate, other than the concentration of the antibody, must be optimized and controlled. As the reaction velocity is at its maximum under optimal conditions, a larger analytical signal is obtained that can be more accurately and precisely measured as compared to a smaller signal obtained under suboptimal assay conditions.

Investigations, of the factors affecting Ag-Ab reactions were extensively studied by Heidelberger and Kendall. In addition to the relative proportions of immune reactants, other conditions such as temperature, ionic strength of the medium, characteristics of the antibody such as avidity and affinity are important for formation of Ag-Ab immune complex. These principles need to be applied to the reagent system optimization for immunoturbidimetry.

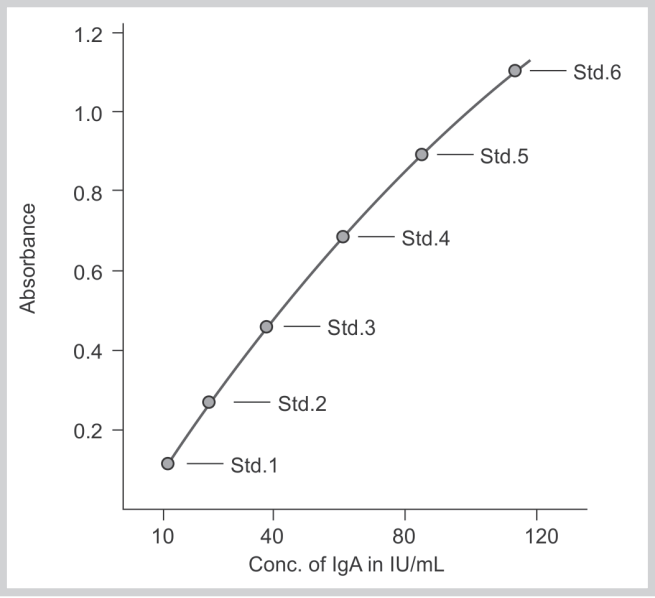


FIG. 23.9: Illustrating standard curve for IgA using lowest standard as 7 IU/mL and highest standard as 120 IU/mL

Ionic Strength

It has been observed that the Ag-Ab reactions are strongly influenced by the nature of ionic medium in which the reaction is carried out. The ionic strength of the reaction environment has a profound effect on the quantum and the rate of the Ag-Ab reaction. As the ionic strength increases, the depth of the electrical double layer that forms around the charged molecules is compressed, reducing the distance over which repulsive forces that keep the molecules apart can act. This in effect leads to the promotion of aggregation. The reduction in charge on the other hand influences the electrostatic attraction between oppositely charged species, thereby reducing the specific binding between antigen and antibody.

pH

The reaction pH also influences the rate of aggregate formation. The rate of reaction is found to be fairly consistent at a pH of 6.0–8.0. Reduction in pH leads to some proteins having net positive charge (those with a pH above the reaction pH) leading to nonspecific agglutination with negatively charged proteins or particles.

Temperature

As it is well known, temperature influences the rate of formation of immune complexes and it should be optimized to obtain accurate results. Assays are usually designed with an incubation temperature of 37°C because it is the most common temperature used in the routine of clinical laboratories for clinical assays.

Enhancers

For enhancing the Ag-Ab reactions, polymeric compounds, such as PEG and BSA, may be included in the buffer system. These compounds facilitate the formation of immune complex and help in amplification of signals and improve the assay system sensitivity.

Interference

Many interfering factors, such as bilirubin and lipids, are normally present in the samples apart from the analyte of interest. High concentrations of some of these interfering factors are frequently encountered in clinical samples. They may influence signal generation and, therefore, can interfere in the assay result. Minimizing the influence of these factors help provide accurate and precise assay results.

Turbidimetric assays usually employ a suitable buffer in the assay design to optimize assay conditions and the desired ionic strength, pH and enhancement required for the reaction medium. In addition, the buffer is also useful in reducing the influence of interfering factors present in the sample. The buffer used in turbidimetric assays is generally referred to as *activation buffer*.

Characteristics of Antibody Used as a Reagent

Intrinsic characteristics of antibody employed as reagents have a profound effect on the Ag-Ab reactions. The specificity and affinity of the antibody to the antigenic sites affect the sensitivity of the assay and signal generation time. Usually, when high specificity and affinity antibodies are used, a strong agglutination reaction will readily result. In contrast, antibodies with low affinity, even if highly specific, tend to react slowly and form a weak immune complex, thereby lowering the detectable signal. The avidity of antibody is also an important consideration in the formation of immune complexes. This characteristic of the antibody determines the degree of stability of the Ag-Ab complexes at the antigen-binding sites. The tendency of the complexes to dissociate and disperse decreases substantially as the avidity of the antibody increases.

An antibody without cross-reactivity and with a good titer is a prerequisite for a reliable turbidimetric assay utilizing antibody as a principal reagent. In addition, the antibody must be formulated as a clear solution to give a low reagent blank and should be free from particulate matter.

Standardization and Calibration

During the course of treatment, individual patients are likely to have tests carried out for the same analyte by

different methods, and to have results checked against reference intervals that were set elsewhere. To achieve agreement between different methods, a single recognized source of reference preparation is needed.

The reference preparation should:

- Have value assignment in meaningful units
- Be stable and identical to the analyte in the test samples
- Be free of interference from the test sample matrix
- Be standardized by a reference method
- Demonstrate intermethod agreements.

Most International Reference Preparations (IRPs) and Certified Reference Materials (CRMs) such as CRM 470 for immunoassay analytes, can be obtained from the main custodians of International Biological Standards such as National Institute for Biological Standards and Controls (NIBSC) or WHO and Community Bureau of Reference of the Commission of European Communities (BCR) or the IFCC.

As the availability of International Standards is limited, it is a practice to prepare sets of secondary standards, from which future lots of calibrators can be assigned values. The secondary standards act as an intermediate between IRP primary standard and future lots of calibrators for assay runs. The calibrator sets are made in bulk and values are assigned with reference to the secondary standards.

As discussed, the immunoturbidimetric assays require a set of 5 or 6 calibrators to obtain a standard curve. The quantitative values of unknown analyte obtained from the standard curve will be highly dependent on correct assignment of values to the calibrator used for preparing the standard curve.

Quality Control

The tendency of most immunological reagents to produce changes in reactivity over time requires the application of quality control procedures to ensure the satisfactory analytical performance of immunometric assays on a day-to-day basis. Similarly, in the case of turbidimetric immunoassays reagent stability within a defined usable time span is a prime requirement of the reagent systems, so is the need for accurate and stable controls to validate reagent functioning, precision and accuracy.

Reading Principles in Turbidimetry

For turbidimetric measurements, both end point and rate measurements are applicable. However, the factor method for calculating the concentration of the unknown is not preferred in the kinetic methods by turbidimetry due to the nonlinear nature of relationship between absorbance and analyte concentrations.

Once the assay system has been designed, the analyzers used for reading must be able to operate according to the principles mentioned below with respect to the addition of reagents and reading of signals (absorbance).

Real Sample Blanking

In this system first the activation buffer (R1) is added to the sample cuvette (S). Then the sample is added, mixed and allowed to stabilize (preincubation period). The first reading (A1) is then taken at the end of preincubation period.

The antibody reagent (R2) is subsequently added, to the above mixture and mixed gently. Turbidity develops due to the reaction between the antigen and the antibody over a short period of time. A second reading is taken at the defined time interval (usually 2–10 minutes).

The difference ΔA_S (Table 23.1) between the two readings represents the absorbance generated as a result of Ag-Ab reaction.

If required, the absorbance due to the reagent ΔA_B can be measured by running in parallel a reagent blank in a separate cuvette (R) using saline in place of sample (Table 23.2).

ΔA_R thus obtained of the reagent blank can be subtracted from ΔA_S of the sample to calculate the absorbance generated due to the Ag-Ab reaction in the sample.

The reagent blank facility may not be available in many semiautomated analyzers. However, the reagent assay system can be optimized to provide a very low reagent blank in order to obviate the need for correcting the reagent blank signals which can contribute to the complete reaction absorbance.

The principle of taking a reading just before the addition of antibody solution (R2) is referred to as '*true sample blanking*' or '*real sample blanking*'.

Immediate Mixed Blanking

In this system initially the activation buffer, sample and the antibody reagent solution are all mixed simultaneously. Then as fast as possible usually 10 to 20 seconds after mixing, the first reading A1 is taken. This 10 to 20 seconds delay time in taking a reading is referred to as lag phase. The reaction is allowed to proceed further and the second reading A2 is measured at the preselected time interval. The increase in absorbance ΔA (A2-A1) represents the signal generated due to the Ag-Ab reaction (Table 23.3).

This method eliminates the need for determination of reagent blank as it measures the increase in absorbance after equilibration of all the reagents and sample. Hence, absorbance generated both due to interfering substances in the sample and the reagent would be blanked during the first reading.

TABLE 23.1: Real sample blanking system: using sample cuvette

	Signals		
	First reading (A1)	*Second reading (A2)*	*ΔA_s (A2-A1)*
	Absorbance due to:	Absorbance due to:	Absorbance due to:
Sample cuvette (S)	• Sample • Buffer	• Sample • Buffer • Reagent • Immune complexes	• Reagent • Immune complexes

TABLE 23.2: Real sample blanking system: using reagent cuvette

	Signals		
	First reading (A1)	*Second reading (A2)*	*ΔA_R (A2-A1)*
	Absorbance due to:	Absorbance due to:	Absorbance due to:
Reagent cuvette (R)	• Buffer • Blank sample (saline)	• Buffer • Blank sample • Reagent	• Reagent

TABLE 23.3: Immediate mixed blanking system

	Signals		
	First reading (A1)	*Second reading (A2)*	*ΔA_s (A2-A1)*
	Absorbance due to:	Absorbance due to:	Absorbance due to:
Sample cuvette	• Reagent • Sample • Buffer	• Reagent • Buffer • Sample • Ag-Ab reaction	• Ag-Ab reaction

In all Ag-Ab reactions in the initial contact phase the reaction kinetics do not follow a systematic pattern. As this initial chaotic phase settles, the reaction pattern and the absorbances move proportionately. This pattern depends upon the intrinsic nature of the antibody, such as affinity, avidity, etc. and also the concentration of the analyte being measured.

Depending on the assay system requirements, it is desirable that the initial chaotic phase is not included in the measurement of absorbance. Typically, a lag phase would vary from ten to thirty seconds from analyte to analyte. It is, therefore, imperative to follow diligently the recommended time assigned for the lag phase for precise blanking in the **"***immediate mixed blanking***"** method.

Reaction Kinetics and its Effect on Blanking

The reaction kinetics of an antigen-antibody also guides as to the appropriateness of the blanking system. As the reaction kinetics is not the same for all Ag-Ab systems, for a system with slow reaction kinetics, e.g. IgA, a first reading 10–20 seconds after mixing with the antibody is not very critical (Fig. 23.10).

However, for a system with fast reaction kinetics, e.g. IgG (Fig. 23.11), half of the reaction would have taken place within 10 to 20 seconds when the first reading is taken. Here, a poorly defined point for the first reading would be obtained.

The implications of '*immediate mixed blanking*' can be demonstrated by comparing the standard curves obtained for the six calibrators of latex enhanced reagent system for measurement of IgA (Fig. 23.12A) and IgG (Fig. 23.12B) at zero seconds and ten seconds, respectively.

The standard curve obtained for IgA (Fig. 23.12A) is practically not affected by the difference between the two ways of blanking indicating that a delay of ten seconds is not very significant.

Whereas for a non-enhanced system with fast reaction kinetics for measurement of analytes such as IgG (Fig. 23.12B), a delay of 10 seconds becomes very critical.

There is considerable signal development during the first ten seconds. This results in decreased difference between A1 and A2 (Fig. 23.11). The loss of signal increases with increasing concentration of IgG in the calibrators.

It can be observed from Fig. 23.12B that the curve for "*immediate mixed blanking*" tends to get flatter with the increasing concentration of IgG, resulting in a decrease in the precision of the analysis.

It would be desirable to optimize both slow reacting systems and assay systems based on particle enhanced turbidimetry (latex-based assays) where the reagent absorbance is very high, based on "*immediate mixed blanking*".

Whereas for systems with fast reaction kinetics such as IgG, the assays should be optimized using the "*real sample blanking*" principle as the sample blanking and the immunochemical reaction can be optimized separately.

Concepts of Assay Optimization

While optimizing reagent system for immunoturbidimetric assays, it is important to optimize the dose-response curve by titerating the amount of sample (antigen) and the antibody concentration in the reagent until a dose-response curve as shown in Figure 23.13, is obtained.

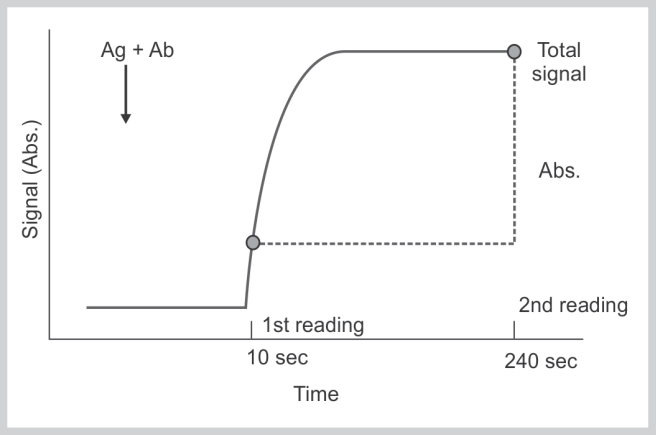


FIG. 23.10: Signal development as a function of time. Figure illustrating 'immediate mixed blanking' using IgA as an example

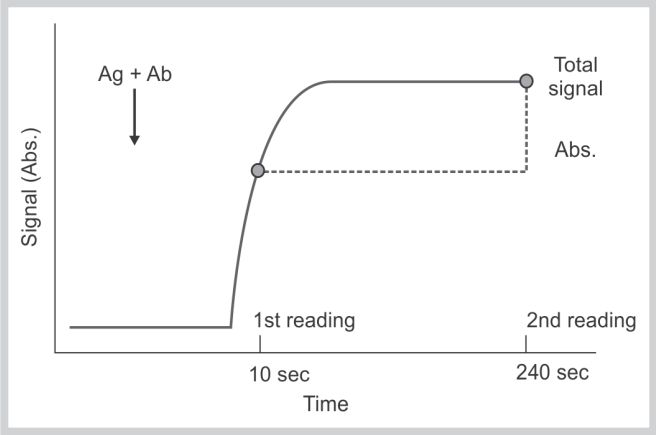


FIG. 23.11: Signal development as a function of time. Figure illustrating 'immediate mixed blanking' using IgG as an example

A portion of the antibody excess zone of dose-response curve is then selected as the "*range for the standard curve*".

Detection Limit

The lowest concentration of an antigen, which gives a detectable signal compared to the background noise, is defined as the detection limit or analytical sensitivity of the analysis. It is defined as the minimum concentration of analyte that is statistically unlikely to form part of the range of signals seen in absence of analyte. Usually, the detection limit is set as the lowest signal where the standard deviation around that signal is less than one third of the signal itself. The lowest concentration selected for the calibration of the assay is usually above the detection limit.

Measuring Range

As long as the analyte signal is higher than the signal of the lowest calibrator and lower than the signal of the highest calibrator, the assay system will operate accurately for the said analyte and a concentration value of the sample can be interpolated.

The interval between the signal generated by the lowest calibrator to the signal generated by the highest calibrator, which gives proportionate and measurable and a linear signal, is referred to as the measuring range of the assay system.

Security Range

The critical point (Cx in Fig. 23.13), in the antigen excess zone of the dose-response curve corresponds to the maximum concentration value of analyte, which gives a signal value higher than the signal value of the calibrator of highest concentration, and just before the value (B_1 in

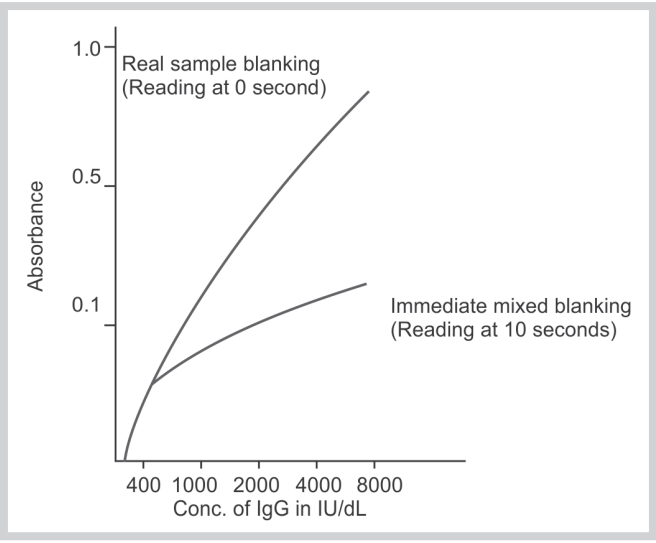


FIG. 23.12A: Standard curves for IgA obtained with 'real sample blanking' and 'immediate mixed blanking'

Fig. 23.13) at which erroneous interpolation begins. The interval between the signal of highest standard and the signal of the critical concentration can be referred to as the security range of the assay system for the analyte.

Reagent Optimization

The risk of obtaining signals in the antigen excess zone are more relevant in analytes like C-reactive protein or immunoglobulins such as IgG where the concentration between normal and pathological sample can differ by manifolds. It is necessary to make sure that the concentration values lying in the antigen excess zone (critical point) are beyond the concentrations which can

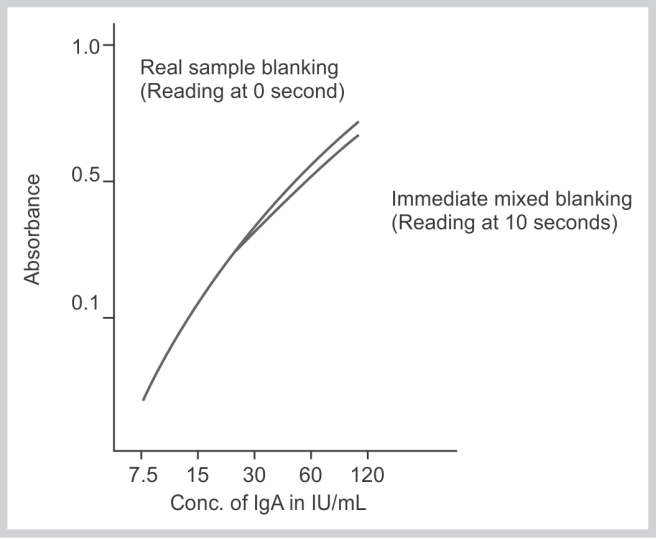


FIG. 23.12B: Standard curves for IgG obtained with 'real sample blanking' and 'immediate mixed blanking'

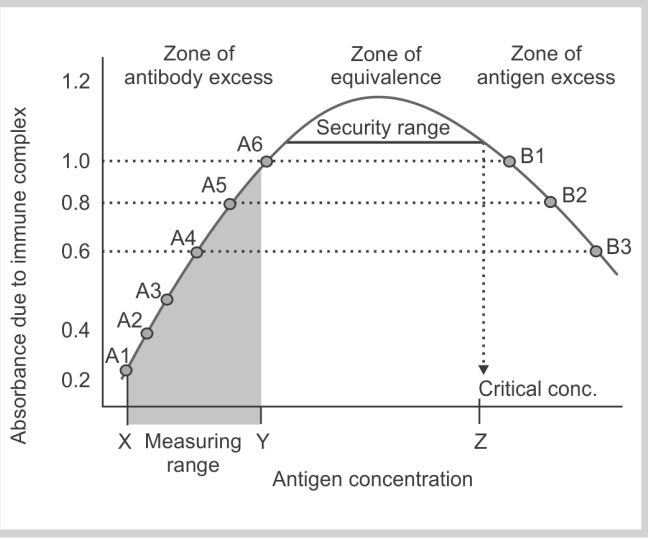


FIG. 23.13: Dose-response curve Figure illustrating measuring range (X-Y), critical concentration (Z), standard curve (A1-A6), erroneous interpolation (where Abs. val.1.0 corresponds to A6 and B1, Abs. val. 0.8 corresponds to A5 and B2 and Abs val. 0.6 corresponds to A4 and B3), security range (Y-Z)

be expected to occur in clinical samples during routine analysis.

The relation between the security range and measuring range is very important in optimizing assay systems. As mentioned earlier the shape of the dose-response curve depends on the ratio between the antigen and the antibody. At a constant antibody concentration, an increase in the measuring range will result in a narrower security range, leading to antigen excess at a lower antigen concentration (Fig. 23.14).

The implications of increasing the antibody reagent concentrations can be practically demonstrated using IgG as an analyte and antihuman IgG as a reagent. The measuring range and the security range can be expanded by increasing the antibody concentration. However, this expansion can only be done to a point where it is still possible to have the desired sensitivity for lower analyte concentration.

Figure 23.15, shows the effect of increasing the antibody concentration on the security ranges while keeping constant, the measuring range of the dose-response curve.

When the volume of an antibody solution of concentration 'X' used is 50 µL, the security range obtained is around 5000 mg/dL. As the volume of the same antibody concentration is increased to 75 µL, the security range increases to >10,000 mg/dL. With a further increase in volume of the same antibody concentration to 100 µL, the security range shifts to >15,000 mg/dL. But this increase in measuring range is possible till a certain limit of increasing volume of antibody solution. If the volume of antibody solution is increased further, there will be a decrease in the absorbance at the lowest concentration of the measuring range due to antibody excess, resulting in compromising with assay sensitivity.

By adjusting the sample dose and the antibody concentration, a measuring range 20 to 25 times the lowest calibrator value can be possibly optimized, with a security range still giving a warning up to the pathophysiological concentration. A wide measuring range combined with a wide security range offer the advantage of a few reruns and maximum security against antigen excess problems.

Standard Curve

Once the dose-response curve for a reagent has been optimized, a standard curve can be obtained by using a number of dilutions of the calibrator (preferably 5–6) covering the optimal measuring range. The lowest calibrator should be chosen to give a signal significantly higher than the background noise. The highest calibrator should be selected to allow measurements for a reasonably wide range of analyte concentrations, and still leaving space for a fair security range (Fig. 23.16).

A curve is fitted to the signals obtained for calibrator dilutions and can be stored in the memory of the instrument. Different curve fitting programs can be made available in instrument software.

Many of the instruments are equipped with a facility to give a 'warning' that indicate reruns of the test with

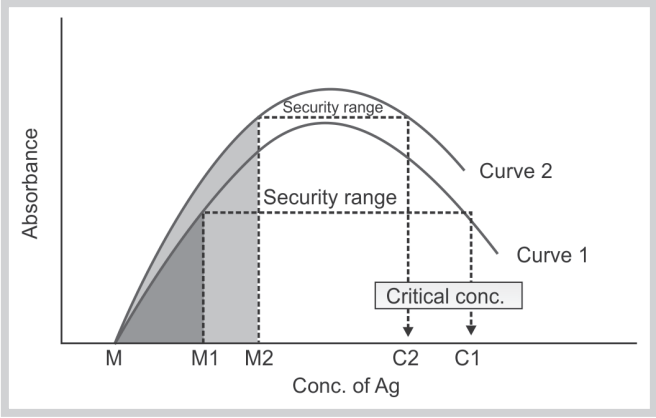


FIG. 23.14: Effect of increasing measuring range on security range. M-M1 = measuring range for curve 1, C1 = critical concentration for curve 1 M-M2 = Measuring range of curve 2, C2 = critical concentration for curve 2

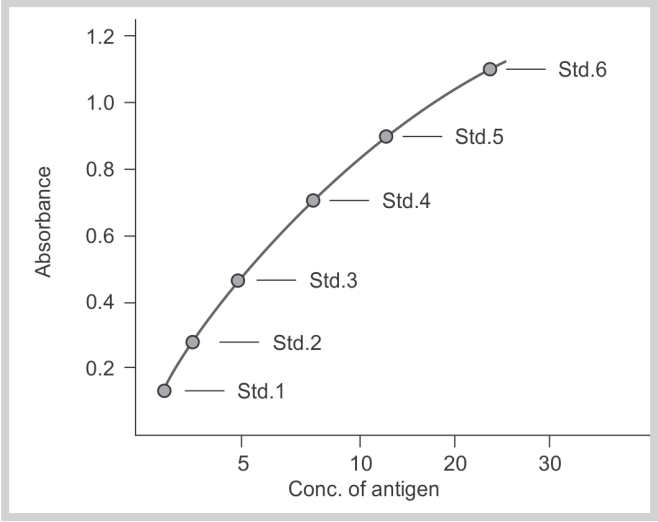


FIG. 23.16: Illustrating standard curve

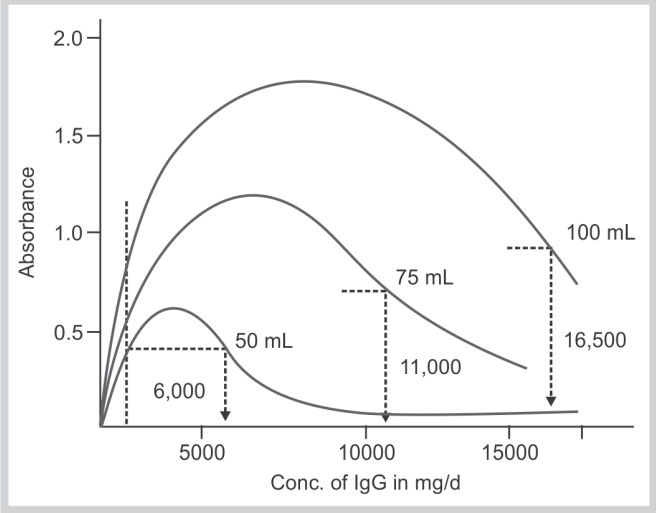


FIG. 23.15: Effect of increasing antibody concentration on security range. Where 50, 75 and 100 µL are volumes of antibody and 6,000, 11,000 and 16,500 are their respective critical concentrations

dilution of the sample with high concentration values of analyte. This warning is given as long as the sample signal is higher than the signal of the highest calibrator.

The validity of the stored standard curve should be checked with known controls at regular time intervals.

Instrumentation for Turbidimetry

The development of automated instruments for the clinical laboratory began in the 1950s at the same time as the demand for test such as IgA, CRP, HBsAg escalated dramatically. One of the benefits of automation is a reduction in the variability of results and errors of analysis by eliminating tasks that are repetitive and monotonous for a human and that can lead to boredom or inattention. The significant improvement in quality of laboratory tests in recent years owes much to the combination of well-designed instrumentation with good analytical methods.

The photometric requirements of turbidimetric analysis are no different from those of photometric biochemistry analysis. However, the photometer used must be provided with means to maintain the contents of the cuvette at a constant temperature during the reaction along with compatible software to run the various steps of the reagent and reagent kinetics appropriately.

The chemistry analyzers (spectrophotometers) available utilize either or both of the two modes of measuring absorbance.

Aspiration Mode

In this mode, the chemical reaction is carried out in a test tube/cuvette. The instrument aspirates the reaction mixture from the test tube/cuvette, which enters into the flow cell (internally built reading chamber), where the absorbance is measured. After the absorbance is measured, the reaction mixture is passed through a different outlet and is collected in a waste collecting bottle. As all the measurements are done in a single flow cell, the flow cell has to be washed after each test. Improper washing may affect the test results of the subsequent tests. Especially, if latex enhanced tests are used, the latex has a tendency to stick and form a permanent coating on the internal walls of the flow cell resulting in variation in the wavelength of the

incident light, and hence, lead to erroneous results. Even non-enhanced antibody reagents are proteinacious and cleaning of the flow cell would remain a critical issue.

When Ag-Ab complexes are aspirated, because of the force of aspiration, the formed immune complexes would be structurally disturbed and may break down into smaller complexes, which would result in lower absorbance values. Moreover, the immune precipitates formed may block the aspiration tube or the flow cell itself.

For turbidimetric assays designed with real sample blanking reading principle, it would be inconvenient to run the test in the aspiration mode. Initially, the sample mixed with the activation buffer would have to be aspirated first and absorbance A1 measured. Then again the activation buffer and the sample will have to be taken in another test tube and the principle reagent added and the reaction read after a fixed time interval. Hence, for running one assay twice, double the amount of activation buffer and sample will have to be used.

Cuvette Mode

In this mode, the reaction of the reagent and sample takes place in a measuring cuvette, and absorbance is read in the same measuring cuvette itself. Hence, assays using any of the reading principles can be conveniently read in cuvette mode without wastage of reagents.

Instruments applying this mode of measuring have an advantage. As the reactions are run in external cuvettes, the instruments are safe from the effects of reagents. Moreover, availability of standardized optically clean disposable cuvettes eliminates the carryover effects of the previous tests.

Classification of Analyzers

There are several types of analyzers available in the market. They may be grouped in two categories:

- Semiautomated analyzers
- Automated analyzers

Semiautomated Analyzers

Instruments with an absorbance linearity of 2.0 are suitable for turbidimetric estimations of both particle enhanced and non-particle enhanced reagent systems. Most of the instruments with the above specification can be used for turbidimetric measurements using the absorbance mode. In the absorbance mode as the calibration curve cannot be stored, it has to be drawn manually.

Among the instruments working on cuvette mode for measuring absorbance, very few instruments have software interface programmed with a facility to store the calibration curve utilizing both the principles of reading, i.e. 'real sample blanking' and 'immediate mixed blanking' in the multistandard mode. Many instruments with cuvette modes are known to have programes in multistandard mode to store calibration curve for assay systems using the real sample blanking techniques only.

Automated Analyzers

The automated analyzers can be grouped in two categories:

- Centrifugal analyzers
- Static instruments (non-centrifugal analyzers).

Centrifugal Analyzers

In these analyzers, the cuvettes are arranged in circle (rotor) that can be rotated at a velocity of about 1000 rpm. The shape of each cuvette allows application of sample and reagent (reaction buffer, antibody) in separate compartments. When the rotor starts to spin, the contents of these compartments are mixed simultaneously and held in place in the cuvette by centrifugal force. Readings of all the cuvettes are performed at essentially the same time (i.e. when the rotating cuvettes are passing the optical measuring device). Two reading systems are used: either parallel to the length of the cuvette where the volume in the cuvette is proportional to the light path or perpendicular to the length of the cuvette where the width of the cuvette equals the light path.

Static Instruments

In these instruments, the cuvettes are mostly arranged in a circle (rotor), and this is slowly rotated in step at a fixed time interval (cycle time). Access to the cuvette is possible only at these intervals for sample or reagent application and reading. Mixing is in most cases performed with a mechanical stirring device. Modern instruments seem more and more to be based on these principles.

All instruments operate under software control. A part of this software is the user interface that makes it possible to program the instrument to perform analysis and calculation according to an optimized protocol. The analytical parameters available for user control vary from instrument to instrument.

Some instruments, however, are "closed instruments" which implies that all parameter settings are read into the instruments by bar coded reagents. In this case, the user cannot control the assay and will have to rely entirely on the manufacturer and their instructions.

The applications and reference values of important clinical analytes are shown in Table 23.4.

Given below are serum proteins with clinical conditions where they are raised and diminished (Table 23.5)

TABLE 23.4: Applications, reference values of important clinical analytes

Analyte	*Description*	*Reference values*	*Applications*
RF	Quantitation of rheumatoid factors	< 10 IU/mL	Detection of RA, differential diagnosis of RA from rheumatic fever and other rheumatic disorders
CRP	Quantitation of C-reactive protein	Adults and children < 0.6 mg/dL	Detection of inflammatory conditions, measuring the severity of conditions, differential diagnosis of bacterial and viral infections Monitoring the response to therapy
CRP US	Quantitation of ultrasensitive levels of C-reactive protein	< 0.05 mg/dL	Prognostic cardiac marker
ASO	Quantitation of antistreptolysin 'O'	Children : < 150 IU/mL Adults : < 200 IU/mL	Detection of Group A streptococcal infections such as sore throat, rheumatic fever, rheumatic heart disease
MA	Quantitation of urinary albumin	< 20 mg/L	Detection of microalbuminuria. Monitoring the effect of ACE inhibitor or intervention strategies for reducing UAE*
IgA	Quantitation of immunoglobulin IgA	70–400 mg/dL	Chronic infections of GI and respiratory tract, anaphylactic transfusion reactions, monitoring progress of IgA myeloma
IgG	Quantitation of immunoglobulin IgG	700–1600 mg/dL	IgG myeloma, IgG deficiency, assessment of the progression and response to treatment of IgG myeloma
IgM	Quantitation of immunoglobulin IgM	40–230 mg/dL	Monitoring patients with Waldenström's macroglobulinemia Estimating frequent, chronic and acute infections
IgD	Quantitation of immunoglobulin IgD	3–14 mg/dL	Screening for congenital infections. Monitoring IgD myeloma
IgE	Quantitation of immunoglobulin IgE	Adult 3–423 IU/mL	Assessment of atopic diseases, dermatologic and parasitic infections
C3	Quantitation of complement component C3	90–180 mg/dL	C3 deficiency, recurrent infections detection and monitoring of immune complex disorders such as SLE, vasculitis, glomerulonephritis, autoimmune hemolytic anemia
C4	Quantitation of complement component C4	10–40 mg/dL	Congenital deficiency in lupus erythromatosus Hereditary angioneurotic edema Recurrent infections
AT III	Quantitation of antithrombin II	17–30 mg/dL	Evaluating patient at risk of developing thrombotic-embolic disease. In surgical patients receiving heparin, assessment of thrombotic risk of contraceptive or estrogen therapy
Apo A-1	Quantitation of apolipoprotein A-1	Males: 105–175 mg/dL Females: 105–205 mg/dL	Independent risk factor for coronary artery disease
Apo B	Quantitation of apolipoprotein B	Males: 60–140 mg/dL Females: 55–130 mg/dL	Elevated Apolipoprotein B levels are associated with atherosclerosis
Lp(a)	Quantitation of lipoprotein(a)	< 300 mg/L	Risk factor for coronary heart disease that is independent of all other lipid parameters
β2-M	Quantitation of β2-Microglobulin	< 60 years : 0.8–2.4 mg/L > 60 years : < 3.0 mg/L	Prognosis of multiple myeloma Early detection of renal transplant rejection, differentiation of glomerular and tubular nephropathies, Monitoring therapeutic response of patients with nonsecretory myeloma or light chain disease
Cp	Quantitation of ceruloplasmin	20–60 mg/dL	Diagnosis of Wilson's disease, Menkes disease, nutritional copper deficiency
Hp	Quantitation of haptoglobin	30–200 mg/dL	Diagnosis and of monitoring of hemolytic diseases

*UAE = Urinary albumin excretion
Note: The above reference values are for guidance only. As the reference values are related to age, geographical and methodological differences and vary widely. Each laboratory should define its own reference range for the relevant population.

TABLE 23.5: Serum proteins in different clinical conditions

Protein	*Increased in*	*Decreased in*
Serum albumin	Rare, usually associated with hemoconcentration	Acute phase response, severe liver disease, nephrotic syndrome, other renal diseases, malnutrition, pregnancy, premature infants
Complement		
Complement C3	APR (infection, inflammation etc.), biliary obstruction, obstructive jaundice, diabetes mellitus, gout, some connective diseases (excluding SLE)	Autoimmune diseases, immune complex diseases, mixed cryoglobulinemia, serum sickness, chronic renal failure, thrombotic thrombocytopenic purpura, neonatal respiratory distress syndrome, genetic deficiency
Complement C4	APR, RA, SLE, rheumatic fever, ankylosing spondylitis, temporal arteritis, acute viral hepatitis, MI, DM, malignancies, thyroiditis, irritable bowel syndrome, pneumonia, pregnancy	Acquired deficiencies resulting from: *Hypercatabolism:* Disease in which circulating immunecomplexes are likely to lead to acquired hypocomplementemia, subacute bacterial endocarditis, mixed cryoglobulinemia, hereditary angioneurotic edema, progressive glomerulonephritis *Hyposynthesis:* Protein calorie malnutrition, liver disease, Sjögren's syndrome *Congenital deficiencies:* Associated with increased frequency of scleroderma, chronic hepatitis, autoimmune hepatitis, Henoch-Schönlein purpura
Lipoproteins		
Apolipoprotein A-1	Familial hyperalphalipoproteinemia, modest alcohol intake, estrogen use, exercise, thyroid hormones androgen use	Chronic renal failure, tangier disease, diabetes, hypertriglyceridemia, liver diseases, familial and non-familial hypoalphalipoproteinemia, certain drugs,
Apolipoprotein B	Premature atherosclerosis, familial defective Apo B, hyperapobetalipoproteinemia, tendon xanthomata, hepatosplenomegaly, diabetes mellitus, chronic renal disease, tangier disease, hypothyroidism, nephrotic syndrome, familial hypercholesterolemia	Familial hypobetalipoproteinemia, abetalipoproteinemia, neuromuscular degeneration, chronic anemia, exercise, liver disease, acute inflammation, certain drugs, neurologic disease
Lipoprotein (a)	Coronary artery disease, cerebrovascular disease, peripheral vascular disease, nephrotic syndrome, DM (variable), cancer, gout, APR, familial hypercholesterolemia	Cirrhosis (particularly primary biliary cirrhosis), certain drugs (nicotinic acid, neomycin, oral estrogen), some steroids such as stanozolol
Immunoglobulins		
IgA class	Polyclonal: Chronic liver disease, alcoholic and non-alcoholic cirrhosis, chronic respiratory infections, GI diseases, some immunodeficiency states, RA, ankylosing spondylitis, nephropathy *Oligoclonal:* May be observed in electrophoresis of IgA myeloma *Monoclonal:* Multiple myeloma (IgA type)	Infancy and early childhood, selective IgA deficiency, protein-losing syndromes, congenital rubella, macroglobulinemia or non-IgA multiple myeloma
IgG class	*Polyclonal:* Autoimmune diseases, chronic liver disease, chronic or recurrent infections, sarcoidosis, some parasitic infections, intrauterine contraceptive devices *Oligoclonal:* Lymphoid or non-lymphoid malignancies, various autoimmune disorders, infections *Monoclonal:* IgG myeloma, lymphoma, Monogammopathies of unknown significance	Agammaglobulinemia, hypogammaglobulinemia, omenn's syndrome, X-linked hyper-IgM syndrome, nephrotic syndrome, non-IgG myelomas, infancy, pregnancy

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Protein	*Increased in*	*Decreased in*
IgM class	*Polyclonal:* Viral infections (hepatitis A, CMV), parasitic infections (filariasis, malaria), chronic liver disease, hyper-IgM dysgammaglobulinemia, collagen vascular disease, primary biliary cirrhosis, primary sclerosing cholangitis *Monoclonal:* Waldenström's macroglobulinemia, malignant lymphoma, reticulosis, cold agglutinin/hemolysin disease	Immunodeficiency states (Wiskott-Aldrich syndrome), non-IgM or IgM myeloma, infancy and early childhood, lymphoma
IgD class	IgD myeloma, chronic infections (pyelonephritis), connective tissue disease, Hodgkin's disease, some forms of liver disease	Various hereditary and acquired deficiency syndromes
IgE class	IgE myeloma, allergic rhinitis, atopic dermatitis, bronchial asthma, hay fever, thymic dysplasia, selective IgA immnodeficiency, eosinophilic gastroenteritis, Wiskott-Aldrich syndrome, Loeffer's syndrome, hyper-IgE syndrome, active SLE nephritis, certain drugs (particularly gold compounds)	Some progressive neoplastic diseases, ataxia-telangiectasia, hypogammaglobulinemia, hypersensitivity
CRP	APR, bacterial infections, viral infections, rheumatic fever, active RA, vascular disorders, MI, Crohn's disease, ulcerative colitis, renal transplant failure, early pregnancy, intrauterine devices, malignancies with widespread metastases	None described
Fibrinogen	APR, nephrotic syndrome, hemodialysis patients, pregnancy, estrogen therapy, contraceptives, acromegaly	Consumption coagulopathies, recurrent pulmonary embolism, recurrent stroke, DIC, incompatible blood transfusion reactions, obstetrical complications, inherited deficiency, prostatic carcinoma, liver disease, certain drugs (e.g. tamoxifen, anabolic steroids, nicotinic acid)
Haptoglobin (Hp)	APR, RA, biliary obstruction, nephritis, ulcerative colitis, aplastic anemia. Major depression, corticosteroid therapy, androgen use	Ineffective erythropoiesis (sickle cell anemia, folic acid deficiency), intravascular hemolysis, progressive tumors of liver and marrow, severe liver disease, pregnancy, estrogen therapy, newborns
Proteinase Inhibitor		
AT III	APR, vitamin K deficiency	Inherited deficiency, acute thrombosis, DIC, consumptive coagulopathies, certain chemotherapeutic drugs, severe liver disease, estrogen therapy, nephrotic syndrome, heparin therapy, some contraceptive medications
Other Markers		
Serum β2-microglobulin	Decreased glomerular filtration, lymphoproliferative disorders, myeloma, RA, viral infections, anticancer drugs, newborns, ESRD, Crohn's disease, Sjögren's syndrome, dialysis related amyloidosis, certain anti-inflammatory drugs	None reported
APR = acute phase reactant, RA = rheumatoid arthritis, SLE = systemic lupus erythematosus, MI = myocardial infarction, DM = diabetes mellitus, ESRD = end-stage renal disease		

AN EXAMPLE OF TURBIDIMETRIC IMMUNOASSAY

Turbidimetric Immunoassay for Determination of Rheumatoid Factors Quantia Rf®

(*Courtesy:* Tulip Group of Companies)

Summary

In rheumatoid arthritis (RA), diagnostically useful autoantibodies termed as rheumatoid factors (RF) can be detected which are immunoglobulins of the class IgG, IgM, IgA and IgE. IgM class IgA with specificity to human IgG Fc is the most useful prognostic marker for RA.

RF play a role in perpetuating the rheumatoid inflammatory process, the severity of joint damage could be predicted according to the strength of RF reactivity. A significant decline of RF with the remission of disease activity has also been demonstrated. Therefore, quantified serial determinations of RF are more meaningful for the diagnosis, prognosis, and assessment of therapeutic efficacy of rheumatoid arthritis. Initial RF positivity has been a sensitive predictor for later joint destruction. Quantified measurement of initial RF level and especially repeated measurements of RF at regular intervals seems to add significantly to the prognostic value of RF in distinguishing between progressive and non-progressive disease in early RA.

QUANTIA-RF is a turbidimetric immunoassay for quantitative detection of rheumatoid factors of the IgM class.

Reagent

1. *QUANTIA-RF activation buffer (R1):* Ready-to-use buffer.
2. *QUANTIA-RF latex reagent (R2):* Ready-to-use uniform suspension of polystyrene latex particles coated with suitably modified Fc fraction of human IgG.
3. *QUANTIA-RF calibrator:* Lyophilized preparation of RF positive serum, which is equivalent to stated amount of RF on IU/mL basis, when hydrated appropriately. The QUANTIA-RF calibrator is traceable to the WHO, International Reference Preparation of Rheumatoid Arthritis Serum.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagents at 2 to 8°C. Do not freeze.
2. The shelf-life of the reagent, activation buffer and the calibrator is as per the expiry date mentioned on the respective vial labels.
3. The reconstituted QUANTIA-RF calibrator is stable for 7 days at 2 to 8°C and 48 hours at 25 to 30°C (RT).
4. The working reagent for QUANTIA-RF can be prepared by mixing R2 and R1 in the ratio 1:5.
5. The mixed stability of the working reagent (R1+ R2) is 7 days when stored at 2 to 8°C.

Principle

QUANTIA-RF is a turbidimetric immunoassay for the determination of rheumatoid factors and is based on the principle of agglutination reaction. The test specimen is mixed with QUANTIA RF latex reagent (R2) and activation buffer (R1) and allowed to react. Presence of RF in the test specimen results in formation of an insoluble complex resulting in an increase in turbidity, which is measured at wavelength 505 to 578 nm. The increase in turbidity corresponds to the concentration of RF in the test specimen.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. All the reagents derived from human source have been tested for HBsAg and HIV antibodies and are found to be non-reactive. However, handle the material as if infectious.
3. Reagents contain 0.1% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal, flush with large quantities of water.
4. The reagents can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagents be verified using known controls periodically.
5. Gently mix the QUANTIA-RF latex reagent well before use to disperse the latex particles uniformly to improve test performance.
6. The working reagent should be mixed gently.
7. Do not use vortex mixers for mixing. Gently mix the reagents and samples during test procedures.
8. As the reagents within lots have been matched, reagents from different lots must not be interchanged.
9. Calibrators of different manufacturers must not be used with QUANTIA-RF reagents.
10. The calibration curve must be validated periodically with known controls.
11. The QUANTIA-RF assay is recommended only for analyzers with cuvette mode. Though any semiautomated analyzer with appropriate programing facility can be used, for best results it is recommended to use; **Quantiamate** analyzer. Fully automated

analyzers may be used, provided the reagent has been standardized on the system.

12. The procedure mentioned here is based on a minimum reading volume of 500 µL (0.5 mL). In case of instruments where minimum volume required for reading absorbance is 1.0 mL, use double the quantity of reagents and samples mentioned in the test procedure.

Specimen Collection and Preparation

No special preparation of the patient is required prior to specimen collection by approved techniques.

Only serum should be used for testing. Should a delay in testing occur, store the samples at 2 to 8°C. Samples can be stored for up to 3 days at 2 to 8°C, provided they are not contaminated. Do not use hemolyzed, icteric or highly turbid sera. Turbid or particulate serum samples must be clarified by centrifugation at 2000 rpm for 15 minutes. Use the clear supernatant for testing.

Additional Material Required

Spectrophotometer with 505 to 578 nm wavelength filters and cuvette mode, stopwatch, well-calibrated micropipettes, disposable tips, isotonic saline, particulate free distilled water, test tube rack, incubator/waterbath set at 37°C, optically clean disposable cuvettes such as **Quantiamate semimicrocuvettes**/glass cuvettes.

Note: Though any filter between the wavelengths 505 to 578 can be used, optimum results are obtained with a filter with 546 nm wavelength.

Test Procedure

Bring reagents and specimen to room temperature before use.

Assay Conditions

Wavelength	546 nm
Reaction temperature	37°C
Cuvette	1 cm path length

Method for Preparation of RF Calibration Curve

The QUANTIA-RF calibrator must be reconstituted exactly with 1.0 mL of distilled water, wait for 10 minutes, gently swirl the vial till the solution attains homogeneity. Once reconstituted, it is ready to use for preparation of RF calibration curve. The concentration (S) of RF in the reconstituted calibrator is as mentioned.

Dilute the calibrator serially as mentioned below for preparation of calibration curve.

Test tube No.	*1*	*2*	*3*	*4*	*5*
Calibrator dilution No.	D1	D2	D3	D4	D5
Isotonic saline	-	100 µL	100 µL	100 µL	100 µL
Calibrator	100 µL	100 µL	100 µL	100 µL	100 µL
Conc. of IgA in IU/mL	120	60	30	15	7.5

The above five dilutions of the calibrator including the highest 120 IU/mL (D1) and lowest 7.5 IU/mL (D5) concentrations of measuring range must be used for the preparation of the calibration curve.

Test Procedure for Preparation of Calibration Curve

1. Zero the instrument with distilled water.
2. Pipette 400 µL of QUANTIA-RF activation buffer (R1) and 100 µL of QUANTIA-RF latex reagent (R2) in the measuring cuvette. Mix well and incubate for five minutes at 37°C.
   or
   Pipette 500 µL of QUANTIA-RF working reagent in the measuring cuvette. Mix well and incubate for five minutes at 37°C.
3. Add 10 µL of calibrator (D1), mix gently and start the stopwatch simultaneously.
4. Read absorbance (A1), exactly at 10 seconds, and absorbance (A2) again at the end of exactly 4 minutes.
5. Repeat steps No. 2 to 4 for each diluted calibrator (D2 to D5) for preparing calibration curve.
6. Calculate DA (A2-A1) for each calibrator (D1 to D5). Plot a graph of DA versus concentration of RF on the graph paper provided with the kit.

"The calibration curve" so obtained is valid only for the same lot of QUANTIA-RF reagents.

Test Procedure for Specimen

For determination of RF concentration in the test specimen:

1. Follow steps 2 to 4 as mentioned in the above procedure for calibration curve using the test specimen in place of the calibrator.
2. Calculate ΔA (A2-A1) for the test specimen.

Validation Criteria

If the ΔA of the test specimen is less than ΔA obtained for the standard of highest concentration (D1) then the concentration of IgA in the test specimen can be determined directly by interpolating ΔA of the test specimen from the calibration curve.

If the ΔA of the diluted test specimen is higher than ΔA of standard with highest concentration (D1), then the

test has to be rerun by carrying out further dilution of test specimen such as 1:10, 1:20, etc. till the DA of the diluted test specimen is less than ΔA of Δ1. Then proceed for calculations.

Calculations

1. Interpolate ΔA of the diluted test specimen on the calibration curve and obtain the RF concentration 'C' of the diluted test specimen.
2. Multiply the RF concentration 'C' with the dilution factor (F) of the test specimen for obtaining the concentration of RF in the neat test specimen.

Concentration of RF in the neat test specimen in IU/mL = C × F

(where 'F' is the dilution factor of the test specimen, i.e. 10 for 1:10 dilution of test specimen and so on).

Specific Performance Characteristics

Measuring Range
The QUANTIA-RF reagent has been designed to measure RF concentrations in the range 7.5–120 IU/mL and is linear between the measuring range.

Detection Limit/Analytical Sensitivity
Detection limit: 7.5 IU/mL.

The detection limit represents the lowest measurable RF concentrations that can be distinguished from zero.

Prozone Limit
No prozone effect was observed up to a concentration of 1250 IU/mL of RF.

Interference
No interference was observed with:

Interference factor	No interference up to
Glucose	500 mg/dL
Albumin	10 g/dL
Bilirubin	50 mg/dL
Hemoglobin	500 mg/dL
Triglycerides	1000 mg/dL

Reference Values
The reference values of RF in normal population are ≤10 IU/mL.

Each laboratory should define its own reference range for relevant population.

Remarks

1. Usage of well-calibrated equipment and accessories and procedures is critical for achieving correct results.
2. When ΔA obtained for the test specimen is greater than the ΔA of the standard with highest concentration then, it indicates that the concentration of IgA in the test specimen is beyond the measuring range of the QUANTIA-RF assay. Such specimens should be rerun with further dilutions.
3. Markedly lipemic, hemolyzed, and contaminated serum samples could produce non-specific values.
4. Use of plasma rather than serum can lead to non-specific values.
5. Do not read results beyond 4 minutes.
6. Rheumatoid factors are not exclusively found in rheumatoid arthritis but sometimes in syphilis, systemic lupus erythromatosus, hepatitis and hypergammaglobulinemia also.
7. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
8. QUANTIA-RF assay is sensitive to the presence of IgM IgA with heterogenous specificity.

C-REACTIVE PROTEIN

C-Reactive protein (CRP) is an abnormal serum glucoprotein produced by the liver during acute inflammation or infections. CRP is synthesized by the liver under regulatory control of cytokines. Interleukins 1b and 6 and tumor necrosis factors are the most important regulators of CRP synthesis. The intact CRP molecule is a pentameric protein with identical subunit arranged in a doughnut shaped polymer.

The function of CRP is felt to be related to its role in the innate immune system. Similar to IgG it activates complement, binds to Fc receptor and acts as an opsonin for various pathogens. Interaction of CRP with Fc receptors leads to the generation of proinflammatory cytokines that enhance inflammatory response. Unlike IgG, which specifically recognize distinct antigenic epitopes, CRP recognizes altered self and foreign molecules based on pattern of recognition. This recognition provides an early defense and leads to a proinflammatory signal and activation of the humoral immune response. CRP binds to apoptotic cells, protects the cells from assembly of terminal complement components and sustains an antiflammatory innate immune response.

All acute inflammatory process (infectious and non-infectious) and certain malignant conditions result in rise in serum CRP, as a non-specific phenomenon. CRP production is a non-specific response to disease and it can never on its own be used as a diagnostic test. However, if the CRP results are interpreted in the light of clinical information on the patient it can provide exceptionally useful information.

Levels of CRP increase very rapidly in response to trauma, inflammation and infection and decrease very rapidly with the resolution of the condition. An activated CRP is always associated with pathological changes. Hence, determination of CRP is of great value in diagnosis, treatment and monitoring of inflammatory condition. Measurement of CRP may be helpful to know whether the patient is getting better, or if there are any complications arising.

CRP Measurements Help in Diagnosis and Management

Rheumatology	The rheumatic diseases exhibit joint or soft tissue symptoms such as back pain, and myalgia. However, such symptoms may also be due to psychogenic factors. The elevation of acute phase proteins confirms the presence of organic disease but a value within the reference range does not exclude a mild local disease. In condition like ankylosing spondylitis, serum CRP may be elevated before the onset of clinical symptoms
Adult rheumatoid arthritis	Increased CRP levels are found in more than 90% of adults with this condition and in established disease, levels relate to severity. Values of up to 5 mg/dL are associated with mild inflammation and values around 10 mg/dL indicate more severe disease. Certainly CRP levels correlate more closely with radiologically determined joint damage than other serological test. Typically, if a patient responds to a particular drug the fall in CRP precedes the improvement in clinical symptoms by about 6 weeks and the radiological improvement by about 6 months
Ankylosing Spondylitis	Back pain is a very common clinical symptom and an elevated CRP is a strong indication of organic disease such as ankylosing spondylitis
Polymyalgia Rheumatica	CRP concentration is markedly raised. If untreated 30% of patients will develop cranial arteritis with a serious risk of eyesight. CRP rapidly falls to normal as the disease responds to therapy with corticosteroids
Connective tissue diseases	SLE, polymyositis, systemic sclerosis, in these cases acute phase response is minimal even in active disease. Hence, CRP levels can be used to distinguish these conditions from other rheumatic diseases
Infections	Bacterial infections are associated with some of the highest CRP levels and its measurement is a sensitive marker for bacterial sepsis. Gram-negative bacteria generally elicit more reproducible responses than gram-positive bacteria, with modest responses to parasitic infestations and minor responses to viruses and fungi. CRP measurement is useful in detecting infections where clinical and microbiological diagnosis is difficult but where infection is suspected. CRP levels relate to the extent and intensity of sepsis and successful treatment leads to decline in levels within about 3 days
Pediatric fever	In children, although fever is most often due to viral infection, this is difficult to distinguish from bacterial sepsis such as otitis media, bronchitis, tonsillitis, and cystitis, and antibiotics are often prescribed unnecessarily. It has been shown that, in children who have been ill for more than 12 hours, a CRP level of greater than 4 mg/dL had a diagnostic sensitivity of 79%, and a specificity of 90% for the diagnosis of bacterial infections
Adults postoperative surgery	Surgery of all types induces inflammation and an acute phase response roughly in proportion to the extent of tissue damage. In uncomplicated cases CRP rises above 1 mg/dL by about 6 hours, reaches a peak rarely greater than 15 mg/dL at about 48 hours, and declines thereafter to baseline values by 7–10 days. Postoperative complications such as infections, tissue necrosis, hematoma, and thrombosis, depending upon when they occur, will maintain a raised CRP level after 48 hours, or result in a secondary increase. In many cases the raised CRP precedes the clinical diagnosis of the complication pathology by up to 24 hours. In such situations, single values are of little value and serial monitoring is essential
Appendicitis	Using a cut off of 1 mg/dL it has been reported that CRP has clinical sensitivity for this condition of 68.2% and a specificity of 75.1%
Meningitis	Some studies using serum CRP have described almost perfect discrimination between bacterial and viral meningitis in children. Bacterial meningitis is associated with higher CRP levels than aseptic or viral meningitis. Appropriate therapy for bacterial and tuberculous meningitis causes fall in CRP levels, and hence, this simple test can be used to monitor response to treatment with many advantages over repeated lumbar punctures especially in children

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Pulmonary infection	Pneumonia can be difficult in the elderly where the febrile response may be lost. A CRP level above 10 mg/dL provides a very strong indication of bacterial infection such as purulent bronchitis or pneumonia. Typically, viral pneumonias do not result in values above 5 mg/dL
Malignant tumors	Increasing levels of CRP imply a poor prognosis and frequently suggest metastalic spread.
Burns	CRP levels increase significantly in patients with extensive burns. A second peak of CRP later implies superadded infection as a late complication of burns
Myocardial infarctions	Peak CRP levels occur about 50 hours after the onset of pain in myocardial infarction, and correlate well with peak serum levels of cardiac isoenzymes such as CKMB. In patients who recover uneventfully the CRP levels fall rapidly towards normal. However, complications such as persistent cardiac dysfuntion further infarction, intercurrent infection, thromboembolism, are associated with either persistently raised CRP levels or secondary increase after initial decrease. Angina without infarction does not stimulate CRP production. Routine assays of CRP in patients with chest pain may thus assist in diagnosis, and management of complications
Immunocompromised patients in acute leukemia	Fever in patients with leukemia and neutropenia can be caused by infection, the underlying disease process, administration of blood products, and cytotoxic therapy. Approximately 40% of cancer patients with fever and neutropenia develop culture proven bacterial infections. Fever can also be caused by viral infections or may be by other non-infectious causes. Because of significant morbidity and mortality in this group, there is aggressive use of antibiotics. Chemotherapy or transfusions do not affect CRP. Pronounced elevations of CRP do not occur in malignancies without other concomitant stimuli for synthesis such as intercurrent infection. If CRP concentration is less than 4 mg/dL for 48 hours after the onset of fever, infection is unlikely, whereas levels above 10 mg/dL should be treated by antibodies even in absence of bacteriological confirmation. If after treatement levels do not fall below then it must be assumed that response has not occured and therapy must be maintained and changed

It is often difficult to diagnose abdominal infection in pregnant women, since CRP is at normal levels in pregnant women, increased CRP concentration indicates infection complication.

Bacterial sepsis is one of the most common diagnostic challenges in neonatal medicine. A definitive diagnosis based on culture of blood, CSF or urine is usually reached only after a delay of a day or two, yet rapid progression of untreated infection may greatly increase morbidity and mortality. Initiation of antibiotic therapy may result in treatment of as many as 30 uninfected infants for every single infant who is determined to have been infected. Attempts to develop a screening test that can identify infected infants, sparing others from invasive diagnostic procedures, intravenous antibiotic therapy, mother infant separation and heightened parental anxiety has led to the observation that CRP levels during these intervals may be useful for early identification of infants for whom antibiotic therapy can be safely discontinued. In addition to better management of disease or disorders, CRP has been known to aid in the differential diagnosis of many illnesses.

The degree of elevation of CRP reflects the mass or activity of the inflamed tissue, which may be secondary to the underlying disease as in myocardial infarction and malignancy, or a primary component as in rheumatoid arthritis.

In many cases, the changes in palsma CRP levels precede changes in clinical symptoms. In every situation sequential measurements provide more information than single determinations.

To summarize, quantitative CRP measurement would be useful in:

- Screening or organic diseases
- Differential diagnosis
- Assessment of disease activity and monitoring of therapy
- Recognition of intercurrent infections
- Prognosis of conditions such as myocardial infarction.

TURBIDIMETRIC IMMUNOASSAY FOR DETERMINATION OF C-REACTIVE PROTEIN

Quantia CRP®

(*Courtesy*: Tulip Group of Companies)

Summary

C-reactive protein (CRP) is an acute phase protein synthesized in the liver. Its rate of synthesis increases within hours of acute injury or the inflammation and may reach as high as 20 times the normal levels. A rapid fall of CRP indicates recovery. The degree of elevation of CRP level directly reflects the mass or activity of inflamed

Role of CRP in Differential Diagnosis

Clinical condition	*Significantly Elevated CRP*	*Normal CRP/mildly elevated CRP*
Rheumatic diseases	In established RA disease—levels relate to severity. Values upto 5 mg/dL are associated with mild inflammation and values around 10 mg/dL indicate more severe disease	Normal in osteoarthritis
Gastrointestinal diseases (inflammatory bowel disease)	Crohn's disease	Ulcerative colitis, normal CRP or mild elevation < 5 mg/dL
Pediatric fever	Children ill for more than 12 hours with CRP > 4 mg/dL generally indicates bacterial infection	CRP level < 4 mg/dL may be bacterial or viral infection
Genital infections	Chlamydial infections when extended into the pelvic organs with acute or chronic pelvic inflammatory disease	Uncomplicated gonococcal or chlamydial infection not elevated
Pulmonary infection	Above 10 mg/dL provide a strong indication of bacterial infection such as pneumonia or purulent bronchitis	Typically viral pneumonia does not result in values above 5 mg/dL
Causes related with chest pain	Elevated in pulmonary embolism, pleurisy, or pericarditis	Not elevated in angina without infarction or invasive investigation
UTI in young children	Values > 5 mg/dL indicate pyelonephritis	Normal to slightly elevated levels indicates uncomplicated UTI

tissue. And its ability to fall to normal levels on resolution of the condition renders quantified CRP values to be a good indicator to allow rapid selection of appropriate anti-inflammatory therapy in several rheumatic diseases, which are, clinically difficult to assess. Apart from indicating inflammatory disorders, CRP levels help in differential diagnosis, in the management of neonatal septicemia and meningitis where standard microbiological investigations are difficult. CRP levels rise invariably after major surgery, but fall to normal within 7–10 days. Absence of this fall is indicative of septic or inflammatory postoperative complications. Serum CRP concentration provides useful information in patients with myocardial infarction there being an excellent correlation between peak levels of CRP and creatine phosphokinase.

TURBIDIMETRIC IMMUNOASSAY FOR ULTRASENSITIVE DETERMINATION OF C-REACTIVE PROTEIN

Quantia CRP-US®

(*Courtesy*: Tulip Group of Companies)

Summary

C-reactive protein (CRP), the classical acute phase protein is an extremely valuable marker for underlying systemic inflammation. The median value for serum CRP in apparently healthy adults is approximately 0.08 mg/dL, the 90th centile of distribution in such subjects is approximately 0.03 mg/dL. The baseline values for CRP in a healthy individual remain stable over a long period of time. The baseline serum concentration of CRP predicts the risk of future myocardial infarction and stroke independent of other risk factors, in apparently healthy subjects.

Increased values of CRP below 0.5 mg/dL previously considered to be within the reference interval are strongly associated with increased risk of atherothrombotic events. Several prospective studies suggest that in apparently healthy individuals, as the concentration of CRP increases from greater than 0.055 to 0.211 mg/dL, the probability for developing AMI increases significantly from a factor of 1 to 2.9. Apparently, healthy individuals in the highest quartile (the upper 25%) of the above-mentioned range have 2 to 3 times higher risk of developing subsequent atherosclerotic diseases compared to those in the lowest quartile. Simultaneous ultrasensitive measurements of CRP and total HDL cholesterol predict future vascular risk better than lipid measurements alone.

Such low levels of CRP in apparently healthy adults can be determined by ultrasensitive immunoassays such as QUANTIA-CRP US.

TURBIDIMETRIC IMMUNOASSAY FOR DETERMINATION OF ANTISTREPTOLYSIN 'O' IN HUMAN SERUM

Quantia ASO®

(*Courtesy*: Tulip Group of Companies)

Summary

The group A, β-hemolytic streptococci produce various exotoxins such as streptolysin O, steptolysin S that can act as antigens. The affected individuals produce specific antibodies against streptolysin 'O' that has clinical significance namely, Antistreptolysin 'O'. Antistreptolysin 'O' can be detected 1–3 weeks after infection, attaining a maximum level at around 3–6 weeks. Determination of these antibodies is very useful for the diagnosis of streptococcal infections and their relative effects such as rheumatic fever and acute glomerulonephritis.

TURBIDIMETRIC IMMUNOASSAY FOR DETERMINATION OF MICROALBUMINURIA

Quantia MA®

(*Courtesy*: Tulip Group of Companies)

Summary

Urinary albumin excretion between 30 and 300 mg/day (microalbuminuria), far below the levels found in clinical proteinuria (> 300 mg/day) is a strong predictor of development of diabetic nephropathy and vascular complications. diabetic nephropathy leads to progressive loss of renal function or end-stage renal disease (ESRD) and may necessitate need for dialysis or transplantation in most cases. The progression of microalbuminuria is closely associated with progressive hypertension and loss of blood glucose control. The early presence of microalbuminuria can be reversed by strict metabolic control and timely intervention of drugs early in the course of disease can arrest the progression of diabetic renal disease. Quantitative values of albumin are useful for differentiating microalbuminuria from clinical proteinuria and the effective monitoring of intervention strategies.

Annual screening of microalbuminuria is recommended by the 'WHO' and 'International Diabetes Foundation' in all patients with IDDM over the age of 12 years and who have had diabetes for five years or more.

Microalbuminuria is also a significant risk marker of cardiovascular diseases. Its presence can be regarded as an index of increased cardiovascular vulnerability and a signal for correction of known risk factors.

Information regarding the concentration of albumin in urine for the detection of microalbuminuria can be obtained by using QUANTIA-MA reagents.

IMMUNOGLOBULINS (Ig)

(Immunoglobulins IgG, IgM and IgA)

Immunocompetent persons have an immune system that can be divided into the following two functionally cooperative but developmentally independent ways:

- Thymus (T) lymphocyte system; it represents a functionally heterogeneous group of cells concerned with immune regulation and antigen elimination
- Bursa or bone marrow (B) lymphocyte system; B lymphocytes differentiate into plasma cells which synthesize and secrete antibodies after an antigenic stimulus.

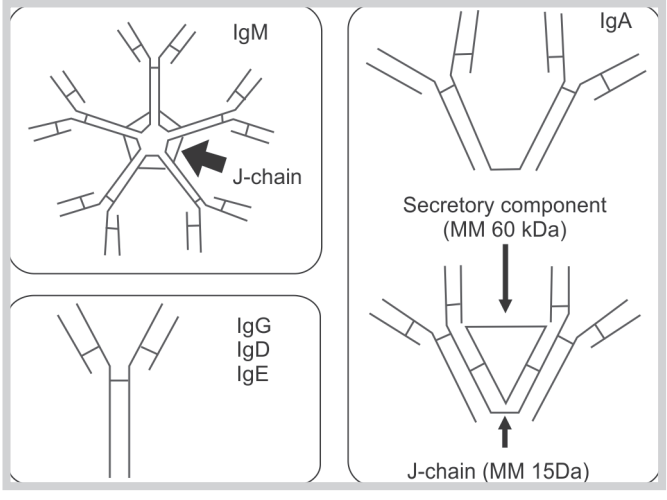
Immunoglobulins represent a heterogeneous group of proteins with antibody function, i.e. they are capable of binding antigen. The structure of antigen binding site is made according to the configuration of the antigen with which the antibody reacts. Immunoglobulins have following effector functions:

- Formation of immune complexes with antigens
- Binding the membrane receptors of defense cells and their activation
- Reaction with plasma proteins, e.g. with complement components, and activation of these proteins in order to eliminate the antigen.

Ig Classes

IgG, IgA, IgM, IgD and IgE are present in descending order of concentration. IgG has subclasses from IgG_1 to IgG_4, IgA and IgM have two subclasses each namely 1 and 2.

Ig Structure



Immunoglobulin G (IgG)

Increased in	*Decreased in*
• IgG myeloma	• Acquired immunodeficiency
• Sarcoidosis	• Hereditary deficiencies
• Chronic liver disease	• Protein-losing syndromes
• Autoimmune diseases	• Pregnancy
• Parasitic diseases	• Non-IgG myeloma
• Chronic infection	• Waldenström's macroglobulinemia

Immunoglobulin M (IgM)

Increased in	*Decreased in*
• Liver disease	• Hereditary deficiency
• Chronic infections	• Acquired immunodeficiency
• Waldenström's macroglobulinemia	• Protein-losing syndromes
	• Non-IgM myeloma
	• Infancy, early childhood

Immunoglobulin A (IgA)

Increased in (in relation to other Ig's)	*Decreased in (alone)*
• Gamma-A myeloma (M-component)	• Normal persons (1:700)
• Cirrhosis of liver	• Hereditary telangictasia (80% of patients)
• Rheumatoid arthritis with high titers of rheumatoid factors	• Type III dysgammaglobulinemia • Malabsorption (some patients)
• SLE (some patients)	• SLE (occasionally)
• Sarcoidosis (some patients)	• Cirrhosis of liver (occasionally)
• Wiskott-Aldrich syndrome	• Still's disease (occasionally)
• Other rare entities	• Recurrent otitis media (occasionally) • Non-IgA myeloma • Waldenström's macroglobulinemia • Acquired immunodeficiency *(combined with other Ig's)* • Agammaglobulinemia *Acquired* Primary Secondary (multiple myeloma, leukemia, nephritic syndrome, protein losing enteropathy) *Congenital* Hereditary thymic aplasia Type I dysgammaglobulinemia (all, IgG, IgM, and IgA decreased) Type II dysgammaglobulinemia (IgA and IgM absent, IgG has normal levels) • Infancy, early childhood

Serum Immunoglobulin Changes in Various Diseases

Disease	*IgG*	*IgA*	*IgM*
Immunoglobulin disorders			
Lymphoid aplasia	D	D	D
Agammalglobulinemia	D	D	D
Type I dysgammaglobulinemia (selective IgG and IgA deficiency)	D	D	N or I
Type II dysgammaglobulinemia (absent	N	D	D
IgA and IgM)			
IgA globulinemia	N	D	N
Ataxia telangiectasia	N	D	N
Hematological neoplasms			
Heavy chain disease	D	D	D
IgG myeloma	I	D	D
IgA myeloma	D	I	D
Macroglobulinemia	D	D	I
ALL	N	D	N
CLL	D	D	D
AML	N	N	N
CML	N	D	N
Hodgkin's disease	N	N	N
Liver disease			
Hepatitis	I	I	I
Laennec's cirrhosis	I	I	N
Biliary cirrhosis	N	N	I
Hepatoma	N	N	D
Miscellaneous			
Rheumatoid arthritis	I	I	I
SLE	I	I	I
Nephrotic syndrome	D	D	N
Trypanosomiasis	N	N	I
Pulmonary tuberculosis	I	N	N

N = normal, I = increased, D = decreased

TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF IMMUNOGLOBULIN IgA IN HUMAN SERUM

Quantia IgA®

(*Courtesy*: Tulip Group of Companies)

Summary

Persistently elevated immunoglobulin levels indicate an ongoing response of the immune system, whereas a decline in immunoglobulin levels hints at a recovery from the

infectious process. The absolute levels of immunoglobulin concentration are a measure of the severity of the inflammatory process, especially in virally induced chronic liver disease, chronic bacterial infections, collagen vascular diseases and other autoimmune diseases. In diseases that cause hypergammaglobulinemia quantitative determination in conjunction with the clinical presentation as well as analysis of serological and clinical chemistry results may not only help in diagnosis, differential diagnosis, but may also prove to be useful for disease monitoring and prognosis.

IgA is the second most abundant immunoglobulin (approximately 10% of the total Ig mass) and is the major immunoglobulin found in mucosal surfaces. Patients with congenital IgA deficiency are prone to autoimmune diseases, and may develop antibodies to IgA and anaphylaxis if transfused. Approximately, 10 to 15% of all myeloma are of the IgA type. Polyclonal increase in serum IgA may be observed in chronic inflammatory disease of gastrointestinal and respiratory tracts and liver. IgA may be decreased in patients with chronic sinopulmonary disease, ataxia-telangiectasia or congenital. Information regarding the concentration of IgA can be obtained by using QUANTIA-lgA reagents.

TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF IMMUNOGLOBULIN IgG IN HUMAN SERUM

Quantia IgG®

(*Courtesy*: Tulip Group of Companies)

Summary

Persistently elevated immunoglobulin levels indicate an ongoing response of the immune system, whereas a decline in immunoglobulin levels hints at a recovery from the infectious process. The absolute levels of immunoglobulin concentration are a measure of the severity of the inflammatory process, especially in virally induced chronic liver disease, chronic bacterial infections, collagen vascular diseases and other autoimmune diseases. In diseases that cause hypergammaglobulinemia quantitative determination in conjunction with the clinical presentation as well as analysis of serological and clinical chemistry results may not only help in diagnosis, differential diagnosis, but may also prove to be useful for disease monitoring and prognosis.

Deficiency of IgG is associated with frequent and occasionally severe pyogenic infections. Increased levels of IgG are associated with chronic or recurrent infections, various autoimmune disorders, lymphoid or non-lymphoid malignancies and IgG myeloma.

IgG is the only immunoglobulin to cross the placenta and provide protection from intrauterine infections to the fetus, and is of importance in defence against infections in newborns.

Decreased levels of IgG are observed in agammaglobulinemia, hypogammaglobulinemia, and nephrotic syndrome. Information regarding the concentration of IgG can be obtained by using QUANTIA-IgG reagents.

TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF IMMUNOGLOBULIN IgM IN HUMAN SERUM

Quantia IgM®

(*Courtesy*: Tulip Group of Companies)

Summary

The absolute levels of immunoglobulin concentration are a measure of the severity of the inflammatory process, especially in virally induced chronic liver disease, chronic bacterial infections, collagen vascular diseases and other autoimmune diseases. In diseases, that cause hypergammaglobulinemia quantitative determination in conjunction with the clinical presentation as well as analysis of serological and clinical chemistry parameters or test results may not only help in diagnosis, differential diagnosis, but may also prove to be useful for disease monitoring and prognosis.

Immunoglobulin IgM comprises approximately 7 to 10% of normal serum immunoglobulins and are the prominent antibody class in primary response to most antigenic stimuli. As IgM does not cross the placenta, presence of virus specific IgM in cord blood or neonatal serum is indicative of congenital infection. IgM levels may be increased in chronic liver disease, infections, Waldenström's macroglobulinemia, and malignant lymphoma. Decreased levels are observed in immune deficiency states, non-IgM myeloma, infancy and early childhood lymphoma.

Quantified IgM measurements are useful for the detection of frequent chronic or acute infections, suspected immunodeficiency, screening for congenital infections and monitoring patients with Waldenström's macroglobulinemia. Information regarding the concentration of IgM can be obtained by using QUANTIA-IgM reagents.

TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF COMPLEMENT C3 IN HUMAN SERUM

Quantia C3®

(*Courtesy*: Tulip Group of Companies)

Summary

Clinically, complement determination helps to detect whether the complement system has been activated. A decrease in complement components due to the activation of complement system or a hereditary deficiency and/

or dysfunction of a complement component is of clinical significance. C3 is a central component of the complement system. C3 is the rate-limiting factor for both the alternate and the classical complement pathways. C3 is often decreased in active forms of SLE and membranoproliferative glomerulonephritis. C3 fixation on red cells and on tissue may result in autoimmune hemolytic disorder or severe tissue damage. Increased levels of C3 are observed in biliary obstruction, nephrotic syndrome and during corticosteroid therapy.

Information regarding the concentration of C3 can be obtained by using QUANTIA-C3 reagents.

TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF COMPLEMENT C4 IN HUMAN SERUM

Quantia C4®

(*Courtesy*: Tulip Group of Companies)

Summary

Clinically, complement determination helps to detect whether the cojmplement system has been activated. A decrease in complement components due to activation of complement system or a hereditary deficiency and/or dysfunction of a complement component is of clinical significance.

C4 is essential for activation of the classical complement pathway. Most of the conditions, which result in decreased levels of C3 also, result in decrease of C4. However, in cases of autoimmune hemolytic anemia (AIHA) and hereditary angioneurotic edema (HAE), C3 is usually normal while C4 is decreased.

Information regarding the concentration of C4 can be obtained by using QUANTIA-C4 reagents.

TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF ANTITHROMBIN III IN HUMAN SERUM

Quantia AT III®

(*Courtesy*: Tulip Group of Companies)

Summary

Antithrombin (AT) formerly referred to as antithrombin III, a proteinase inhibitor is the most important inhibitor of the plasma coagulation system. The action of AT III in presence of heparin is directed against thrombin, Xa and IX leading to an effective inhibition of coagulation. Concentration of AT III is useful in differential diagnosis of congenital AT deficiencies. In type I congenital deficiency, the AT III concentration is reduced, whereas in type II AT deficiency the AT III concentration is normal. Estimation of AT III is useful in diagnosis of disseminated intravascular coagulation (DIC) and monitoring of the course of treatment of DIC, monitoring of AT III replacement therapy and in cases of heparin resistance. Information regarding the concentration of AT III can be obtained by using QUANTIA-AT III reagents.

QUANTITATIVE IMMUNOTURBIDIMETRIC ASSAY FOR ESTIMATION OF FIBRINOGEN

Quantia Fibrinogen®

(*Courtesy*: Tulip Group of Companies)

Summary

Fibrinogen (Factor I) is a high molecular weight glycoprotein synthesized in the liver, which plays an important role in hemostasis. For normal hemostasis to occur in response to injury or tissue damage, a sufficient concentration of fibrinogen must be present in plasma.

Low levels of fibrinogen are found in:

- Liver disease
- Increased fibrinogen consumption due to the prolonged presence of disseminated intravascular coagulation
- Hyperfibrinolysis in patients with neoplasia, acute promyelocytic leukemias and obstetric complications such as premature detachment of placenta or abruptio placentae, amniotic fluid embolism, retention of dead fetus
- Dysfibrinogenemia (functionally defective fibrinogen due to an abnormal molecular form, but the levels remain normal) found either congenitally or acquired in liver disease.

Studies such as the Framingham study and Northwick Park Heart Study have demonstrated that an increased fibrinogen concentration is an independent risk factor for atherosclerotic diseases, e.g. myocardial infarction or stroke.

Quantia-Fibrinogen is an antigen immunoassay for the quantitative determination of fibrinogen in human plasma.

TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF LIPOPROTEIN (A) IN HUMAN SERUM

Quantia LP (a)®

(*Courtesy*: Tulip Group of Companies)

Summary

Coronary artery disease (CAD) is emerging as a major public health problem. Of all the ethnic groups, people of Indian origin have one of the highest incidences of

CAD and diffuse and severe CAD frequently occurs in Indians at an early stage. The prevalence of premature CAD in Indians is upto three times higher when compared with people of similar age group in the Western world.

Lipoprotein (a) [Lp (a)] is a genetically determined lipoprotein molecule having a protein moiety apolipoprotein (B)-100 (the protein associated with low-density lipoprotein) disulfide linked to apolipoprotein (a) the distinctive glycoprotein that is homologous to plasminogen. Lp (a) functions as a dual pathogen that is highly atherogenic and also prothrombotic. The Apolipoprotein B-100 part of Lp (a) binds to LDL receptors and acts as an atherogenic protein. The Apolipoprotein (a) moiety competes with plasminogen for binding to fibrinogen and fibrin monomer and thus acts as a prothrombotic agent.

Several studies have demonstrated that Lp (a), is one of the most powerful and most prevalent independent risk factors for premature CAD. Early therapeutic interventions and lifestyle modifications at lower levels of total cholesterol and LDL cholesterol, particularly in persons with a family history of premature CAD and in persons with high Lp (a) levels has been suggested.

The CHD (Coronary heart disease) risk prediction is high, especially when Lp (a) and LDL concentrations are elevated simultaneously. Lp (a) elevations have also been linked to restenosis after angioplasty and progression of angiographically documented coronary heart disease.

In normolipidemic subjects those with Lp (a) levels greater than 30 mg/dL may have a risk for myocardial infarction 1.7 times that of subjects with LP (a) levels below this level have been documented.

Information regarding the concentration of Lp (a) can be obtained by using Quantia-Lp (a) reagents.

QUANTITATIVE TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF APOLIPOPROTEIN A-I

Quantia Apo A-I®

(*Courtesy*: Tulip Group of Companies)

Summary

The risk of premature atherosclerotic coronary heart disease (CHD) has direct correlation with plasma concentration of LDL cholesterol and inverse correlation with HDL cholesterol. Apolipoprotein A-I (apo A-I) is the major anti-atherogenic apolipoprotein found in HDL cholesterol and plays an important role in HDL metabolism. Apo A-I is an activator of lecithin cholesterol acyltransferase (LCAT) enzyme present in HDL, which catalyzes the reaction forming cholesterol esterase (CE). This CE rich HDL cleans up the cholesterol from peripheral tissue and transport it to liver [reverse cholesterol transport (RCT)]. Increased rate of apo A-I production causes high plasma HDL concentration and may have a protective effect from premature CAD. Genetic defects that cause the inability to synthesize apo A-I cause very low plasma concentrations of HDL-C thereby increasing the risk of atherosclerotic CHD.

Estimation of apo A-I levels is useful in determining the cholesterol clearing capacity of the blood in an individual and thereby predicting the relative risk of CHD. Studies have demonstrated that in patients with known CAD on treatment with lipid lowering drugs, levels of apo A-I and apo B were a significant perdictor for recurrent cardiovascular events as compared to plasma LDL-C and TC (Total cholesterol) levels.

Quantia Apo A-I is a turbidimetric immunoassay for the quantitative determination of apolipoprotein A-I in human serum.

QUANTITATIVE TURBIDIMETRIC IMMUNOASSAY FOR ESTIMATION OF APOLIPOPROTEIN B

Quantia Apo B®

(*Courtesy*: Tulip Group of Companies)

Summary

The risk of premature atherosclerotic coronary heart disease (CHD) has direct correlation with plasma concentration of LDL cholesterol and inverse correlation with HDL cholesterol. Apolipoprotein B is a major apolipoprotein of VLDL and LDL the atherogenic lipoproteins. Apo B helps in solubilizing the cholesterol within the LDL complex, which in turn increases the transport capacity of LDL for subsequent deposition of cholesterol on the arterial wall thereby promoting heart disease. Only one molecule of apo B exists per lipoprotein particle. The quantity of apo B is therefore, a direcct measurement of VLDL and LDL particles. However, due to wide variations in the amount of cholesterol in these lipoproteins, measurement of apo B has better relevance to the concentration of atherogenic lipoprotein particles than LDL cholesterol or non-HDL cholestrol levels. Subjects with the greatest risk of mortality from heart attack tend to have the highest ratios of apo B/apo A-I.

Studies have demonstrated that in patients with known CAD on treatment with statins (lipid lowering drugs), levels of apo B and apo A-I were a significant predictor for

recurrent cardiovascular events as compared to plasma LDL–C and TC (Total cholesterol) levels.

Quantia Apo B is a turbidimetric immunoassay for the quantitative determination of apolipoprotein B in human serum.

AUTOMATION IN TURBIDIMETRY

Quantimate Turbidimetry Analyzer (Fig. 23.17)

Optimized Measuring System

- Preprogramed for Quantia reagents
- Adaptable turbidimetric immunoassay reagents
- Backup analyzer for routine chemistries
- End point, kinetic, fixed time, multistandard (MSD) and absorbance modes
- 90 open locations including 40 open locations for MSD
- Six programing modes in MSD
- Automatic flagging of outlying results
- Plug and play system.

Instrument Specifications

- Measuring system : Cuvette mode
- Filter range : 340, 405, 505, 546, 578, 630
- Temperature : 37°C
- Measuring range : –200 to +2.0 OD
- Photometric accuracy : 2% from 0 to 2.0 OD
- Photometric linearity : ±1%
- Reading volume : Minimum 1.0 mL in macrocuvettes, Minimum 0.3 mL in semimicrocuvettes
- Keyboard : 10 digital keys and 8 functional keys
- Display : Back illuminated LCD with 32 characters
- Thermal printer : 20 columns

FIG. 23.17: Quantimate turbidimetry analyzer

Troubleshooting

Immunoturbidimetry Tests

For Product Range

Quantia-RF, Quantia-ASO, Quantia-CRP, Quantia-CRP US, Quantia-MA, Quantia-IgG, Quantia-IgM, Quantia-IgA, Quantia-C3, Quantia-C4, Quantia-AT III, Quantia-Lp(a), Quantia-Fibrinogen, Quantia-Apo-A1, Quantia-Apo-B

Problem: Testing errors

Possible causes	*Solutions*
Pipettes and Pipetting	
1. Use of wet, contaminated or damaged pipettes	Before testing, check whether the pipettes are wet, contaminated or damaged to avoid errors in testing
2. Improperly calibrated micropipettes	Prior to testing, check if the micropipettes are being used for a long-time without being recalibrated
3. Inadequate pipette volumes	Ensure that small volumes of reagent or sample are not pipetted out with large volume pipettes
4. Contamination of reagent or sample pipetted	Use fresh tips during pipetting of reagent as well as sample to avoid contamination of the same

Contd...

Contd...

Temperature and Time	
1. Inadequate reagent temperature	Prior to commencing of the testing procedure, ensure that the reagents and samples are at the assay temperature so recommended
Equipments	
1. Contaminated cuvettes	Check whether the cuvettes are free from foreign matter. Also, ensure that the solution in the cuvettes is free of other foreign matter and air bubbles Cleanlines of cuvettes will help in eliminating contamination
2. Incorrect wavelength	Ensure that correct wavelength is used in testing
3. Hardened or clogged filters	Filters need to be checked periodically to avoid hardening and loss in efficacy over a period of time
Storage and Procedure	
1. Inadequate storage conditions for longer life of the reagents	Ensure proper storage of kits before and after use
2. Error in performing the test	Ensure that the procedure and addition sequence is followed as indicated in the package insert

Problem: Errors in real sample blanking and immediate mixed blanking methods

Possible causes	*Solutions*
Low absorbance values	
1. Change in the time interval for measuring absorbance A_1 and A_2 can lead to early or delayed mesurement than the actual reaction time	Ensure correct time interval for measuring absorbances A_1 and A_2 to avoid erroneous results
2. Improper incubation temperature	Temperature influences the rate of formation of immune complexes; therefore, it should be optimized to obtain accurate results
3. High or low absorbance of calibrator during calibration	Ensure that the sample is added properly while performing the test
4. Increase in concentration of calibrator	Avoid evaporation of the calibrator in order to obtain desired concentration.
5. Deterioration of reagent	Partial deterioration of the reagent can give low reactivity and hence low absorbance values Ensure that proper storage conditions are adhered to
6. Incorrect sample used	If plasma is used instead of serum, low absorbance values may be obtained. Follow proper procedural steps for use of sample
7. Sample has analyte concentration beyond the prozone limit	Dilute the sample and rerun the test
8. Differences in reactivity between antigen and antibody are observed leading to variations in readings obtained	If the latex based reagents are not mixed well before use, variations in readings are observed. Therefore, it is of utmost importance to mix the latex based reagents well before pipetting to avoid variation in readings. Besides, dispensing and mixing well it is also important to mix the reagent well with the sample to trigger optimum reaction between antigen and antibody to obtain correct absorbance values
High absorbance values	
1. Deterioration of calibrator	Deterioration of the calibrator can give high absorbance values. Ensure that proper storage conditions are adhered to

CHAPTER 24

The Endocrine System

INTRODUCTION

The science concerned with the structure and functions of the endocrine glands and the diagnosis and treatment of disorders of the endocrine system is called endocrinology. This term comes from the Greek words *'endo'* (within), *'crine'* (to secrete) and *'logos'* (study of).

The endocrine system consists of glands situated in different areas of the body as shown in Figure 24.1. Each gland produces different hormones, which regulate the activity of other organs and tissues in the body. These hormones are released directly into the blood flowing through the gland. This is in contrast to exocrine glands, which release hormones down a tube or duct.

Glands are functional units of hormone-secreting cells located in various regions of the body making up the endocrine system. Each gland has specific functions that help to maintain the normal internal environment and promote the survival of the organism. Although, there are some diffuse endocrine tissues, as in the gastrointestinal epithelium, there are several major glands or control centers within the endocrine system.

PITUITARY GLAND

The pituitary gland, which lies in a small depression in the sphenoid bone of the skull called the sella turcica, has often been termed the *'Master Gland'* because many of the hormones, it releases affect the release of other hormones. However, the pituitary is really not the master. It is controlled by a brain region called the hypothalamus via the release of releasing factors into a special blood vessel

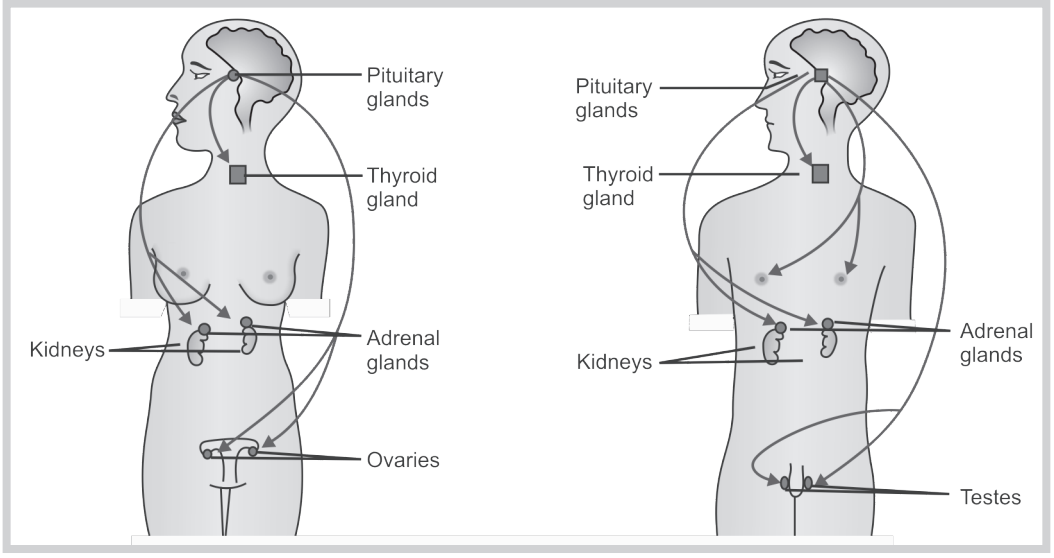


FIG. 24.1: Endocrine glands

network (hypothalamic-hypophyseal portal system) that feeds the pituicytes. These releasing factors then cause or inhibit the release of pituitary hormones, which travel via the circulatory system to the target organ (Fig. 24.2).

Anatomically and functionally the pituitary can be divided into three portions.

Anterior Pituitary (Adenohypophysis)

Six peptide hormones are secreted by the adenohypo-physis:

- Growth hormone (somatotropin, GH)
- Corticotropin (ACTH)
- Thyroid-stimulating hormone (TSH)
- Follicle-stimulating hormone (FSH)
- Luteinizing hormone (LH)
- Prolactin (PRL).

All hormones except growth hormone and prolactin regulate the activities of other glands. Somatotropin, PRL and ACTH are polypeptide hormones and LH, FSH, and TSH are glycoproteins having very similar structures.

The ACTH is an anterior pituitary hormone that stimulates cortisol and androgen production by the adrenal gland. Diurnal variation of ACTH are typical, with peak levels occurring from 0600 to 0800 hours and trough levels occurring from 1800 to 2300 hours.

Hormones Secreted by the Pituitary Gland

Anterior pituitary	*Posterior pituitary*	*Intermediate lobe*
Growth hormone	Oxytocin	MSH
TSH	ADH	
FSH		
LH		
ACTH		
Prolactin		

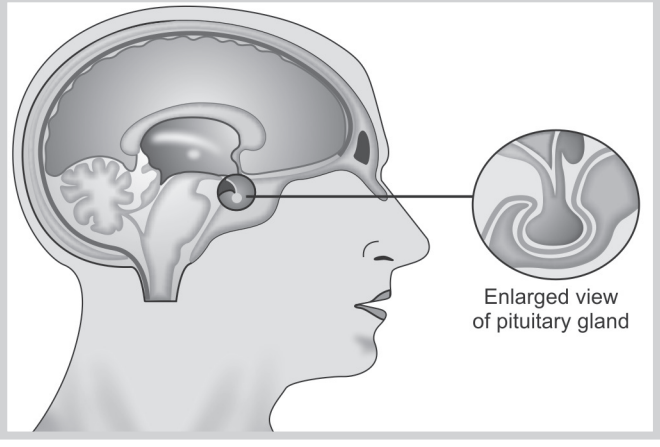


FIG. 24.2: Pituitary gland

The regulation of hormones elaborated by the endocrine glands is complex and varied. The tropic hormones of the anterior pituitary are regulated by CNS factors (hypothalamic-releasing hormones) as well as by products of their target organs. A delicate balance is thus maintained. Antidiuretic hormone (ADH) is regulated by osmotic pressure and volume. Parathormone is controlled by calcium and phosphorus concentrations, norepinephrine and epinephrine by direct neural stimuli; and insulin secretion by blood glucose concentrations. The MSH secretion is inhibited by glucocorticoids and causes skin pigmentation when cortisol is absent.

Clinical tropic hormone deficiencies may be single or multiple. If single, deficiency is most often of gonadotropins and leads to failure of development or involution of the sexual organs. Less often, TSH or ACTH is diminished. Hypothalamic-releasing factors for corticotropin (CRF), thyroid-stimulating hormone (TRF), and luteinizing hormone (LH-RF) have been identified as well as a probable growth hormone-releasing factor and prolactin and MSH inhibitory factors.

Causes of Hypopituitarism Resulting in Multiple or Single Deficiencies

Intrinsic Pituitary Disease

1. Neoplasms:
   - Chromophobe adenomas
   - Craniopharyngiomas
   - Carcinoma
   - Metastatic carcinoma.
2. Histiocytosis
3. Infections
4. Metabolic disorders, e.g. hemochromatosis
5. Vascular disorder:
   - Infarction (postpartum necrosis)
   - Intracerebral vascular malformation.

Extrinsic Pituitary Disorders

1. Surgical, heavy particle, or other forms of pituitary ablation
2. Hypothalamic neoplasms or metastatic deposits.

ANTERIOR LOBE: GROWTH HORMONE (GH)

Growth hormone has no specific target tissue. All cells of the human body are affected by this hormone. It is very important in the growing child, but it remains essential to many bodily functions throughout life. The GH has effects on the growth of bone and cartilage, protein metabolism, RNA formation, electrolyte balance, fat and glucose metabolism.

Actions

The GH stimulates growth of all nonendocrine tissues in the body and may affect secretions of the medulla and pancreas. Normal plasma levels are less than 3 ng/mL (females, higher than males), and after insulin hypoglycemia rise to approximately 25 ng/mL. It increases nonesterified fatty acids. Also, in diabetic and acromegalic patients it is diabetogenic. The GH produces nitrogen retention in man and monkeys. It may stimulate the growth of malignant craniopharyngioma.

Clinical Disorders

1. **Deficiency:** Dwarfism
2. **Excess:** Gigantism (prepubertal), acromegaly (postpubertal).

METHOD OF EVALUATION: STREPTAVIDIN-BIOTIN ELISA

Expected Ranges of Values

Because of the pulsatile and sporadic nature of growth hormone secretion, reference intervals for basal values are without meaning. However, normal levels rarely have been reported above 50 ng/mL. The well rested, fasting (12 hours) subjects should have GH values of 20 ng/mL or less.

With this caveat in mind, 75 apparently healthy adults were assayed the hGH immunoassay. The results are depicted below.

Expected values for the gh iema test system (in ng/mL)

	N	*Mean*	*Range*
Specimens	75	2.8	0-17

Provocative tests for hGH response are normally used to access the function of the anterior pituitary. Stimulatory procedures measure the secretion ability of the anterior pituitary to release hGH. Children suspected of growth retardation are common subjects for stimulatory testing. Several dynamic tests are available to induce GH release: exercise (3), L-dopa administration (4), insulin tolerance test (5), and arginine infusion (6). Each laboratory should assess the normal response, but a peak GH release in excess of 8 ng/mL is probably normal in all cases.

Inhibitory testing measure the suppression of hGH release from the anterior pituitary. Inhibitory tests are useful in ascertaining growth hormone excess and the resulting conditions of gigantism and acromegaly. The glucose tolerance test is a dynamic test to measure growth hormone excess. The failure of hGH levels to fall below 1 ng/mL within 60–120 minutes suggests excess hGH secretion.

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

Growth Hormone (hGH)

Chemiluminescence Immunoassay

(*Courtesy: Lilac Medicare)*

Determination of Growth Hormone Concentration in Human Serum by a Microplate Immunoenzymometric Assay

Summary and Explanation of the Test

Growth hormone (hGH, somatotropin), secreted from the anterior pituitary, is a polypeptide with two intra-chain disulfide bridges, which circulates free or bound to number of different GH-binding proteins. Several forms of growth hormone have been identified with the major being of molecular weight 22,000 daltons containing 191 amino acid residues. A 20,000-dalton variant, which posseses all known biological functions of GH, has also been demonstrated to be important. The primary biological actions of the hormone are in direct growth promoting. GH exerts its effect directly on target organs such as bones and muscles and indirectly through the release of somatomedins, a family of insulin-like growth factor (IGF) hormones, produced in the liver. In particular, somatotropin C (IGF-1) is essential for bone growth during childhood.

The clinical usefulness of the measurement of growth hormone (GH) in children has been well established in ascertaining linear bone growth along the epiphyseal plate. Abnormal elevated levels lead to gigantism while complete absence slows the rate of growth to one-third to one-half of normal. In adults, the epiphyseal growth plates have fused; GH excess gradually produces acromegaly, a coarse thickening of the bones of the skull, hands and feet.

In this method, GH calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of GH)

are added and the reactants mixed. Reaction between the various GH antibodies and native GH forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme-growth hormone antibody bound conjugate is separated from the unbound enzyme-growth hormone conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce light.

The employment of several serum references of known growth hormone levels permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with growth hormone concentration.

Principle

Immunoenzymometric Assay

The essential reagents required for an immuno-enzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-GH antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$EnzAb_{(x\text{-}GH)} + Ag_{GH} + {}^{Btn}Ab_{(m)} \underset{Ka}{\overset{K_a}{\uparrow\downarrow}} EnzAb_{(x\text{-}GH)}\text{-}Ag_{GH}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

Ag_{GH} = Native Antigen (Variable Quantity)

$EnzAb_{(p)}$ = Enzyme labeled Antibody (Excess Quantity)

$EnzAb_{(x\text{-}GH)}\text{-}Ag_{GH}\text{-}{}^{Btn}Ab_{(m)}$ = Sandwich Complex

Ka = Rate Constant of Association

K_a = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$EnzAb_{(x\text{-}GH)}\text{-}Ag_{GH}\text{-}{}^{Btn}Ab_{(m)} + Strept_{CW} \Rightarrow \text{immobilized complex}$$

$Strept_{CW}$ = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the well.

After equilibrium is attained, the antibody bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve is generated from which the antigen concentration of an unknown is ascertained.

Clinical Condition

Deficiency

In children, careful attention to growth records and X-ray of the skull and hands (especially of wrists) for bone age, looking primarily for retarded bone age. Hypopituitarism must be differentiated from isolated growth hormone deficiency, where normal pubertal development occurs. Other causes of short stature must be considered, e.g. constitutional causes, hypogonadism, hypothyroidism, and gonadal dysgenesis. Eunuchoid habitus eventually emerges only in the hypogonadal disorder. Normal plasma levels are less than 3 ng/mL, after insulin hypoglycemia the level rises to approximately 10–25 ng/mL within 60 minutes. Caution should be used in the dose of insulin administered. If hypopituitarism is suspected, doses of 0.05 units/kg are recommended; the dose can be increased if insufficient hypoglycemia is achieved. The infusion of arginine 0.5 g/kg for 30 minutes, can also increase GH levels to greater than 10 ng/mL in 60–90 minutes. This test has the advantage of not producing hypoglycemia. Absence of a normal response to these provocative tests is diagnostic.

Excess

Growth records, changes in soft tissue mass, enlargement of sella turcica, increased sweating, tufting of phalanges, vertebral overgrowth and spur formation, and organomegaly are all useful indications of GH excess. Serial studies of visual fields are essential. While increases in BMR and serum phosphorus concentration are helpful, the diagnosis is established by the level of GH and its response to suppressive maneuvers. Serum GH levels are greater than 10 ng/mL and are not suppressed after oral glucose loads. The availability of serum GH levels makes possible the evaluation of surgical or radiologic treatment.

In Brief

Clinical Disorders

1. Deficiency: Dwarfism
2. Excess: Gigantism (prepubertal)
   Acromegaly (postpubertal)

Interfering Factors

1. Increased levels are associated with use of oral contraceptives and estrogens.
2. Decreased levels are associated with obesity and use of corticosteroids.

CORTICOTROPIN (ACTH)

Actions

This stimulates production of all adrenocortical hormones (transient stimulatory effect on aldosterone) and causes hyperplasia of adrenal cortex. In the adrenal, it promotes increased protein synthesis, accelerates glycolysis and increases steroidogenesis. Extra-adrenal actions include mobilization of nonesterified fatty acids from fat depots. Normally, the amount of circulating ACTH is controlled by the levels of cortisol in the blood, individual biorhythms and stress.

Methods of Evaluation

The X-ray of sella turcica should be taken, study basal excretion of 17-hydroxycorticosteroids, assess diurnal variation patterns, study plasma cortisol levels. *Corticotropin lack is indicated by:*

- Failure of adrenocortical function
- Low 24 hours urinary excretion of 17 ketosteroids and 17-hydroxycorticosteroids, which increase stepwise in response to daily corticotropin administration
- Failure of hydroxycorticosteroid excretion to increase following administration of metyrapone, which blocks the production of cortisol and results in increase of corticotropin production by the intact hypophysis
- Normal or slightly reduced aldosterone excretion
- Plasma ACTH levels—normally barely detectable are markedly increased in adrenal insufficiency and Cushing's disease.

		SI units
0800 hours, peak	25-100 pg/mL	25-100 ng/L
1800 hours, trough	0-50 pg/mL	0-50 ng/L

Clinical Disorders

Increased Addison's disease, ectopic ACTH syndrome, pituitary adenoma, pituitary Cushing's syndrome, primary adrenal insufficiency, and stress. Drugs include amphetamine sulfate, calcium gluconate, corticosteroids, estrogens, ethanol, lithium carbonate, and spironolactone (corticotropin-like substances elaborated by malignant tissue, particularly in the lung, pancreas or prostate, may also lead to Cushing's disease).

Decreased primary adrenocortical hyperfunction (due to tumor or hyperplasia) and secondary hypoadrenalism.

OTHER ANTERIOR PITUITARY HORMONES

TSH

This hormone stimulates the synthesis and secretion of thyroid hormones. It is a glycoprotein hormone controlled by feedback from thyroid hormones.

The TSH controls production and release of T3 and T4 at three levels:

- The entry of I- into the follicle
- The entry of Tg bound T3 and T4 from follicular space into the lumen
- The release of T3 and T4 from Tg with the help of protease enzyme.

FSH

The target organs for FSH are the testes, in men, and the ovaries in women. The hormone stimulates the germinal epithelium in the testes to cause and facilitate the making of sperm. In women, it stimulates the growth and development of the follicle. It stimulates the production of testosterone in men and estrogen and progesterone in women. Its release from the pituitary is governed by a negative feedback mechanism involving these steroids.

LH

The male target organ is the testes and the testosterone producing interstitial cells of Leydig in particular. In women the target of LH is the developing follicle within the ovary where it is necessary for ovulation to occur and a corpus luteum to develop.

LH, FSH—Recommendations for Testing

- Irregular menstrual periods—amenorrhea.
- Primary and secondary hypogonadism in male and female
- When a female complains of masculinizing features or a male complains of feminine features
- Infertility cases
- IVF centers—assisted conception

- Monitor LH, FSH levels after LHRH stimulation
- LH/FSH ratio is a useful parameter in diagnosis of PCO (polycystic ovary)
- FSH is a good indicator in menopause
- In children where they seem to grow faster than their age or otherwise (precocious and delayed puberty).

Prolactin

This hormone is involved in breast development and lactation. In concert with estrogen, it prepares the mammary gland for lactation and then causes the synthesis of milk. Secretion is regulated by a release inhibiting factor and suckling may cause the release of prolactin from the pituitary. It steadily increases during pregnancy, reaching 200 ng/mL in the 3rd trimester and returns to normal in nonlactating women 2-3 weeks postpartum. In lactating women 6 months postpartum. It also increases with breast stimulation, exercise, sleep, and stress.

Prolactin—Recommendations for Testing

- Diagnosis of hyperprolactinemia and monitoring the effectiveness of treatment
- When a male complains of impotence, decreased libido
- Female complains of irregular menstrual cycle—amenorrhea
- Pituitary tumor (microadenoma or macroadenoma)
- Used alone or with LH and FSH for detecting pituitary dysfunction.

INTERMEDIATE LOBE (PARS INTERMEDIA)

In the adult human, this lobe is diminished with poor vascular and neural connections such that secretion is not facilitated. Cells in the pars intermedia may secrete MSH (melanocyte-stimulating hormone) which stimulates the activity of melanocytes in the skin.

POSTERIOR PITUITARY (NEUROHYPOPHYSIS)

This portion of the pituitary is really an extension of the hypothalamus. Neurons with their cell bodies in the hypothalamus and their terminal portions in the neurohypophysis release two hormones. Antidiuretic hormone (ADH) and oxytocin are stored there within the terminal processes of neurons until the signal to release them is received.

ADH

It is also known as vasopressin. In the presence of ADH, the kidneys reabsorb more water from the forming urine within renal tubules. Without ADH the kidney tubules are almost completely impermeable to water such that a very dilute urine is excreted (diabetes insipidus). The ADH has a direct effect on vascular smooth muscle causing vasoconstriction and an increase in blood pressure when present in large doses. They are stimulated by a high blood osmolarity (increased concentration) causing the release of ADH. The hormone then causes the kidney tubules to reabsorb more water to return osmolarity to normal. Volume receptors also play a role when they sense a low blood pressure. Alcohol inhibits ADH secretion.

Normal Values

Serum osmolarity	*ADH level*	*SI units*
270–280 mOsm/kg	<1.5 pg/mL	<1.4 pmol/L
280–285 mOsm/kg	<2.5 pg/mL	<2.3 pmol/L
285–290 mOsm/kg	1–5 pg/mL	0.9–4.6 pmol/L
290–295 mOsm/kg	2–7 pg/mL	1.9–6.5 pmol/L
295–300 mOsm/kg	4–12 pg/mL	3.7–11.1 pmol/L

The ADH elaboration is initiated by increase in the extracellular fluid osmotic pressure, by direct nervous system stimulation of the hypothalamus; and to a minor degree, by extracellular fluid volume. It is formed by the neurosecretory cells in the supraoptic and paraventricular nuclei of the hypothalamus, and travels along axons to the posterior lobe, where it is stored. Lesions at any of these sites interfere with ADH release to the body.

Clinical Disorders

A. *Deficiency of ADH:* ADH deficiency produces diabetes insipidus if the anterior pituitary is still functioning.
B. *Excess ADH:* Inappropriate ADH secretion syndrome.

Methods of Evaluation

Deficiency

Study for intracranial lesion (lumbar puncture, skull film, EEG), STS, chest X-ray (metastasis), bone marrow examination (multiple myeloma, eosinophilic granuloma). However, 45% are classified as idiopathic. Differentiate diabetes insipidus (10 to 15% cases) by administration of vasopressin. The simple measurement of a urine volume of more than 5 liters/day is strong presumptive evidence of deficiency.

1. Water restriction

Though quite simple, careful supervision is necessary so that losses of 3 to 5% of body weight are avoided. Greater care has to be exercised in children. Volume and concentration (specific gravity or mOsm/kg) are

determined at each voiding. Urine flow should reach less than 0.5 mL/minute, and urine concentration should be greater than 800 mOsm/kg (specific gravity 1.020).

2. Hypertonic saline test
(Carter-Robbins test, Hickey-Hare test)

This test is used to differentiate psychogenic polydipsia from diabetes insipidus. Here again caution is needed, since dehydration may cause vasomotor collapse in patients with diabetes insipidus. Administration of hypertonic saline solution may be hazardous in cardiac or renal disease.

Antidiuretic therapy is stopped until urine output reaches its original level. The patient may be cautiously dehydrated for 8-12 hours or this step may be omitted. Just before the test, the patient drinks 20 mL of water per kg of body weight in 1 hour. Urine is collected at 15 minutes intervals.

When the urine flow exceeds 5 mL/min, 2.5% saline solution is given IV at a rate of 0.25 mL/kg body weight/min for 45 minutes.

In normal subjects and in psychogenic polydipsia, a marked reduction in urinary flow will occur during the saline infusion or during the two 15 minutes intervals immediately following it.

In 85 to 95% of patients with true diabetes insipidus, the urine flow does not decrease with the saline infusion, but administration of 0.1 unit of vasopressin will inhibit diuresis in the absence of renal disease.

3. Response to vasopressin

This test also differentiates diabetes insipidus from vasopressin-resistant polyuria due to other causes, e.g.

a. Potassium depletion
b. Hypercalcemia
c. Chronic renal disease
d. Congenital nephrogenic diabetes insipidus
e. After renal transplantation
f. Sjögren's syndrome
g. Obstructive uropathy.

Urine volume and specific gravity, and symptoms of polyuria and polydipsia are observed before and after repeated subcutaneous injections of 0.2 mL (4 units) vasopressin every 3 or 4 hours day and night for 24 hours, or before and after a 1 hour of infusion of aqueous vasopression (5 μm/minute).

Patients with chronic nephritis or vasopressin-resistant diabetes insipidus experience no relief of symptoms during test period. In diabetes insipidus or psychogenic polydipsia, symptoms may improve, urine volume may decrease, urine specific gravity may increase to 1.015 or more, and urine osmolality may rise above serum osmolality.

4. Nicotine stimulation

Various side effects (nausea, vomiting and sweating) limit the usefulness of this test. Give 0.5–1 mg IV of nicotine base to non-smokers and doses as high as 3 mg IV to habitual smokers undergoing water diuresis. The normal response to intravenous nicotine is secretion of vasopressin, 80% reduction in urine flow, and rise in osmolality. Responsiveness to nicotine but not to hypertonic saline stimulus suggests that osmoreceptor centers are functionally separate from vasopressin sensory centers.

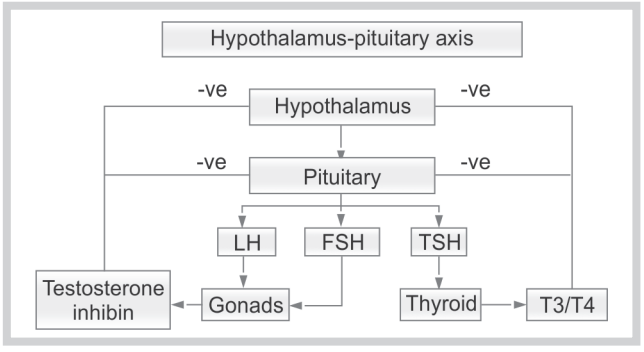
Syndrome of Inappropriate ADH Secretion (SIADH)

Hyponatremia is a frequently observed laboratory finding in severely ill patients. The recognition of SIADH is critical to their management. Findings consist of hyponatremia, normal BUN, urinary sodium loss, competent circulatory system, and increased urinary osmolality. These findings are similar to the observations made after exogenous administration of ADH to man. This syndrome is seen most frequently in pulmonary neoplasms, but it is reported in CNS disorders, tuberculous meningitis, head trauma, pneumonia, intrathoracic tumors, myxedema, acute intermittent porphyria, sickle cell anemia, cerebral thrombosis and postoperative ADH release after morphine or barbiturates. Neoplastic tissue in some cases has been shown to possess ADH activity.

Oxytocin

A major role of this hormone is the stimulation of smooth muscle cells in the pregnant uterus. When labor begins, stretching of the cervix and vagina stimulates a reflex production and release of oxytocin. Oxytocin then travels in the blood to the uterus where it causes more forceful contraction of the smooth muscle. This hormone is also involved in lactation. It causes milk ejection by acting on the smooth muscle surrounding the milk producing cells. Again, its production and release is mediated by a neural reflex, the suckling reflex. Emotion, anxiety and pain can inhibit oxytocin release.

Feedback Mechanism



DISORDERS OF THE PITUITARY SYSTEM

It is of two types:

Underproduction of hormones—hypopituitarism.

Overproduction of hormones—pituitary adenoma (micro and macroadenoma)

The effects of these disorders vary with the hormone and the target organ of action.

1. TSH Disorders

Hypothyroidism: Secondary or subclinical hypothyroidism.
Hyperthyroidism: TSH-secreting tumor leading to hyperthyroidism.

2. Prolactin Disorders

Hyperprolactinemia: Consistently elevated serum prolactin (PRL>20 ng/mL) in the absence of pregnancy or postpartum lactation.
Etiology: Prolactinoma, acromegaly, Cushing's disease, lactotroph hyperplasia, empty sella syndrome, other pituitary tumors, hypothalamic disease, pharmacologic agents, macroprolactinemia.

3. Gonadotropin Disorders

Excess and reduced production of gonadotrophs manifests as fertility disorders. The hormones control gametogenesis in males and females at different levels together with negative feedback mechanism of the hypothalamus.

4. Growth Hormone Disorders

The symptoms of GH deficiency in adults are subtle, consisting of decreased muscle strength and exercise tolerance and a reduced sense of well-being (e.g. diminished libido, social isolation). Patients with GH deficiency have increased body fat, particularly intra-abdominally, and decreased lean body mass compared with normal adults. Some patients have decreased bone mineral density, which may improve with GH replacement.

HYPOTHALAMUS

Anterior pituitary functions are controlled by the region of the brain called the hypothalamus via the secretion of releasing and inhibiting factors. Specialized neurons in the hypothalamus, controlled by feedback and other communication methods release factors that cause the release of hormones from the anterior pituitary. The pituitary trophic hormones then control the release of other hormones from a target gland. With the exception of prolactin, release promoting factors are more important to the release of pituitary hormones.

- Somatostatin (inhibits GH release)
- Prolactin-inhibiting factor (PIF, dopamine)
- LH-releasing factor (LHRF)
- FSH-releasing factor (FSHRF)
- Prolactin-releasing factor (PRF)
- Corticotropin-releasing factor (CRF)
- Thyrotropin-releasing hormone (TRH).

These hormones control the release of anterior pituitary hormones. The release of these factors is controlled by feedback from the target organ hormone to maintain the proper hormonal balance.

ADRENAL (SUPRARENAL) GLAND

The suprarenal glands are located on top of each of the kidneys. The adrenal cortex (outer portions) produces four major groups of hormones:

- **Glucocorticoids:** Cortisol, cortisone.
- **Androgens:** Androstenedione, dihydroepiandrostenedione (DHEA)
- **Mineralocorticoids:** Aldosterone, deoxycorticosterone, corticosterone
- **Estrogens and progesterones.**

The *adrenal medulla* is actually an extension of the nervous system. The adrenal medulla produces norepinephrine and epinepherine (adrenaline) that are released in response to stress or a fright.

MINERALOCORTICOIDS

The major mineralocorticoid, which is secreted almost independently of ACTH from the pituitary, is aldosterone. Aldosterone secretion is controlled mostly by the levels of potassium and sodium in serum and a blood pressure control system called the renin-angiotensin system. Aldosterone has the opposite effect on serum levels of potassium as it is lost in the urine in exchange for sodium in the renal tubules. Salivary and sweat glands are also influenced by aldosterone to save sodium and the intestine increases the absorption of sodium in response to aldosterone.

Clinical Relevance

1. Elevated levels occur in primary aldosteronism as in:
   - Aldosterone-producing adenoma
   - Adrenal cortical hyperplasia
   - Glucocorticoid remediable hyperaldosteronism.
2. Elevated levels also occur in secondary aldosteronism when aldosterone output is elevated due to external stimuli or because of greater activity in the renin-angiotensin system as in:

- Salt depletion
- Potassium loading
- Large doses of ACTH
- Cardiac failure
- Hepatic cirrhosis with ascites
- Nephrotic syndrome
- Barter's syndrome
- Postsurgical syndrome
- Hypovolemia and hemorrhage.

GLUCOCORTICOIDS

The major glucocorticoid is cortisol. Cortisol has important actions in the control and metabolism of carbohydrates, lipids, and proteins and assists in the metabolic reaction to stress, especially chronic stress. It causes glucose to be liberated from the liver by increasing glucose production from fatty acids (by-products of lipid breakdown) and amino acids. Cortisol causes the tissues to take up less glucose from the blood and mobilizes fat breakdown. The net effect is to increase serum glucose concentrations, which is protective for the brain in that it cannot use any other fuel source than glucose. It also stimulates protein breakdown for glucose formation in all tissues except the liver where it stimulates protein synthesis.

Cortisol

Plasma Cortisol

Unconjugated cortisol (free and protein-bound) concentration vary diurnally. At 8 am, the average concentration in plasma is 120 ng/mL (range 60–230 ng/mL). Diurnal variation is striking. In normal humans observing customary day-night activity, the highest levels occur at about 8 am and the lowest level shortly after midnight cortisol plasma or serum norms. Peak occur at about 0800 (8 am) and troughs occur in late afternoon.

Normal Values

Cortisol interpretation: Normal values in ng/mL		
Age/time		
Adult:	8–10 am	60–230
	4–6 pm	30–130
	8 pm	< 50% of am value
Child:		
	8–10 am	180–230
	4–6 pm	60–120
	8 pm	< 50% of am value
Patient treated with ACTH		280–600
Patient treated with dexamethasone		0–50

Cortisol Levels are Increased in

Burns, Cushing's disease, Cushing's syndrome, eclampsia, exercise, hepatic disease (severe), hyperpituitarism, hypertension, hyperthyroidism, infectious disease, obesity acute pancreatitis, pregnancy, severe renal disease, (severe heat, cold, trauma, psychological), surgery, and virilism. Drugs include corticotropin, estrogens, oral contraceptives, and vasopressin.

Cortisol Levels are Decreased in

Addison's disease, adrenal insufficiency adenogenital syndrome, chromophobe adenoma, craniopharyngioma, hypoglycemia, hypophysectomy, hypopituitarism, hypothyroidism, liver disease, postpartum pituitary necrosis, and Waterhouse-Friderichsen syndrome. Drugs include dexamethasone, dexamethasone acetate, and dexamethasone sodium phosphate.

Interfering Factors

1. Pregnancy will cause an increased value
2. There is no normal diurnal variation in patients under stress
3. Drugs, such as spironolactone and oral contraceptives will give falsely elevated results.

Cortisol Suppression (Dexamethasone Suppression)

Normal values

8 am : 60-230 ng/mL
4 pm : 30-130 ng/mL
Morning following administration of dexamethasone: 50 ng/mL.

Test Significance

This is screening test for Cushing's syndrome and depends on the fact that ACTH production will be suppressed in normal persons after a low dose of dexamethasone, whereas it is not in Cushing's syndrome.

Method

1. Venous blood sample is obtained at 8 am, 4 pm and again at 8 am the next day after dexamethasone has been administered.
2. At 4 pm, dexamethasone tablets are given orally. The dosage varies according to weight.

All medications should be discontinued for 24 to 48 hours before the study. Especially important are aldactone, estrogens, contraceptive pills, cortisol, tetracyclines, stilbestrol and dilantin.

Clinical Relevance

No diurnal variation or suppression will occur in:

1. Cushing's syndrome
2. Conditions causing extreme stress

3. Failure to take dexamethasone
4. If dilantin has been administered.

Cortisol Stimulation (Cortrosyn Stimulation)

Normal values
Rise : > 70 ng/mL
Peak : > 200 ng/mL

Test Significance
This is a good test to detect adrenal insufficiency. Cortrosyn is a synthetic subunit of ACTH that exhibits full corticosteroid-stimulating effect of ACTH in normal persons. Failure to respond is an indication of adrenal insufficiency.

Method
1. A fasting venous sample is obtained
2. Cortrosyn is administered intramuscularly
3. Additional blood samples are obtained 30 and 60 minutes after administration of cortrosyn.

Clinical Relevance
Absent or diminished response occurs in:
1. Adrenal insufficiency
2. Hypopituitarism
3. Prolonged steroid administration.

Tests for Adrenocortical Insufficiency

Water Excretion Test (Soffer)
Method: The patient fasts overnight. In the morning, he empties his bladder and drinks 1500 mL of water (about 20 mL/kg body weight) over a period of 15-45 minutes. A 5 hours urine specimen collected from the beginning of the test is measured. During the 5 hours period, the patient reclines or sits except while voiding. The test may be repeated 2 hours after the oral administration of 50 mg of cortisone.

Interpretation
Normal individuals excrete 1200 mL or more of urine over the 5 hours collection period. Patients with Addison's disease may excrete less than 800 mL of urine. False positive results may be obtained if the rate of absorption of water from the gastrointestinal tract or its elimination by the kidney is decreased, e.g. in patients with nephritis, cirrhosis, celiac disease, or cardiac failure.

Patients with adrenal insufficiency (primary, or secondary to hypopituitarism) show substantial increase in diuresis when retested following cortisone.

Corticotropin (ACTH) Response Test (Thorn test)

If responsive adrenocortical tissue is present, the administration of potent corticotropin results in an increased secretion of adrenocortical steroids, and increase in plasma cortisol, producing eosinopenia and increased urinary excretion of 17-ketosteroids and 17-hydroxy-corticosteroids. If ACTH has been absent because of pituitary insufficiency, its daily administration leads to a stepwise increase in adrenocortical response over a period of 2–3 days.

Adrenal response to corticotropin is retarded in myxedema as well as in hypopituitarism. Allergic eosinophilia may mask a fall in eosinophils. The patient should be free of the effects of large doses of androgens, cortisone, and corticotropin before urinary steroids are measured.

Method
1. The 4 hours corticotropin test may be used for screening. The eosinophil count or plasma cortisol is measured before 25 USP units of corticotropin are administered in a 4 hours infusion. Four hours later, another eosinophil count is done or plasma cortisol measured.
2. Eight hours intravenous corticotropin test. 2–5 USP units of corticotropin in 500 mL of normal saline are administered IV as a continuous 8 hours infusion. An eosinophils count or plasma cortisol level is determined at the beginning and at the end of the 8 hours period. 24 hours urine collections are made on a control day prior to the test and on the day of corticotrophin administration. Urinary excretion levels of 17-ketosteroids, 17-hydroxycorticosteroids, ketogenic steroids, or urinary-free cortisol on each specimen are compared with the control value.
3. As an alternative to the intravenous test, 40-80 USP units of corticotropin gel (repository corticotropin injection) or corticotropin zinc may be given intramuscularly twice daily over the testing period. Corticotropin gel should not be used in suspected adrenal insufficiency.
4. The patient with Addison's disease may be protected from an untoward reaction to ACTH by the administration of 0.1-0.25 mg of fludrocortisone, urinary steroid levels are not significantly altered.
5. A synthetic 24 amino acid ACTH compound has made possible a rapid intramuscular test. 0.25 mg IM will take more than double normal plasma cortisol in less than 1 hour (given earlier).

Interpretation
The 4 hours corticotropin screening test normally decreases circulating eosinophils by more than half. In test (2) or (3) above, normal subjects respond with an 80-100% fall in eosinophil levels, a 2-fold to 5-fold increase in 17-hydroxycorticosteroids, and 2-fold increase in

17-ketosteroid excretion levels. Plasma cortisol increases by 3 or 4 times.

When Cushing's syndrome is present due to adrenocortical hyperplasia, 17-hydroxycorticosteroid excretion levels may reach 75–100 mg/24 hours. The response is usually absent in the presence of the usually more autonomous adrenal carcinoma. The ACTH stimulation of patients with the adrenogenital syndrome produces an excessive response in 17-ketosteroid levels, not noted in cases of idiopathic hirsutism.

In Addison's disease, the 4 hours corticotropin test elicits a fall of less than 50% in circulating eosinophils. In the 8 hours IV corticotropin test and the corticotropin gelatin solution alternate, the addisonian patient shows little or no change in circulating eosinophils or urinary or plasma hormone levels. In order to rule out adrenal insufficiency unequivocally, at least 3 days of method –2 are recommended. The synthetic ACTH method is of value in identifying a normal adrenal response. If no response is observed, method –2 should be applied.

Patients with hypopituitarism and ACTH insufficiency show varying responses depending upon the degree of adrenocortical involution. Repetition of the test on 3–5 consecutive days shows a gradual rise in 17-ketosteroid and 17-hydroxycorticosteroid output and an increasing eosinopenia. Plasma cortisol usually shows an increase of 3–4 times in 4 hours.

All tests other than the IV administration of corticotropin are subject to occasional false-negative responses due to extravascular inactivation of corticotropin.

Adrenocortical Inhibition Test

1. Test with dexamethasone

Tests to determine the suppressibility of ACTH by glucocorticoids may be done using dexamethasone. Dexamethasone has little effect on sodium or potassium balance and does not interfere with corticosteroid determination.

Method: 24 hours urine specimens are collected for analysis on 3 successive days. After the first specimen is obtained, 0.5 mg dexamethasone is given every 6 hours by mouth for 2 days. Excretion level is measured on each 24 hours urine specimen. In normal individuals, the repeated administration of the 0.5 mg dose reduces by 50% the basal excretion of 17-hydroxycorticosteroids, urinary cortisol, and plasma cortisol per 24 hours by the end of the second day. If suppression is not obtained, the test is repeated with a dose of 2 mg every 6 hours for 2 days.
Interpretation: The increased corticosteroid levels, which occur in patients with adrenocortical hyperplasia, is usually suppressed only by the larger (2 mg) doses, whereas the corticosteroid levels in patients with autonomous adrenocortical neoplasms may not be suppressed even with higher doses.

2. Rapid dexamethasone test

1 mg of dexamethasone at midnight will reduce normal plasma levels to less than 5 ng/ml by 8.00 am. This will effectively rule out adrenal overactivity.

3. Tests with metyrapone (an endogenous ACTH test)

Inhibition of 11 β-hydroxylase by metyrapone results in reduced blood levels of cortisol and loss of cortisol inhibition by ACTH. The ability of the pituitary to respond to this stimulus by release of ACTH may be measured by noting the increment in 17-ketosteroids, 17-hydroxycorticosteroids, or the metabolite of 11-deoxycortisol (compound-S) produced by the adrenal and excreted in the urine. Compound-S is formed by the adrenal cortex after inhibition of cortisol (hydrocortisone) formation. The rise in urinary 17-hydroxycorticosteroids is a result of increased amounts of this metabolite.

Method

1. *IV-Metyrapone,* 30 mg/kg body weight in 1 liter of normal saline is given IV for 4 hours, starting between 8.00 and 10.00 am. The same method is followed on another day, except that 25 USP units of ACTH are added to metyrapone infusion to compare the functional capacity of maximally stimulated adrenals with the response evoked by the endogenous ACTH released after administration of metyrapone alone.
2. *Oral:* A basal 24 hours urine 17-hydroxycorticosteroid measurement is obtained. 0.75 g of metyrapone are given every 4 hours for 6 doses. A second 24 hours urine for 17-hydroxycorticosteroid levels is obtained the day following the drug administration.
   Interpretation: Normal subjects may double their basal 24 hours 17-hydroxycorticosteroid excretion after metyrapone, and ACTH adds nothing to this response. Hypopituitary patients show no increase in excretion with metyrapone, but their response to exogenous ACTH is adequate. Addisonian patients respond to neither stimulus. IV metyrapone causes a vigorous response in patients with adrenal hyperplasia, but adrenal tumors fail to respond. Chlorpromazine blocks metyrapone responses.
   Direct measurements of the metabolite of compound-S and its plasma level are also available, and the direct measurement of their increase provides a more direct assessment of ACTH release.

Cortisol Abnormalities

Excess
Cushing's syndrome.

Deficiency
Congenital adrenal hyperplasia.

At high concentrations (greater than physiologic), glucocorticoids (such as hydrocortisone or prednisone) are useful for the treatment of allergies and inflammation. Each step of the inflammatory process is blocked by glucocorticoids when given systemically (an IV injection or orally). Topical application of glucocorticoids have anti-inflammatory effects for the local area. The anti-inflammatory activity of glucocorticoids is thought to be due primarily to the stabilization of cell membranes and the inhibition of phospholipases and therefore prostaglandin synthesis. The immune response can also be suppressed by the use of glucocorticoids. Eosinophils and lymphocytes decrease in the circulation affecting both cellular and humoral immunity. The glucocorticoids are used for many other conditions including asthma, renal diseases, rheumatic disorders such as lupus and inflammatory bowel disease.

ADRENAL MEDULLA

The adrenal medullary hormones are catecholamines—epinephrine and norepinephrine, the parent compound from which epinephrine is formed by addition of a methyl group.

Epinephrine is sympathomimetic, increases cardiac output and rate, systolic blood pressure, blood glucose, hepatic glycogenolysis, basal metabolic rate, sweating and causes mydriasis and skin-vessel constriction. By contrast, norepinephrine causes bradycardia, peripheral vasoconstriction and rise in diastolic blood pressure, and has much less prominent metabolic effects.

Clinical Disorders

Deficiency
Hypotension. Idiopathic spontaneous hypoglycemia (failure of epinephrine response to hypoglycemia).

Excess
Paroxysmal or persistent hypertension, headache, sweating, tachycardia and elevated blood glucose.

THYROID

The thyroid is a large endocrine organ that functions mostly to control metabolism. It is located in the neck between the trachea and larynx and is bilobed with a connecting isthmus (Fig. 24.3).

The gland is composed of many tiny follicles, that are in effect, each a separately functioning gland with a single-layer epithelial lining. Each follicle accumulates a storage form of the circulating thyroid hormones, thyroglobulin. Thyroglobulin is a large protein molecule that contains multiple copies of the amino acid, tyrosine. The thyroid hormones are very simple modifications of the amino acid, tyrosine. Both T4 and T3 enter into cells and bind to an intracellular receptors whereby they increase the metabolic capabilities of the cell. Thyroid hormones are necessary for normal growth and development. They have metabolic effects on protein synthesis, lipid and carbohydrate metabolism.

The polypeptide hormone calcitonin is also produced by the parafolicular cells within thyroid. It functions in calcium maintenance to decrease the levels of calcium in the blood. When serum calcium levels are excessive, calcitonin is released. It inhibits bone resorption (by inhibiting osteoclast activity), allows the loss of calcium in the urine and, therefore, decreases calcium in the blood. It opposes the action of parathyroid hormone and has been used clinically for the treatment of osteoporosis.

Markers of the Gland

Hormones	T3, T4, FT3, FT4
Structural	Tg–(thyroglobulin)
Antibodies	Anti-Tg, Anti-TPO
Carrier protein	TBG (thyroxine-binding globulin)
Pituitary marker	TSH (thyroxine-stimulating hormone)

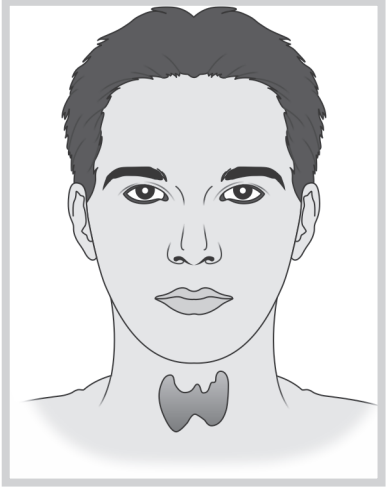


FIG. 24.3: Thyroid gland—anatomical position

Role of Carrier Protein

The hormone synthesized in the gland is transported to various parts by carrier proteins.

TBG: Thyroxine-binding globulin protein is the major carrier protein. It has more affinity to T4 than T3.

TBPA: Thyroxine-binding prealbumin, binds to T3 than T4.

Thyroxine-binding Globulin (TBG) Test

Almost all the thyroid hormones in the bloodstream are bound to proteins, and these thyroxine-binding proteins play an important role in regulating the free thyroxine (FT4) in the circulation. Thyroxine-binding globulin (TBG) is by far the most important determinant of the overall binding of T4. A measure of TBG should provide a good approximation of the thyroxine-binding function of the blood. This can be a valuable aid in clarification of many clinical conditions. The TBG test is a direct measurement of the total thyroxine-binding capacity of the specific thyroxine-binding interalpha (alpha1-alpha2) globulin in serum.

Free Hormones

Some portions of the hormone remain, unbound to the carrier protein. These are called **free hormones:**

Hormone	*Bound %*	*Free %*	*Carrier protein*
T3	97	3	TBPA
T4	99.7	0.3	TBG

Free T3 and Free T4 are the "Physiologically active hormones"

Measurement of these hormones is more relevant in clinical conditions where the levels of total hormones does not correlate.

Feedback Mechanism

Thyroid hormones have a negative feedback on the pituitary. Whenever, the concentrations of T3 and T4 are high, the release of TSH is inhibited from the pituitary. When the thyroid hormones levels are low, it is stimulated. But this need not be true always (Fig. 24.4).

Neonatal Thyroxine

Newborn infants normally have circulating levels of T4 that are considerably higher than normal adults; but within the first week of life, the values would have decreased markedly. Failure or extreme deficiency of T4 production occurs at a frequency of approximately 1 in 4000 live births. If this deficiency is left untreated, growth deficit, neurologic impairment and mental retardation (cretinism) result. Such infants are characterized by low circulating levels of T4 and, in thyroid gland failure (primary hypothyroidism), by very high levels of TSH. The disorders are markedly affected by the stage of development during which the defect arises. Later-phase development results in transient abnormalities, especially in premature infants, whereas permanent disorders result from early-stage defects.

Since early diagnosis on clinical grounds is difficult and since initiation of treatment before three months of age appears to be necessary to prevent neurologic defects, neonatal screening for hypothyroidism has become a key test in neonatal patient management.

Anti-TPO (Thyroid Peroxidase Antibodies)

The anti-TPOs are autoantibodies directed against the enzyme peroxidase. Because the antibodies bind to the microsomal part of the thyroid cells, they are known as thyroid microsomal antibodies.

Clinical Application

- TPO antibody tests are used to distinguish between different types of goiters.
- It is positive in Hashimoto's disease whereas in nontoxic goiter, it is normally negative.
- Twenty percent of patient's thyrotoxic patients have high titers of anti-TPO antibodies.
- The presence of anti-TPO antibodies and elevated TSH is a predictor of future hypothyroidism.

Anti-Tg (Anti-Thyroglobulin Antibodies)

Antibodies are produced against thyroglobulin. These autoantibodies gradually destroy the thyroid tissue and prevent the production of thyroid hormones, causing hypothyroidism. Presence of Tg-antibodies indicates Hashimoto's disease. However, they are less often present and less pathogenic than anti-TPO antibodies.

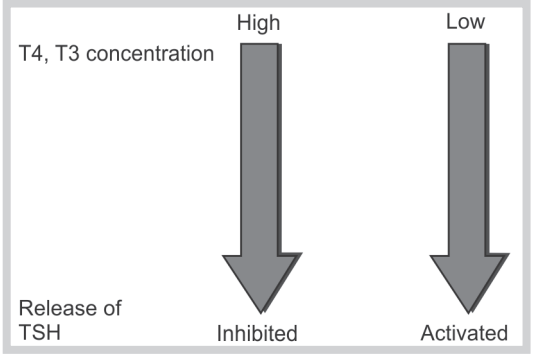


FIG. 24.4: Thyroid hormones—feedback mechanism

LATS (Long-acting Thyroid Stimulator)

In 1956, Adams and Purces demonstrated in the sera of some hyperthyroid patients, the existence of a thyroid-stimulating factor which had a longer-acting effect than TSH. The name applied to this factor was long-acting thyroid stimulator (LATS). It does not appear to have its origin in the pituitary and has been classified as a 7S γ-globulin. The LATS is now considered to be a thyroid autoantibody. Since, it may persist for years following total thyroidectomy, the nature of the antigen is unknown. It is considered one of a group of thyroid-stimulating immunoglobulins. These antibodies are stimulators and do not destroy thyroid tissue as do the anti-thyroglobulin and antimicrosomal antibodies. The presence of certain HLA antigens (HLA-B8 and HLA-DR3) indicated an approximate fourfold increase in risk for Graves' disease.

Clinical Significance

The frequent finding of LATS in the sera of patients with malignant exophthalmos and Graves' disease makes its detection an important part of thyrodiagnostic evaluations. The LATS has been overwhelmingly associated with a complex of symptoms known as Graves' disease. Studies showed that positive LATS findings in patients without Graves' disease are "low positive". In patients with active Graves' disease or patients who are presently in remission, "low" as well as unequivocally "high" results are found. Infants born of mothers with Graves' disease may also suffer from this illness because of the transplacental passage of LATS from the maternal to fetal circulation. Fortunately, prenatal testing can warn the physician of this potential threat; under alert management such infants will recover since the LATS will undergo metabolic destruction.

Disorders of the Gland

Hypothyroid

Low levels of thyroid hormones.

Primary Hypothyroidism—Where the low levels of thyroid hormones are due to failure of the thyroid gland.

Secondary Hypothyroidism—Where the low levels of thyroid hormones are caused by failure of the hypothalamic-pituitary system to produce TRH or TSH or both.

Subclinical Hypothyroidism—Elevated TSH levels in the absence of any clinical symptoms.

Hyperthyroid

High levels of thyroid hormones.

Autoimmune disease—Graves' disease.

Thyroiditis—Inflammation of the thyroid gland.

Tumors in the Thyroid—T3 toxicosis, T4 toxicosis.

- Due to a high dose of T4 therapy.
- Induced in patients with goiters if iodine is administered.

Nonthyroidal Illness (NTI)

Abnormal thyroid hormones due to other disorders. Severe illness or injury can induce changes in thyroid hormone levels. In seriously ill patients TSH, T3 and T4 levels may decrease, and a severe decrease often signifies that the patient is dying.

Clinical Manifestations

Overactivity (Hypothyroidism)	*Underactivity (Hyperthyroidism)*
1. Nervousness, increased activity	Decreased energy, physical and mental stability
2. Weight loss without loss of appetite	Weight gain but decreased appetite
3. Warm moist skin	Dry rough skin
4. Tachycardia	Bradycardia
5. Increased bowel movements, diarrhea	Constipation, disturbed equilibrium
6. Muscle weakness	
7. Osteoporesis	

Tests for Thyroid Function Generally Recommended in

- Abnormal weight loss or gain.
- Typical symptoms of hypothyroidism—tiredness, lethargy, intolerance to cold.
- Constipation, bradycardia, increased menstruation.
- In children—if they fail to grow normally. If ability to comprehend is less and mental growth is found to be insufficient. If puberty is delayed.
- Symptoms of hyperthyroidism—tiredness, hot, tachycardia, short of breath, increased appetite but lose weight, muscular atrophy, characteristic features of protruding, starring eyes and enlarged thyroid gland.
- Infertility.

Different Markers of Thyroid Function are Tested in Combination for Assessment of Thyroid Status

- Total tri-iodothyronine (T3)
- Total thyroxine (T4)
- Thyrotropin (TSH) (by ultra-sensitive method)
- Free tri-iodothyronine (FT3)
- Free thyroxine (FT4)
- Anti-thyroglobulin (Anti-Tg) antibody
- Anti-thyroid peroxidase (Anti-TPO) or anti-microsomal antibody
- T-uptake.

Information to be Taken before Sample Collection

The answers to the following questions will aid in the accurate diagnosis of the patient:

- Whether male or female?
- Age?
- Symptoms?
- Is the patient on treatment—Replacement therapy (thyroxine, cytomel), antithyroid drug?
- Does the patient suffer from any other illness—HIV, hepatitis, tumor, and nephrotic syndrome?
- Has the patient had any major surgery, trauma, stress?
- Is the patient hospitalized?
- Is the patient on any other drugs—oral contraceptives, estrogens, androgens, anabolic steroids, glucocorticoids, propranolol, dopamine, and metaclopromide?
- Family history—Does anyone in the patient's family suffer from autoimmune disorders?
- Does the patient suffer from autoimmune or connective tissue disorder.
- Has the patient had any neck/whole body irradiation?
- If the patient is a female—Does she have a regular menstrual cycle? Pregnant or postmenopausal?
- Geographical location—Does the patient come from an iodine deficient/excess region?

Tests for FT3/FT4 are gaining more importance in the diagnosis of thyroid disorders. All limitations of total T3, total T4 due to bound proteins is overcome by free T3 and free T4. FT3 and FT4 are considered to be the "**physiologically active hormones**".

FT3, FT4—Recommendations for Testing

Generally recommended in

- Monitoring treatment—thyroid replacement or suppression.
- Hospitalized patients, where patients may show symptoms of non-thyroidal illness.
- Pregnant women suffering from thyroid disorders.
- Patients known to take certain drugs which will interfere with total T3 and total T4 test results.
- Elderly patients.
- Particularly if a patient's thyroid function test does not correlate with clinical history.

Free Thyroxine Index (FTI)

An estimate (index) related to free T4 levels in serum can be calculated as the product of a T4 result and a T3 uptake ratio (T3UR) test. The reasoning is based on the premise that the T3UR result is inversely proportional to unsaturated thyroxine-binding globulin (UTBG) in serum, and that free T4 varies directly with total T4 and inversely with UTBG levels.

Thus, **FT4 Index = T4 (total)× T3UR**

Results may be expressed in any arbitrary terms and may even be related to actual FT4 levels by calculation, using actual values for FT4, T4, and T3UR on a number of normal specimens. It has been observed that the FT4 index is not as discriminatory as the actual estimation of FT4 by equilibrium dialysis. In particular, aberrant results may occur in patients whose TBG is abnormal and in patients whose concentration of TBG is markedly increased or decreased.

T3 Uptake Ratio (T3UR)

(An Index of the Unsaturated Thyroxine-binding Globulin Fraction of Serum)

The test has nothing to do with the actual T3 serum level in spite of its name which, unfortunately, is sometimes abbreviated to "T3 test". It must be emphasized that the T3 uptake ratio and the true T3 (T3 by EIA) are entirely different tests.

Clinical Significance

The T3 uptake ratio, in conjunction with the T3 measurement in serum, is used as a screening test of thyroid function.

Pregnancy

Elevated T3 values are to be expected in euthyroid patients. TBG and unsaturated TBG are both increased in normal pregnancy. This appears towards the end of the first trimester and is caused by increased estrogen secretion. In such patients low-T3UR test values are to be expected because of the increase in unsaturated TBG. This is important because the normally expected low-T3UR values are not found in certain cases of habitual threatened abortions as well as in pregnancy complicated by hyperthyroidism.

High or Low-T4 in a Euthyroid Individual

In rare cases this is caused by a hereditary abnormality in the level of TBG. Thus, in a eumetabolic person with hereditary absence of TBG, a low-T3 will be found and the T3UR test value will be high; the converse applies to the person with a hereditary excess of TBG.

A Normal T3 in a Hypothyroid Patient

This can occur when the unsaturated TBG is elevated.

Thyroid Function Tests

Introduction

When a patient is referred by a clinician for thyroid function tests, he may be symptomatic or asymptomatic.

Symptomatic patients present vague symptoms and complain that they just do not feel like themselves—may be they are a little tired, are experiencing hot or cold spells, or feel that their heartbeat is too fast or too slow. These symptoms could mean that the patient is suffering from thyroid disease or these could be symptoms related to nonthyroidal diseases.

At the same time, the asymptomatic patient may be suffering from thyroid illness and may be in need of immediate diagnosis and therapy. In such conditions, it is very crucial for the physician to accurately diagnose and treat the patient or monitor treatment.

It is here that the clinical laboratory plays an important role. The physician is totally dependent on the interpretation of the pathologist. It is based on the pathologist's interpretation that the physician can successfully diagnose and treat the patient.

Thyroid assays in general are extremely sensitive assays as they involve measurement of hormones in microgram and nanogram quantities. Also, the thyroid function and thyroid hormones interact as part of a multiple gland feedback loop, and hence are often interpreted together to detect disease and understand its etiology.

In the past decade, important changes have taken place in the strategy of thyroid function testing. Previously, thyroid tests were dominated by T3 (total T3), T4 (total T4) and TSH tests. People believed that if total T3 and total T4 are low, then TSH has to be high and vice versa. Here, they are considered *only* the feedback mechanism between the pituitary hormone TSH and thyroid gland hormones total T3 and total T4. What they failed to consider here is that there are other factors which affect total T3 and total T4 levels. Moreover, total T3 and total T4 being in bound form (bound to TBG) and are not the physiologically active hormones participating in metabolic functions; but it is the free hormones, free T3 and free T4, which are physiologically active.

Throughout the world, there is a trend for TSH and free T4 tests to replace the conventional total T3, total T4, TSH tests for screening thyroid functions with conventional thyroid screening strategy, total T3, total T4 and TSH. When laboratory results are correlated with clinical findings, the relations may be either concordant or discordant.

Laboratarians often face the problem of physicians complaining that the reports do not match with the clinical history of the patient, for example 'abnormal' total T4 in absence of thyroid disease. Here the laboratarian only correlates total T3, total T4 and TSH values; and as a result, in such situations, clinicians are in a dilemma and often doubt the methodology used.

If the laboratory results are discordant, a distinction needs to be made between a previously unsuspected diagnosis, subclinical disease, anomalous assay results, and a discrepancy caused by specific or nonspecific assay interference.

Tests for Thyroid Function

Laboratory tests of thyroid function are required to assist in the screening, diagnosis and monitoring of thyroid disease. Most laboratories offer a standard 'profile' of T3/T4/TSH. Measurement of plasma total T4 concentration was formerly widely used as test of thyroid function, but has a major disadvantage in that it is dependent on binding proteins concentration as well as thyroid activity. For example, a slightly elevated plasma total T4 concentration, compatible with mild hyperthyroidism, can occur with normal thyroid function, if there is an increase in plasma binding protein concentration. With the introduction of more reliable assays for free T4 (FT4), there is now little if any justification for laboratories continuing to measure total T4 as a test of thyroid function.

Plasma total T3 concentration is almost always raised in hyperthyroidism (usually to a proportionately greater extent than total T4, hence, it is the more sensitive test for this condition) but may be normal in hypothyroidism. However, total T3 concentrations, like those of total T4, are dependent on the concentration of binding proteins in plasma and their measurement is being superseded by measurements of free T3 (FT3).

Binding Proteins

For both, the clinician and pathologist, protein binding can provide a major obstacle to the laboratory assessment of thyroid status.

It is a known fact that both T3 and T4, when they are released into the blood, are extensively bound to plasma proteins. There are two types of plasma proteins, which are present in large concentration in blood: TBG—which has a very high affinity for T3 and T4 and TBPA—which has low affinity but high capacity for binding. Therefore, maximum T4 is bound to TBG and very little to TBPA. The precise physiological function of TBG is unknown. It has been suggested that the extensive binding of thyroid hormones to TBG provides a buffer, which maintains the free hormone levels constant in the face of any tendency to change. The binding may also reduce the amount of thyroid hormones lost through the kidneys.

Total thyroid hormone concentration is dependent upon the concentration of binding proteins present in the blood. If these were to increase, the temporary fall in free hormone concentration caused by increased protein

binding would stimulate TSH release and this would restore the free hormone concentrations to normal. Conversely, if the protein concentrations were to fall, the reverse would occur. In either situation, there would be a change in the concentrations of the total hormones, but the free hormone concentrations would remain normal. Thus, measurement of total hormone concentrations can give misleading information.

This is a matter of considerable practical importance since changes in the concentrations of the binding proteins occur in many circumstances. Further, certain drugs, for example, salicylates and phenytoin, will displace thyroid hormones from their binding proteins, thus reducing the total, but not the free hormone concentrations, once a new steady state is attained. If an attempt is made to assess thyroid status in a patient who is not in a steady state, the results may be bizarre and misleading.

Only small amounts of T4 and T3 are excreted by the kidneys due to the extensive protein binding. The major route of thyroid hormone degradation is by deiodination and metabolism in tissues, but they are also conjugated in the liver and excreted in bile (Fig. 24.5).

a. In the initial steady state, TBG is one-third saturated with T4.
b. TBG levels increase causing more T4 to be bound, thus reducing the free T4 concentration. This stimulates TSH secretion which leads to an increase in the release of T4 from the thyroid.
c. The new T4 is redistributed between the bound and the free states leading to a new steady state with the same free T4 level but an increased total T4.

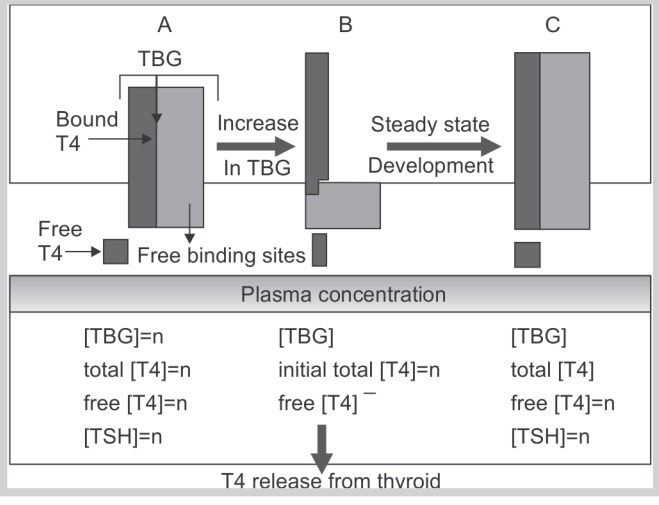


FIG. 24.5: Effect of an increase in TBG concentration on plasma T4 levels

Importance of Free T4 and Free T3

1. In pregnancy, the normal range for FT4 in euthyroid women decreases as the pregnancy progresses. There is an increase in TBG, due to the increased estrogen levels, and in total T4, but these are disproportionate, causing the level of free T4 to fall. Thyroid status, as assessed clinically, does not change.
2. Free T3 is a sensitive test for hyperthyroidism. In hyperthyroid patients, both FT3 and FT4 are usually elevated (FT3 to a proportionately greater extent) but there are exceptions to this.
3. In a small number of patients with hyperthyroidism the FT3 concentration is elevated but the FT4 is not (though it is usually high-normal)—a condition called 'T3 thyrotoxicosis'.
4. Occasionally, FT4 is elevated but not FT3. This is usually due to concomitant nonthyroidal illness resulting in decreased conversion of T4 to T3, and FT3 concentration increases when this illness resolves.
5. One can encounter abnormal total T4 test results in the absence of thyroid disease. Free T4 (FT4) in these circumstances remains constant and is a more useful indicator.
6. Free T4 provides reliable results in patients displaying abnormalities in serum T4 binding particularly if alterations are caused by severe nonthyroidal illness or hereditary dysalbuminemias.
7. Free T3 and free T4 are important in patients with suspected thyrotoxicosis in whom serum T4 is normal and serum TSH is low, to distinguish T3 thyrotoxicosis from subclinical thyrotoxicosis.
8. In the estimation of the serum FT3 : FT4 ratio, a high ratio(>0.024 on a molar basis or >20 calculated as ng/mg) that persists during antithyroid drug treatment may indicate that patients with hyperthyroid Graves' disease are unlikely to achieve remission. This ratio usually is lower in patients with iodide-induced thyrotoxicosis or thyrotoxicosis caused by thyroiditis than in those with thyrotoxicosis caused by Graves' disease.
9. To detect early recurrence of thyrotoxicosis after cessation of antithyroid therapy.
10. To establish the extent of active hormone excess during high-dose replacement or suppressive therapy with T4 or when an intentional T4 overdose has been taken.
11. For diagnosis of amiodarone-induced thyrotoxicosis, which should not be based on T4 excess because of the occurrence of euthyroid hyperthyroxinemia in many amiodarone-treated patients.

Alterations in the Concentrations or Affinity of Thyroid Hormone-Binding-Proteins (Fig. 24.6)

Increases in

a. TBG concentration (or affinity):
   1. Genetic (inherited) determination
   2. Nonthyroidal illness (HIV infection, infectious and chronic active hepatitis, estrogen-producing tumors acute intermittent porphyria)
   3. Physiology (pregnancy, newborn)
   4. Drug use (oral contraceptives, estrogens, tamoxifen, methadone)

b. Prealbumin concentration

c. Albumin binding (familial dysalbuminaemic hyperthroxinemia)

d. T4 binding by antibodies (autoimmune thyroid disease, hepatocellular carcinoma).

Decreases in

a. TBG concentration:
   1. Genetic (inherited) determination
   2. Nonthyroidal illness (major illness or surgical stress, nephrotic syndrome)
   3. Drug use (androgens, anabolic steroids, large doses or glucocorticoids).

b. TBG-binding capacity (drugs bound to TBG, such as salicylates and phenytoin)

c. Prealbumin concentration.s

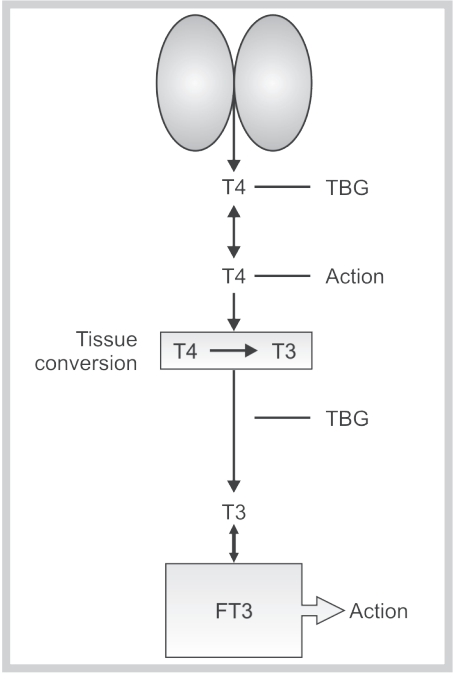


FIG. 24.6: Thyroid hormones and TBG

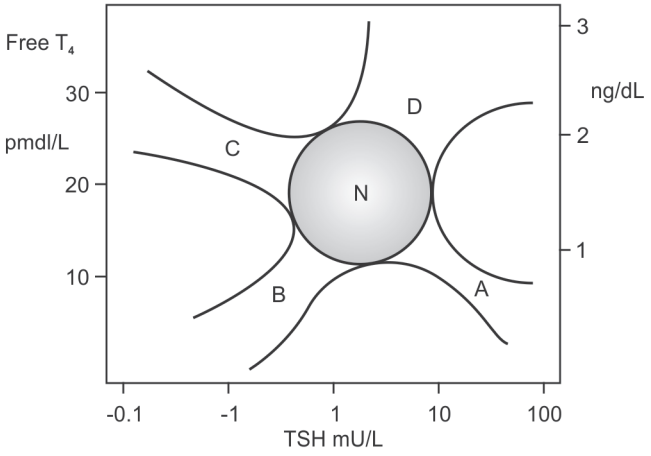


Fig. 24.7: TSH-FT4 relationship

The TSH-Free T4 Relationship

If a sensitive serum TSH assay is used together with a valid serum free T4 estimate, a sensitive and specific assessment of thyroid status can usually be made from the general relation between the two hormones.

Figure 24.7 emphasises the distinction between primary target gland failure (high serum TSH, low free T4: A), failure of TSH secretion (both low: B), autonomous or abnormally stimulated target gland function (high serum free T4, low TSH: C), and primary excess of TSH or thyroid hormone resistance (both high: D).

The relationship between serum TSH and free T4 concentrations in normal subjects (N) and patients with various abnormalities of thyroid function: A, primary hypothyroidism; B, central (secondary) hypothyroidism; C, thyrotoxicosis (excluding TSH-induced thyrotoxicosis). Results in area D are uncommon but suggest a possible methodologic artefact, an unrecognized binding abnormality, generalized thyroid hormone resistance, or TSH-induced thyrotoxicosis. Findings that fall in the undefined areas suggest that an additional factor may be modifying the feedback relationship or that samples have been taken in nonsteady-state conditions. Serum free T4 is shown on a linear scale, whereas the scale for serum TSH is logarithmic.

Problems in the Interpretation of Thyroid Function Tests

It is difficult to guarantee reliable thyroid function results in patients with nonthyroidal illness. Abnormal results may occur in patients with infections, malignancy, myocardial infarction, following surgery, etc. who do not have thyroid disease.

Typically, during the acute phase of an illness, free T3 (FT3) concentration and less often, free T4 (FT4) concentration is decreased. The TSH is usually normal but may be undetectable in the severely ill. During recovery, TSH may rise transiently into the hypothyroid range as free hormone concentrations return to normal. In chronic illness, for example, chronic renal failure, free hormone concentrations are decreased (to an extent that may reflect the severity of the underlying disease); TSH is usually normal, but it is occasionally decreased.

The occurrence of abnormalities of thyroid function tests in patients with nonthyroidal illness has been termed the 'sick euthyroid syndrome'. Causes include decreased peripheral conversion of T4 to T3; changes in the concentration of binding; increased plasma concentrations of free fatty acids, which displace thyroid hormones from their binding sites, and nonthyroidal influences on the hypothalamic-pituitary-thyroid axis, for example, by cortisol, which can inhibit TSH secretion.

Furthermore, many drugs can influence the results of tests of thyroid function. Many times, the levels of FT3, FT4 and TSH do not correlate.

Common Causes of TSH/FT4/FT3 Discrepancies

- Over replacement of thyroid hormone (TSH low, free T4 normal)
- Recent dose adjustment (TSH high, free T4 normal)
- Patient taking T3 (TSH low, free T4 normal)
- Patient noncompliant with hormone replacement (TSH high, free T4 normal)
- Nonthyroidal illness
- Drugs affecting thyroid hormones: Glucocorticoids, dopamine
- Thyroid hormone resistance (TSH high, free T4 high, patient euthyroid)
- TSH-secreting tumor (TSH high, free T4 high, patient hyperthyroid)
- During antithyroid drug therapy, there can be patients who have persistent serum T3 excess, despite normal or low serum T4 values.

Effects of Drugs on Thyroid Function

- Altered hypothalamic or pituitary function
- Altered biosynthesis or release of thyroid hormones
- Displacement of T4 and T3 from binding proteins
- Reduced peripheral conversions of T4 to T3
- Inhibition of peripheral hormone activity.

Drugs that Affect Results of Thyroid Function Tests

Drugs	*Effect*	*Cause*
Salicylates, Phenylbutazone, Diphenylhydantoin	Total T4 and T3 reduced, Free T4 normal	Inhibition of serum protein binding
Propylthiouracil, Methimazole, Lithium, Iodides	Total T4 and T3 reduced	Inhibition of TSH production or release
Propylthiouracil	Total T3 reduced both T4 and TSH high	Inhibition of conversion of T4 to T3
Propranolol	Total T3 reduced, T4 normal, TSH normal	Inhibition of conversion of T4 to T3
Glucocorticoids	Total T3 reduced, T4 and TSH low or normal	Inhibition of conversion of T4 to T3
Oral radiographic dyes	Total T3 reduced, both T4 and TSH high	Inhibition of conversion of T4 to T3
Dopamine, L-dopa, Glucocorticoids	Basal TSH and response to TRH reduced	Direct effect to inhibit TSH production in pituitary gland
Amiodarone, Bensamide (transient), Metopramide, Sulpiride	Basal TSH increased TRH	Increased TSH production

Relationship Between Serum Total T4 and Total T3 Concentrations in Various Disorders

		Serum T4 Concentration		
Serum T3 Concentration	High	*Low*	*Normal*	*High*
		Iodide deficiency, T3 treatment, Antithyroid drug therapy	T3 – thyrotoxicosis, T3 – binding autoantibodies,	Thyrotoxicosis of any cause, Excess T4 ingestion Thyroid hormone resistance, TBG excess,
	Normal	Iodine deficiency, T3 treatment, Hypothyroidism		T4 treatment, Euthyroid hyperthyroxinemia, Thyrotoxicosis with acute or moderate nonthyroidal illness, T4 binding autoantibodies
	Low	Severe hypothyroidism, TBG deficiency, Drugs, Severe nonthyroidal illness,	Acute and chronic non-thyroidal illness, Drugs, Fetal life, Restricted nutrition	Thyrotoxicosis with severe nonthyroidal illness,

Relationship Between Serum FT4, FT3 and TSH Concentrations in Various Disorders

		Serum T4 Concentration		
Serum TSH Concentration	High	*High*	*Normal*	*Low*
		TSH-secreting tumor (rare) (FT3 = ↑)	Borderline/ compensated hypothyroidism,	Hypothyroid (primary), Recovery from sick euthyroid state,
	Normal	Euthyroid with T4 autoantibodies (uncommon)	Euthyroid	Sick euthyroid (FT3 = ↓), Hypopituitarism (other pituitary hormones = ↓,
	Low	Hyperthyroidism (FT3 = ↑)	T3 thyrotoxicosis (FT3 = ↑), Subclinical hyperthyroidism (FT3 = N/↓),	Hypopituitarism (other pituitary hormones = ↓), Sick euthyroid (severe) (FT3 = ↓),

Free Thyroxine Measurements in Common Conditions Affecting Thyroid-binding Proteins

Clinical conditions	*Free T4 Levels*
Near Normal Concentration of Serum-Binding Proteins	
Hypothyroidism	Low
Hyperthyroidism	High
Hyperestrogenism	Low
Abnormal Concentration of Serum-Binding Proteins	
TBG excess	Normal
TBG deficiency	High
Dysalbuminemia	Normal
Hypoalbuminemia	Normal
T4 autoantibody	Normal
Low total T4 non-thyroidal illness	Normal or High
High total T4 non-thyroidal illness	Normal or High

Free T4 and Free T3 in Various Disease Conditions

1. Hyperthyroidism

- Hyperthyroidism produces a primary increase in free T4, whereas estrogens and idiopathic or genetic conditions may produce a primary increase in TBP. In both cases [T4 and TBP] increase, but in the former, the patient is ill and requires treatment; in the latter, the patient is euthyroid. Likewise, a low serum [T4 and TBP] may be due to a primary decrease in [FT4] or to a primary decrease in [TBP]. It is, therefore, clinically important to differentiate between changes in [T4 and TBP] that are due to primary changes in [FT4] (e.g. hyper-or hypothyroidism) and those that are due to primary changes in [TBP].
- Serum TSH level is low in all forms of hyperthyroidism except in rare cases in which hyperthyroidism is mediated by TSH itself. When TSH level is low, free T4 concentration should be measured and will be elevated in most cases of hyperthyroidism. Finding a low TSH level and an elevated free T4 level is usually sufficient to establish the diagnosis of hyperthyroidism. If TSH level is low but free T4 level is normal, a T3 measurement should be performed, since serum T3 concentration is often elevated earlier in the course of hyperthyroidism and to a greater degree than is T4 concentration. Because only the free fraction of T3 is active, the estimation of free T3 is helpful in adjusting the total T3 for variations in binding proteins. It should be remembered that numerous medications as well as both acute and chronic illness may cause a transient lowering of T3 concentration as well as a reduction in TSH level.
- In Graves' disease or toxic adenomas, serum total T3 and free T3 levels are typically elevated to a greater degree than total T4 and free T4.
- T3 toxicosis—encountered in about 5% of hyperthyroid population—total T3 and free T3 values increase.
- Serum total T4 and free T4 are disproportionately elevated to a greater degree than total T3 and free T3 values in most patients with toxic multinodular goiter.
- Monitoring total T3 and free T3 values may also be of importance in evaluating both the severity and the response therapy in patients being treated for thyroid storm or crisis in that the antithyroid drug therapies are focused on reducing both thyroid gland T3 secretion and peripheral tissue T3 production from T4.

2. Nonthyroidal Illness

- In nonthyroidal illness (NTI) and altered states of nutrition there are two categories:
  Low T3 state: Decrease in total T3 and free T3 while maintaining normal total T4 and free T4. Observed in mild or moderate NTIs or states of caloric deprivation (< 400 cal)
  Low T3-T4 state: Total T4 also decreased, a case of severe NTI.
- Free T4 levels remain within or near the normal range of values as serum total T4 levels decline.

- Decreased total T3 or normal free T4 or increased free T4 results from acquired defect in serum T4-binding proteins which accompany NTI.
- Also common are increases in the levels of the free fraction of T4 and T3 which are caused by decrease in serum concentrations of thyroid hormone-binding proteins, changes in binding properties induced by circulating inhibitors and drugs, or both. Low levels of total T4 may be seen in nonthyroidal illnesses, but total T4 concentrations in these patients are usually normal or above normal as determined using reference methods.
- Thus in nonthyroidal illnesses, abnormal thyroid test results are not necessarily indicative of thyroid disease but may demonstrate adaptations to the catabolic state, many of these changes revert to normal when the patient recovers.
- Several test abnormalities may be seen in nonthyroidal illnesses in euthyroid patients (the 'euthyroid sick syndrome'). The most common abnormalities are a reduction in the serum total T3 concentration and an elevation in the serum level of free T3. Also common are increases in the levels of the free fraction of T4 and T3 which are caused by decrease in serum concentrations of thyroid hormone-binding proteins, changes in binding properties induced by circulating inhibitors and drugs, or both. Low levels of total T4 may be seen in nonthyroidal illnesses, but total T4 concentrations in these patients are usually normal or above normal as determined using reference methods.

3. Hypothyroidism

- If total T4 (or free T4) level is normal, hypothyroidism is most unlikely: however, a low T4 concentration is often seen in the euthyroid sick.

Assay Choice Application

For definitive diagnosis, assessment of both serum TSH and free T4 is required, but a more limited approach can be used for initial case finding and follow-up. In the interests of cost effectiveness, evaluation of thyroid status may often begin with an assay for either serum TSH or free T4, followed by further algorithm-based assessment if the initial result is abnormal. As an initial test, serum total T4 measurements give an unacceptable rate of abnormal results, due to the frequency of abnormalities in serum thyroid hormone-binding proteins.

Four distinct clinical situations in which evaluation of thyroid function is done can be considered: testing of unselected populations for case finding or screening, testing of untreated patients who have clinical features that suggest thyroid disease, assessment of the response to treatment for thyroid dysfunction, and evaluation of patients in whom associated illness or drug therapy are likely to complicate clinical and laboratory assessment or whose initial results are atypical or unclear.

Screening and Case Finding

About 2 to 7% of women over age 40 years may have slightly elevated serum TSH concentrations. The case for routine assessment of thyroid status is strongest in elderly women who have any symptoms that could be consistent with hypothyroidism. Among hospitalized patients, the large majority of abnormal results are due to nonthyroidal illness or medications.

Most persons found to have either high or low serum TSH values in screening or case-finding studies have subclinical disease. That is, they have no clinical manifestations of thyroid dysfunction and normal serum free T4 and T3 concentrations.

Regardless of which initial test is used, assessment of thyroid status has a high priority in patients at increased risk of having thyroid dysfunction, as for example in those with goiter, those treated previously for thyrotoxicosis or receiving lithium or amiodarone, and patients with associated autoimmune disease or connective tissue diseases or a history of neck or whole body irradiation.

Untreated Patients

In untreated ambulatory patients, a normal serum TSH concentration has high negative predictive value in ruling out thyroid disease. If serum TSH is abnormal, serum free T4 is done. Diagnostic strategies have been evaluated in which serum T4 measurements are done routinely only if the serum TSH is abnormal, unless pituitary disease is suspected. Long-term assessment of this approach will need to balance cost savings against potentially serious adverse outcomes; for example, if thyrotoxicosis is missed because of normal serum TSH values, or central hypothyroidism is missed on the basis of normal serum TSH values.

The following groups of patients will be incompletely or incorrectly assessed if either serum TSH or free TSH or free T4 alone is measured.

- Patients with subclinical hypothyroidism (high serum TSH, normal free T4) in whom replacement therapy may be beneficial.
- Those with subclinical thyrotoxicosis (low serum TSH, normal free T4) in whom treatment with an antithyroid drug or thyroid ablation may be beneficial.

- Those being treated for thyrotoxicosis, in whom suppression of TSH secretion may persist for weeks or months after normalization of serum T4 and T3 on drug.
- Those with central (secondary or hypothyrotropic) hypothyroidism (low serum free T4 low or normal TSH), who should be evaluated for adrenal insufficiency before T4 therapy is initiated.
- Those with binding abnormalities such as familial dysalbuminemic hyperthyroxinemia (FDH) or T4 or T4 binding autoantibodies in whom some serum free T4 estimates are invalid.
- Those with thyroid hormone resistance with high serum T4 and T3 concentrations and normal or high serum TSH concentrations, who are often not recognized until after inappropriate treatment has been given.
- Those with thyrotoxicosis caused by excess TSH secretion caused by a pituitary tumor or selective pituitary resistance to thyroid hormone.

Not withstanding the widespread acceptance of serum TSH as a single initial test, some still advocate an estimate of free T4 as the best initial test for suspected thyrotoxicosis.

Assessment of the Response to Treatment

In the testing of ambulatory patients with known thyroid disease, the use of serum TSH alone can also be considered. In a study of ambulatory patients attending a thyroid clinic, hyperthyroid patients taking T4 for either replacement or suppression, seldom needed a serum free T4 measurement of the serum TSH was greater than 0.05 mU/L; although at lower values, the magnitude of hyperthyroxinemia did influence management. In contrast, in patients with newly diagnosed thyrotoxicosis, measurements of serum free T4 or free T3, or both, were necessary in addition to serum TSH not only to establish the degree of hormone excess but also to evaluate the response to treatment. This study included a few new cases of hypothyroidism, in whom serum T4 measurement also would be required to establish the degree of hormone deficiency. In patients with thyroiditis and pituitary-hypothalamic disease, combined assessment was required.

In evaluating patients receiving T4 therapy, some have suggested that hormone measurements add little to a clinical assessment made by experts, but there is justification for periodic serum TSH assessment to avoid subtle tissue effects of thyroid hormone excess of deficiency. A serum TSH value in the low-normal range is, probably, the best single indicator of appropriate dosage and is certainly of more use than a serum free T4 value alone, which may be increased slightly depending on the time interval between dose and sampling. In some situations (e.g. patients with ischemic heart disease and hypothyroidism), the appropriate dose of T4 should be based on clinical judgement rather than laboratory findings.

Difficult Diagnostic Situations

The prevalence of abnormal serum T4 or TSH values in patients with acute medical or psychiatric illness is high, but there is controversy as to the value of thyroid function testing in these situations, because most of the abnormalities do not indicate the presence of thyroid disease in acutely ill patients because of the potential importance of intercurrent thyroid disease and the difficulty in assessing clinical features of thyroid dysfunction, others suggest that testing should not be done without some clinical indication.

In patients hospitalized for acute illness one or more of the assumptions outlined above may not be justified; for example, when there are wide fluctuations from the steady state. Serum TSH values frequently are subnormal in the absence of thyrotoxicosis and serum free T4 estimates are subject to multiple interfering influences, depending often on the particular method. Dual assessment clearly is necessary to identify the serum free T4-TSH combinations that indicate true thyroid dysfunction. When a patient has both thyroid dysfunction and a severe nonthyroidal illness, assessment becomes especially difficult because the effects of the illness, medications, or changes in nutrition can alter the expected changes in serum free T4 or TSH. Only clinical re-evaluation and repeated sampling may resolve the dilemma.

Thyroid Diagnosis and Treatment

There are three general principles upon which the physician should focus when evaluating thyroid function in a patient. These principles are:

- The thyroid gland is the principal site of thyroid dysfunction
- Autoimmune thyroid disease is the most common etiology producing the dysfunction
- Thyroid status is best determined by a combined measurement employing a serum free thyroxine (FT4) estimate and thyrotropin (TSH).

For both hypothyroidism and hyperthyroidism, TSH and an estimate of free T4 (FT4) are recommended.

T3 or free T3 may be needed to confirm hyperthyroidism if free T4 is within limits. Anti-thyroid antibodies, preferably antithyroid peroxidase (anti-TPO), may establish an autoimmune mechanism.

Recommendations for Thyroid Testing

- *Hyperthyroid*
  - Symptomatic — Free T4, TSH
  - Post-therapy — Free T4 (Free T3)
- *Hypothyroid*
  - Symptomatic — TSH, free T4 (anti-TPO)
  - Subclinical — TSH-first (T4, anti-TPO)
- *Monitor replacement* — TSH
- *Hypopituitary* — TSH and free T4
- *Acutely Ill* — None without suspicion
- *Pregnant*
- *Diagnosis* — TSH, free T4
- *Hypo, treated* — TSH
- *Elderly*
  - Healthy — None
  - Ill — None
- Women > 60 years — TSH
- High risk* — TSH
- Healthy adults — None without suspicion

*Ambigious symptoms, concurrent illness associated with thyroid disease, drugs associated with thyroid dysfunction.

Diagnostic Approach to Anomalous Serum T3, T4, and TSH Values

- Clinical re-evaluation, with particular attention to long-term features suggestive of thyroid disease and to the medication history.
- Measurement of serum TSH by a third-generation method to identify conclusively the degree of TSH suppression.
- Measurement of the serum T3 concentration with appropriate binding correction (free T3)
- An authentic estimate of serum free T4 (particularly in euthyroid hyperthyroxinemia).
- Follow-up to establish whether the abnormality is transient or persistent.
- Search for evidence of unusual binding abnormalities or hormone resistance in the propositus and family members.

*Typical Reference Ranges for Serum Thyroid Hormones and TSH in Humans**

Hormone	*Reference ranges*	*Variations unrelated to thyroid disease*
Total T3	69–202 ng/dL	Binding protein changes, binding competitors, age-related changes, nutrition, illness, surgery, drugs
Free T3	1.4–4.2 pg/mL	Methodologic factors and influences on total T3
Total T4	4.4–11.6 µg/dL	Binding protein changes, binding competitors
Free T4	0.8–2.0 ng/dL	Methodologic factors, pregnancy
TSH	0.28–6.82 µIU/mL	Diurnal variation, pulse secretion, age-related changes, drugs

*These ranges should be determined for the particular methods used in each laboratory. The neonatal period is excluded. Higher values in childhood.

CALCITONIN

Calcitonin is produced by the parafollicular cells of the thyroid. The main effect in man is to inhibit bone resorption, it lowers serum calcium and phosphorus. Hypocalcemia decreases calcitonin secretion; hypercalcemia increases calcitonin secretion. A syndrome of calcitonin excess—medullary carcinoma of the thyroid—is recognized in man. Little effect on calcium homeostasis is observed. However, the finding of elevated levels of the hormone is useful in the early detection of tumors.

Clinical Relevance

a. Increased levels are associated with:
   1. Medullary thyroid cancers
   2. C cell hyperplasia
   3. Chronic renal failure
   4. Pernicious anemia
   5. Zollinger-Ellison syndrome
   6. Cancers of lung, breast and pancreas.

b. In a small proportion of patients who do have medullary cancer, the fasting level of calcitonin is normal. In these instances, a provocative test using calcium or pentagastrin should be followed by an abnormally large increase in calcitonin levels.

Procedure

1. A pentagastin injection is administered. Blood samples are drawn before the injection and 1½ and 5 minutes after injection.
2. Another method is to infuse calcium (15 mg/kg) over a 4 hours period and collect blood samples before infusion and again at 3 to 4 hours.

Interfering Factors

Levels are normally increased in
- Pregnancy at term
- Newborns.

Thyroid levels in different disease conditions

Disease	*T3*	*T4*	*TSH*	*FT3*	*FT4*	*T-Uptake*	*Tg*	*Anti-TPO*	*TRH*	*TBG*
Primary Hypothyroidism	↓	↓	↑	↓	↓	↓			R	
Hyperthyroidism	↑	↑	↓	↑	↑	↑	↑			
Pituitary insuffiency, tertiary hypothroidism		↓	↓		↓	↓			R*	
T3, thyrotoxicosis				↑	↓				R	
Subacute thyroiditis		↑	↓		↑	↑a , ↓b	↑			
Nontoxic nodular goiter					↑		↑			
Graves' disease				↑	↑			↑		
Hashimoto thyroiditis				↑		↑d, ↓e		↑		
Lymphadenoid goiter								↑		
Iatrogenic hyperthyroidism					↑					
Myxedema			↑		↓					
Estrogen therapy, oral contraceptives, pregnancy		↑	↓c			↓				↑
Androgen therapy, steroid, hypoproteinemia (nephrosis, cirrhosis)		↓				↑				
Hypothyroidism treated with thyroxine				↓						
Hypothyroidism treated with Tri-iodothronine					↑					
Anti-thyroid drug for thyrotoxicosis		↓, N	↓			↓				
Benign adenoma							↑			
Untreated and metastatic carcinoma of thyroid							↑			
Acute psychiatric illness,acute medical illness, hepatic disease, malnutrition, Addison's disease, acromegaly		↓								

a–recovery stage, b–active stage, c–1st trimester, d–early, e–late, R–response, R*–delayed response, ↓–decrease, ↑–increase

PARATHYROID

The four parathyroid glands lie on top of the thyroid gland in separate nodes spread out to the four quadrants of the thyroid. Parathyroid hormone is under direct feedback control of circulating levels of calcium. If calcium levels fall, then parathyroid hormone is released. As calcium levels rise, release of the hormone is reduced. Parathyroid hormone acts on bones, kidneys and intestines to reabsorb calcium.

Hyperparathyroidism

It includes increased levels of parathyroid hormone. It is usually rare and occurs as a result of tumor. It leads to osteitis cystica fibrosis.

Hypoparathyroidism

It includes low levels of parathyroid hormone, can result due to trauma or removal during thyroid surgery.

The production of parathormone varies inversely with the plasma levels of ionized calcium, which is ordinarily maintained within normal limits.

Actions

Parathormone acts by controling metabolic reactions, which:

1. Increase calcium and phosphorus reabsorption from bones
2. Increase calcium reabsorption and phosphate excretion in the renal tubule
3. Increase absorption of calcium from the gastrointestinal tract
4. Decrease calcium secretion in the lactating breast (secondary hyperparathyroidism may follow renal insufficiency).

Clinical Disorders

A. *Deficiency:* Tetany (acute deficiency), hypoparathyroidism (chronic deficiency), often with epileptiform seizures.
B. *Excess:* Hyperparathyroidism with symptoms of hypercalcemia, renal calculi, bone resorption, sometimes peptic ulcer, hypertension, pancreatitis.

Methods of Evaluation

The X-ray of the bones of the hands, teeth, and skull, intravenous urography, serum calcium (repeated), serum phosphorus, urine calcium, serum alkaline phosphatase, bone biopsy, calcium and phosphorus tolerance, reabsorption and excretion tests. Test response of elevated calcium level to cortisone administration. Reduced blood magnesium levels (1.5-1.8 mg%) are frequent in hypoparathyroidism. Serum protein should be determined, as half of serum calcium is protein bound (while withdrawing blood, no tourniquet or pressure should be applied). Some of the important tests are mentioned below:

Serum Calcium

A finding of serum calcium levels above 11 mg% repeatedly suggests hyperparathyroidism. Hypercalcemia also occurs in multiple myeloma, sarcoidosis, milk alkali syndrome, vitamin-D intoxication, acute osteoporosis, Addison's disease, after electroshock therapy, in the presence of metastatic malignant disease with or without bone involvement, and in thyrotoxicosis.

On a diet containing about 100 mg of calcium per day, the normal person excretes 125 ± 50 mg of calcium per 24 hours. If milk or cheese is not present in the diet, the urine normally forms a slight cloud when Sulkowitch reagent is added. In hyperparathyroidism, which may be intermittent, hypercalcemia is usually associated with a daily urinary excretion of calcium greater than 200 mg. Hypercalcemia due to this cause is usually unaffected by corticosteroids (hydrocortisone 100 mg/day, or prednisone 20 mg/day, for 1 week), which decrease hypercalcemia in sarcoidosis, infantile hypercalcemia, metastatic malignancy, the usual case of vitamin-D intoxication, and miliary tuberculosis. Bone biopsy and tracer studies are diagnostic.

Tubular Reabsorption of Phosphate (TRP)

This test may indicate hyperparathyroidism in patients with good renal function and a daily phosphate intake of 800 mg or more. False positives may occur with uremia and in some cases of renal tubular disease, sarcoidosis and osteomalacia.

Method: A constant diet containing moderate amounts of calcium and phosphate is given for 3 days. Fasting blood is drawn in the morning when a timed 4 hours urine specimen is collected.

Urine phosphate (UP) and creatinine (UC) (in mg excreted/minute) and serum phosphate (SP) and creatinine (SC) (in mg/100 mL) are determined. Calculate TRP as:

$$\text{TRP (in\%)} = 100 \times 1 - \frac{\text{UP} \times \text{SC}}{\text{UC} \times \text{SP}}$$

Interpretation: TRP is about 78% on a normal diet, higher on a low-phosphate diet (430 mg/day for 3 days). In hyperparathyroidism, the TRP is 74% or less after a normal diet, 85% or less on a low-phosphate diet.

Calcium Infusion Test

Method: On a constant diet, 3 consecutive 24 hours urines are collected and measured for phosphate. On the second day, a 4 hours infusion of 1 liter of normal saline solution containing calcium gluconate-glucoheptonate (in a quantity enough to provide 15 mg of calcium per kg ideal body weight) is given.

Interpretation: A normal response consists of a marked reduction of urinary phosphate on the day of calcium infusion and a rebound increase on the third day. In hyperparathyroidism, minor alteration in urinary phosphate excretion is observed. Changes in urinary cyclic AMP parallel phosphate changes.

Ellsworth-Howard Test

This test distinguishes hypoparathyroidism from pseudohypoparathyroidism in which the level of parathyroid hormone is adequate, but the renal tubules are unresponsive. Anaphylactoid reactions to parathyroid extract may occur. Be sure the extract used is phosphuretic in humans and renal function is adequate.

Method: The fasting patient is given 2 mL (200 units) of parathyroid extract intravenously. The urinary phosphorus content is determined hourly for 3 hours prior to and for 3-5 hours following the injection.

Interpretation: Following the injection of parathyroid extract in normals, there is a 5-fold to 6-fold increase in urine phosphorus excretion. In hypoparathyroidism, following the injection of parathyroid extract, there is a 10-fold or greater increase in urine phosphorus excretion; with pseudohypoparathyroidism, there is utmost a 2-fold increase and urinary cyclic AMP does not increase in proportion.

Serum Parathyroid Hormone

Radioimmunoassay, ELISA and chemiluminometry methods have been developed and accurate results can be obtained rapidly. The relationship of parathyroid hormone assay to serum or ionized calcium levels are the best discriminators of parathyroid activity.

PARATHYROID HORMONE (INTACT) ELISA

(*Courtesy:* MD Biosciences)

Intended Use

The intact-PTH ELISA is intended for the quantitative determination of intact-PTH (parathyroid hormone) in human serum. This assay is intended for in vitro diagnostic use.

Summary and Explanation

PTH (parathyroid hormone, parathormone, parathyrin) is biosynthesized in the parathyroid gland as a preproparathyroid hormone, a larger molecular precursor consisting of 115 amino acids. Following sequential intracellular cleavage of a 25-amino acid sequence, preproparathyroid hormone is converted to an intermediate, *a* 90-amino acid polypetide, proparathyroid hormone. With additional proteolytic modification, proparathyroid hormone is then converted to parathyroid hormone, an 84 amino acid polypeptide. In healthy individuals, regulation of parathyroid hormone secretion normally occurs via a negative feedback action of serum calcium on the parathyroid glands. Intact PTH is biologically active and clears very rapidly from the circulation with a half-life of less than 4 minutes. PTH undergoes proteolysis in the parathyroid glands, but mostly peripherally, particularly in the liver but also in the kidneys and bone, to give N-terminal fragments and longer lived C-terminal and midregion fragments. In subjects with renal insufficiency, C-terminal and midregion PTH assays typically give elevated PTH results, as reflected by impaired renal clearance.

Clinical Significance

Intact PTH assays are important for the differentiation of primary hyperparathyroidism from other (non-parathyroid-mediated) forms of hypercalcemia, such as malignancy, sarcoidosis and thyrotoxicosis. The measurement of parathyroid hormone is the most specific way of making the diagnosis of primary hyperparathyroidism. In the presence of hypercalcemia, an elevated level of parathyroid hormone virtually establishes the diagnosis. In over 90% of patients with primary hyperparathyroidism, the parathyroid hormone will be elevated. The most common other cause of hypercalcemia, namely hypercalcemia of malignancy, is associated with suppressed levels of parathyroid hormone or PTH levels within the normal range. When intact PTH level is plotted against serum calcium, the intact PTH concentration for patients with hypercalcemia of malignancy is almost always found to be inappropriately low when interpreted in view of the elevated serum calcium. Unlike C-terminal and midregion PTH, which typically are grossly elevated in subjects with renal insufficiency, intact PTH assays are less influenced by the declining renal function. PTH values are typically undetectable in hypocalcemia due to total hypoparathyroidism, but are found within the normal range in hypocalcemia due to partial loss or inhibition of parathyroid function.

Principle of the Test

The intact PTH immunoassay is a two-site ELISA [enzyme-linked immunosorbent assay] for the measurement of the biologically intact 84 amino acid chain of PTH. Two different goat polyclonal antibodies to human PTH have been purified by affinity chromatography to be specific for well-defined regions on the PTH molecule. One antibody is prepared to bind only the mid-region and C-terminal PTH 39–84 and this antibody is biotinylated. The other antibody is prepared to bind only the N-terminal PTH 1-34 and this antibody is labeled with horseradish peroxidase [HRP] for detection.

Streptavidin Well - Biotinylated Anti-PTH (39-84) -Intact PTH —HRP conjugated Anti-PTH (1-34)

Although mid-region and C-terminal fragments are bound by the biotinylated anti-PTH (39-84), only the intact PTH 1-84 forms the sandwich complex necessary for detection. The capacity of the biotinylated antibody and the Streptavidin coated microwell both have been adjusted to exhibit negligible interference by inactive fragments, even at very elevated levels.

In this assay, calibrators, controls, or patient samples are simultaneously incubated the enzyme labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added to stop the reaction and converts the color to yellow. The intensity of the yellow color is directly proportional to the concentration of intact PTH in

the sample. A dose response curve of absorbance unit vs. concentration is generated using results obtained from the calibrators. Concentrations of intact-PTH present in the controls and patient samples are determined directly from this curve.

Test Significance

Investigation is of importance in distinguishing nonparathyroid from parathyroid causes of hyper-calcemia. A decrease in the level of ionized calcium is the primary stimulus for PTH secretions, while a rise in calcium inhibits secretions. This relationship is lost in hyperparathyroidism, and PTH will be inappropriately high in relation to calcium. The C assays tend to have higher values and are more widely accepted as better indication of hyperparathyroidism. Creatinine is usually determined with all PTH assays to determine kidney function.

Clinical Relevance

1. **Increased PTH values are seen in:**
   a. Chronic renal failure. This is a cause of secondary hyperparathyroidism
   b. Pseudohyperparathyroidism. There is a primary defect in renal tubular responsiveness to PTH (slight increase)
   c. Vitamin D deficiency (moderate)
   d. Malabsorption (moderate)
   e. Rickets (moderate)
   f. Osteomalacia (moderate).
2. **Decreased PTH values occur in nonparathyroid hypercalcemia, as in:**
   a. Use of thiazide diuretics
   b. Milk alkali syndrome
   c. Vitamin A and D intoxication
   d. Hematologic malignancies (some of them)
   e. Sarcoidosis
   f. Graves' disease
   g. Permanent postoperative hypoparathyroidism.
3. **Increased PTH-N values occur in:**
   a. Pseudohypoparathyroidism
   b. Secondary hyperparathyroidism
   c. Primary hyperparathyroidism.
4. **Decreased PTH-N values are seen in:**
   a. Hypoparathyroidism
   b. Neoplasms
   c. Nonparathyroid hypercalcemia.
5. **Increased PTH-C values are seen in:**
   a. Pseudohypoparathyroidism
   b. Secondary hyperparathyroidism
   c. Primary hyperparathyroidism
   d. Neoplasms.
6. **Decreased PTH-C values occur in:**
   a. Hypoparathyroidism
   b. Nonparathyroid hypercalcemia.

Interfering Factors

A. Fasting sample should be obtained:
   1. Elevated blood lipids interfere with results
   2. Milk ingestion will lower PTH levels.

PANCREAS

The pancreas is a mixed exocrine and endocrine gland. The exocrine portion makes many of the digestive enzymes necessary for gastrointestinal function. The endocrine portion is comprised of discrete islands of cells called the islets of Langerhans. Cells within the islets produce two hormones that regulate the concentration of glucose in the blood.

Insulin

It is a small protein (MW 6000 Daltons) and is composed of two chains (A and B) held by disulfide bonds. It is secreted only by the β-cells of the pancreas. The α-cells of the pancreas secretes glycogen.

Insulin is secreted in a precursor form, *Proinsulin*. This is cleaved to release *Insulin and C-peptide*. C-peptide is the connecting peptide between the A and B chains of insulin. All the three—proinsulin, insulin and C-peptide are found in the blood. Estimation of these is important in different conditions (Fig. 24.8).

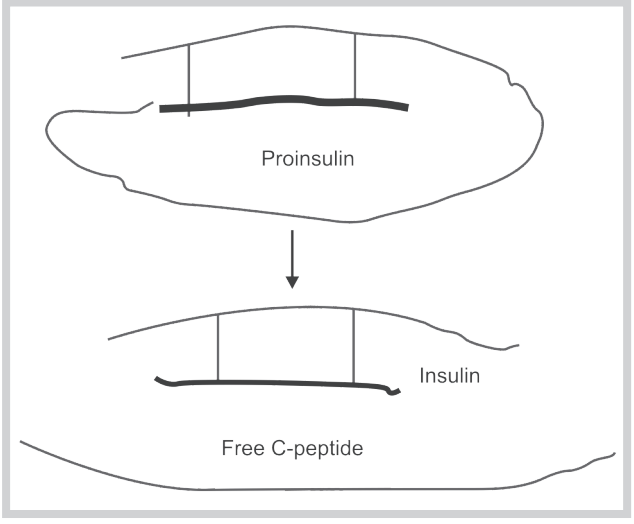


FIG. 24.8: Proinsulin and cleaved products

Functions of Insulin

- Reduces blood sugar level by stimulating uptake of sugar by various tissues
- Stimulates liver to store glucose in the form of glycogen.
- Promotes synthesis of fatty acids in liver
- Stimulates uptake of amino acids, contributing to overall anabolic effect
- Increase the permeability of many cells to potassium, magnesium and calcium ions.

Clinical Relevance of C-peptide

Indicators of β-cell function than peripheral insulin concentration.

Primary indicator for evaluation of fasting hypoglycemia.

Condition	*Insulin*	*C-peptide*
Insulinoma	Increased	Increased
Insulin producing	Normal	Increased
	β-cell tumors	
Hypoglycemia (injected/exogenous)	Increased	Decreased

Anti-insulin Antibodies

Antibodies against insulin hormone have been observed in many conditions. These can be against endogenous or exogenous insulin. It is found in Type 1 diabetes. These are developed under insulin therapy. There are two types of antibodies.

Against Endogenous Insulin
Antibodies against insulin, produced in the body (human insulin).

Against Exogenous Insulin
Antibodies against insulin, taken by medication—insulin injection (bovine, porcine and recombinant human insulin).

Both types are present in the body. Detection of both these types are important.

Glucagon is a small protein produced by alpha cells within the islets that cause the level of blood glucose to increase. Its release is controlled by blood levels of glucose. As levels fall, glucagon release is increased causing the release of stored glucose and the synthesis of glucose until levels are increased and glucagon release is then reduced via negative feedback. Glucagon opposes the metabolic actions of insulin. This opposition plus the negative feedback control of glucose levels maintain very tight control on blood glucose levels.

TESTES

Testosterone is the principal hormone of the testes and is synthesized from cholesterol by the Leydig cells. The secretion of testosterone is under the control of LH from the pituitary. The LH secretion is decreased by increased levels of testosterone in the blood via negative feedback. Testosterone develops and maintains the male secondary sex characteristics, is anabolic and growth promoting and participates in the formation of sperm. It also causes aggressive behavior and increased libido. Body hair is increased by androgens while scalp hair is decreased.

Like other steroids, testosterone enters cells and binds to an intracellular receptor and then causes the production of mRNA coding for proteins that manifest the changes induced by testosterone. In some target tissues a form of testosterone, DHT, is produced that has greater stability in combination with the receptor and, therefore, produces a longer lasting effect. The DHT is needed for the maturation of the accessory glands and external genitalia, while testosterone is more important in the growth of muscle mass, development of the internal genitalia and maintenance of the male libido and sex drive.

Another hormone produced by the testes is the polypeptide hormone, *inhibin*, produced by the Sertoli cells. It inhibits FSH secretion by a direct action on the pituitary.

Androgen Abnormalities

Androgen Excess
Males
- Children—precocious puberty
- Adults—infertility.

Females
- Hirsuitism and virilization
- Pseudohermaphroditism.

Androgen Deficiency
Males
- Improper growth—eunuchoid features
- Disappearance of body hair
- Muscular atrophy
- Infertility
- Testicular feminization.

Females
- Generally low in females.

OVARY

The ovaries produce the steroid hormones (estrogens and progesterone) that cause the development of secondary

sexual characteristics and develop and maintain the reproductive function in the female. Specifically, the estrogens are secreted by the theca interna cells and the granulosa cells of the ovarian follicle, the corpus luteum and the placenta. The LH from the anterior pituitary binds to receptors on theca interna or granulosa cells to cause the production of estradiol from cholesterol or a downstream precursor androstenedione that is passed from the thecal cells to the granulosa cells. Progesterone is secreted mostly by the corpus luteum and the placenta, but some are made by the developing follicle. Negative feedback from progesterone decreases LH secretion and large doses can prevent ovulation.

Estradiol is the most potent and major secreted estrogen although estrone and estriol can be found in circulation as well. Like other steroid hormones, estrogens enter target cells, combine with a nuclear receptor and cause the production of mRNAs that, when translated into proteins, modify cell function. Estrogens are metabolized by the liver and secreted in bile where some are reabsorbed back into the body. Metabolites of estradiol are excreted in the urine.

Estrogens in the bloodstream inhibit the release of FSH and LH, in some circumstances, via negative feedback. At other times, as in the preovulatory LH surge, estrogens increase the release of LH, via positive feedback. Estrogen also increases the excitability of uterine smooth muscle, myometrial sensitivity to oxytocin and increases the libido in women by a direct action on hypothalamic neurons.

Estrogens lower plasma cholesterol, inhibit atherogenesis (plaque formation in blood vessels), and are protective against myocardial infarction as suggested by the lower incidence of heart attacks and atherosclerosis in premenopausal women.

Synthesis

Pregnant women: Placenta (mainly estriol—E_3)
Nonpregnant women: Ovaries (mainly estradiol—E_2).

Estrogen Abnormalities

Excess

- Menstrual irregularities
- Amenorrhea
- Hermaphroditism
- Hashimoto's thyroiditis
- Addison's disease
- Turner's syndrome.

Deficiency

- Tumor of the ovary
- Hirsutism
- Infertility.

Progesterone has the principal targets of the uterus, breasts and the brain. It promotes the development of breast tissue, causes changes in the endometrial lining during the luteal phase of the cycle, decreases the excitability of myometrial cells and decreases uterine sensitivity to oxytocin.

Progesterone Abnormalities

Excess

- Congenital adrenal hyperplasia
- Hirsutism
- Amenorrhea
- Infertility.

Deficiency

- Menstrual irregularities
- Hermaphroditism
- Corpus luteum deficiency.

PINEAL GLAND

The pineal gland can be found deep in the brain at the top of the third ventricle where it is in close communication with the cerebrospinal fluid. In the adult, the pineal gland can often be seen in X-rays of the brain because of the accumulation of radiopaque calcium phosphate and carbonate into small granules called pineal sand. The cells of the pineal gland secrete the hormone Melatonin in a diurnal cycle (the amount changes throughout a 24 hours period) where the amount remains low during the daylight hours but increases during the dark hours. This diurnal variation is controlled by norepinephrine from sympathetic nervous input that is regulated by the light-dark cycle in the environment.

Although some people use melatonin supplements to treat insomnia, this effect has not been proven in scientific trials. There have been reports of increased insomnia and depression as well as other side effects associated with its use.

HORMONES AND FERTILITY

Disturbance in the hormonal system is a major cause of male and female fertility problems.

The brain plays a key role in regulating the hormones that affect the development of sperm (spermatogenesis) in males and regulation of menstrual cycle (ovulation) in females. The process begins when the hypothalamus (a part of the brain) emits a substance (gonadotropin-releasing hormone, or GnRH) that stimulates the pituitary gland, located at the base of the brain. The pituitary

gland then emits LH (luteinizing hormone) and FSH (follicle-stimulating hormone). These stimulate testicular development and sperm production in males and regulate the menstrual cycle and release of ovum in females.

The LH and FSH also regulate the production of steroid hormones responsible for male and female sexual characteristics.

MALE FERTILITY

The Four Factors of Male Fertility

Pretesticular Function (Hormones)

Disturbances in the hormonal system cause about 10% of male fertility problems.

Testicular Function

Testicular failure represents about 55% of male fertility problems.

To respond to hormone stimulation properly, the testicles, or testes, must be capable of producing sperm (spermatogenesis).

Post-testicular Function

Tubal obstruction including vasectomy accounts for about 6% of male infertility.

The post-testicular system of ducts must be capable of storing and delivering sperm. Sperm delivery system problems include obstruction or interruption of the tubes as a result of congenital malformation, disease, surgery, or trauma.

Ejaculatory Disturbance, Impotence, and Sexual Problems

Ejaculatory disturbances, impotence, and sexual problems may prevent the delivery of sperm.

These disorders represent about 10% of male fertility problems.

Problems associated with male infertility

Problem %	*Infertile population %*
Hormone:	
Endocrine	9
Hyperprolactinemia (elevated prolactin)	10-40
Congenital adrenal hyperplasia	1
Stress	?
Sperm production:	
Varicocele	40
Testicular failure	14
Smoking, heat, drugs	?
Sperm delivery:	
Obstructed ducts	7
Congenital obstruction/absence of ducts	2
Erection, orgasm, ejaculation:	
Sexual problems	5
Ejaculation problems	2

The Male Hormone System

The Hypothalamus and Pituitary Start the Action

Approximately every 90 minutes a specialized area in the brain (hypothalamus) secretes GnRH (gonadotropin-releasing hormone). GnRH signals the pituitary gland, located at the base of the brain, to produce LH (luteinizing hormone) and FSH (follicle-stimulating hormone). The LH tells the testes to secrete the male hormone testosterone. Testosterone stimulates the sexual desires and develops and maintains male secondary sex characteristics such as hair growth and deep voice. Together, testosterone and FSH stimulate the testes to produce sperm (spermatogenesis). The body's ability to make and regulate these hormones is vital for maintaining virility and sperm production (Fig. 24.9).

Feedback Hormones from Testicles

There are feedback hormones—testosterone and inhibin—that keep a check and balance on GnRH, LH, and FSH levels. Once the Leydig cells in the testicles produce enough testosterone, the hormone control systems cut back on GnRH and LH production. When the Sertoli cells, which respond to FSH stimulation, produce enough inhibin, the pituitary cuts back FSH production.

Where Can Things Go Wrong?

Fertility Factor: The Hormone Balancing Act

Several things can go wrong with the hypothalamus—pituitary endocrine system:

- The brain can fail to pulse GnRH properly
- The pituitary can fail to produce enough LH and FSH to stimulate the testes
- The testes Leydig cells may not produce testosterone in response to LH (pituitary) stimulation
- The body may produce other hormones and chemical compounds which interfere with sex-hormone balance.

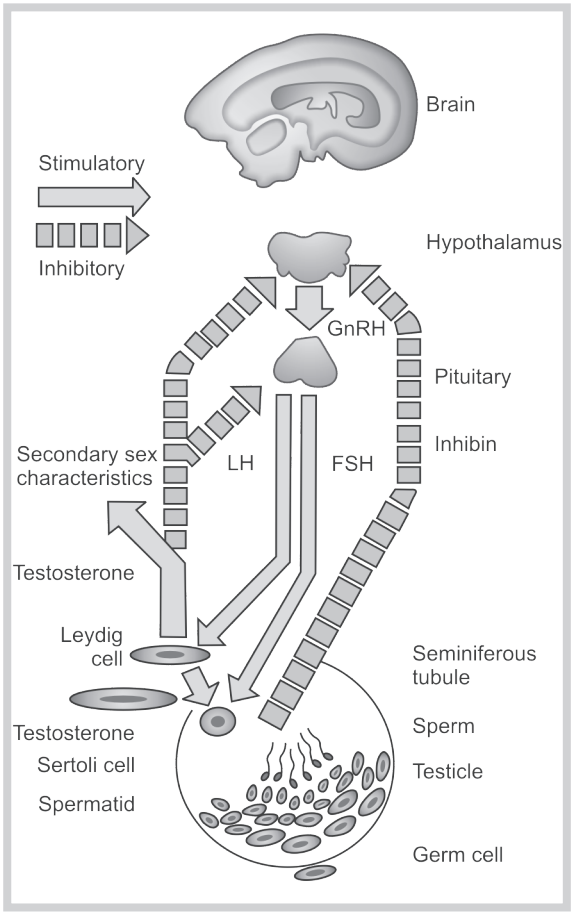


FIG. 24.9: Male hormones, feedback and effects

Any one of these conditions can impair sperm production.

An Overview of Hormonal Treatment

If the pituitary hormones (LH and FSH) are low, but the hypothalamus and pituitary gland are working fine, then clomiphene citrate (cc) is administered to stimulate the hypothalamus to pulse GnRH at regular intervals. When the hypothalamus properly releases GnRH, the pituitary gland will respond by producing LH and FSH. If clomiphene citrate does not improve LH and FSH levels, then one can suspect that the pituitary gland may be malfunctioning.

If the pituitary cannot manufacture the missing sex hormones, one has to take hormone supplements—hCG or Pergonal (LH + FSH).

Diagnosing and Beating Specific Hormonal Problems

Hyperprolactinemia

Hyperprolactinemia (elevated prolactin) can be difficult to diagnose because FSH, LH, and testosterone levels will be normal. We find elevated prolactin, a hormone associated with nursing mothers, in 10 to 40% of infertile males. Mild prolactin elevation produces no symptoms; however, greater elevations can reduce sperm production, impair sex drive, and cause impotence. Hyperprolactinemia responds well to bromocriptine. A prolactin-secreting tumor will also respond to bromocriptine; however, surgery and/or radiation therapy may be necessary.

Hypothyroidism

Found in 1% of infertile men, hypothyroidism (low thyroid hormone) can cause poor semen quality, poor testicular function, and/or disturbances in sex drive. The person will be lethargic, intolerant of cold, and overweight. Because the pituitary gland is trying its best to stimulate the unresponsive thyroid gland, the pituitary-produced TSH (thyroid-stimulating hormone) level will be elevated. Elevated prolactin levels, frequently found with this disorder, may cause impotence.

Correcting the diet or beginning thyroid hormone replacement therapy should elevate sperm count to previous levels.

Congenital Adrenal Hyperplasia

Found in 1% of infertile males, congenital adrenal hyperplasia may be suspected when a semen analysis shows a low sperm count, an increased number of immature sperm cells, sperm with long tapered heads, and low motility. These abnormalities occur when the pituitary is suppressed by increased levels of adrenal androgens. Men with this disease may also have hypertension (high blood pressure) and edema (water retention). Early onset of the disease may result in ambiguous genitalia at birth or reaching puberty at an early age. Adult onset may be characterized by infertility, high blood pressure, and/or water retention.

Cortisone replacement therapy will lower the androgens and allow the pituitary to function normally. Therefore, indirectly, cortisone replacement therapy will elevate sperm count.

Hypogonadotropic Hypopituitarism

Hypogonadotropic hypopituitarism is a spectrum of diseases with a complicated name that means low (hypo) pituitary gland output of LH and FSH. Other stages of this disease are called isolated gonadotropin defect and panhypopituitarism, in which the entire (pan-) pituitary gland is affected.

These diseases arrest sperm development and cause the progressive loss of germ cells from the testes. In addition, the seminiferous tubules and Leydig cells (which produce

testosterone) also deteriorate. If the condition persists for a long time, there will be no sperm production at all. When the disease is associated with a pituitary tumor, elevated prolactin levels may also cause impotence.

Panhypopituitarism

Complete pituitary gland failure (panhypopituitarism) lowers growth hormone, ACTH level, thyroid-stimulating hormone (TSH), and LH and FSH levels. Person having this rare disease will have multiple symptoms that include impotence, decreased sex drive, loss of secondary sex characteristics, and normal or undersized testicles. Hypothyroidism (low thyroid hormone) will cause to gain weight, be intolerant of cold, and feel lethargic. If the disorder began early enough in your life it may even cause dwarfism. The hormonal deficiency is often caused by a tumor, surgery, or trauma to the pituitary gland.

Kallmann's Syndrome

Kallmann's syndrome is a congenital hypothalamic dysfunction. A person born with this unusual condition will have underdeveloped testicles and possibly a harelip, cleft palate, color blindness, and/or the inability to smell. Affected men have varying degrees of sexual infantilism (prepuberty) and no sperm production. Since the hypothalamus fails to stimulate the pituitary adequately, FSH, LH, and testosterone levels are low.

Kallmann's syndrome is treated similarly to hypogonadotropic hypopituitarism. Although at first it seems hopeless, men afflicted with Kallmann's syndrome can achieve normal puberty and eventually become fertile.

Delayed Puberty

Individuals with isolated pituitary growth hormone deficiency do not sexually mature until their mid-to-late twenties. Hormone supplements can make them look virile, but until they go through puberty, they would not be fertile. The LH/FSH and/or hCG injections can bring on puberty, although if left alone, sexual maturity and fertility will be achieved in time.

Fertile Eunuch

Although virilization (acquisition of adult sex characteristics) will be moderately advanced, but the individual will not have completed sexual maturation and testicular growth. Here, the arrest of sperm production and low testosterone levels are caused by an LH deficiency.

Fertility Factor 2
Treating Unresponsive Testicles

What Causes Testicular Failure?

Let us suppose that the hypothalamus and pituitary are working well. The fact is that some conditions prevent the testicles from responding to pituitary hormone stimulation. Testicular failure, as it is called, can be caused by genetic abnormalities or by damage from drugs, injury, radiation, excess heat, adult mumps, a varicocele, or toxins from your environment. Sensing abnormal testicular function, your brain responds by telling your pituitary to pump out more FSH to stimulate sperm production. In fact, elevated FSH is the primary diagnostic indicator for testicular failure.

Varicocele

Varicocele is a varicose vein that allows blood to pool in your scrotum. It is thought that poor circulation may lead to a build-up of blood toxins or increase your scrotal temperature. Either of these conditions may result in infertility.

Cryptorchidism

Undescended testicles occur in 8 out of 1,000 boys. Hence, it causes infertility.

Infection

Mumps, tuberculosis, brucellosis, gonorrhea, typhoid, influenza, smallpox, and syphilis can cause the testes to atrophy. With some of these infections, LH and testosterone (virility) levels may remain normal. However, if FSH is high, then prognosis for testicular recovery is poor.

Torsion

Torsion of the testis and/or blood vessels supplying the testis (spermatic cord) is a common problem that threatens fertility.

Klinefelter's Syndrome

Each cell in a normal man's body has only one Y (male) and one X (female) chromosome. People with Klinefelter's syndrome, however, have one Y and two X chromosomes in each cell. In the beginning stages of this rare disorder FSH is only slightly elevated, indicating minimal testicular failure. However, eventually all other active testicular structures will atrophy, including germ cells, tubules, Leydig cells, and Sertoli cells; the testes themselves actually

shrink. After testicular failure occurs (causing FSH levels to rise dramatically), improving fertility is impossible.

Cushing's Syndrome

Cushing's syndrome occurs when the adrenal gland secretes excessive amounts of cortisol. People with this rare disorder will have a moon-shaped face and will suffer from water retention, obesity, impotence, feminized characteristics, loss of sex drive, and infertility. The condition may be due to an adrenal tumor or to excessive stimulation of the adrenal gland by ACTH (adrenocorticotropic hormone) from the pituitary. If ACTH is high, either the pituitary is overactive or an ACTH-secreting pituitary tumor is present (called Cushing's disease). Elevated adrenal androgens suppress LH and FSH production and spermatogenesis. Cortisone replacement therapy will reduce cortisol levels and restore natural LH, FSH, and sperm production. If a tumor is present, surgery and/or radiation therapy is required.

Germ Cell Aplasia (Sertoli Cell Only)

Germ cell aplasia (Sertoli cell only) is an inherited condition. Testes have normal Leydig cells, no germ cells. Because their Leydig cells continue to produce testosterone, these men remain virile, but they cannot produce sperm. Germ cell aplasia can also be caused by exposure to large doses of radiation and prolonged exposure to toxic substances.

Testicular Enzyme Defects

Testicular enzyme defects prevent the testes from responding normally to hormonal stimulation. These rare genetic defects can cause multiple genital abnormalities, incomplete virilization, small testes, and low or no sperm production. The LH and FSH will both be high, since, the brain is doing its best to stimulate the unresponsive testicles.

FEMALE FERTILITY

The Five Female Fertility Factors

Fertility Factor—1: Ovulation

Any woman complaining of very heavy menstrual flow, very light menstrual flow, no menstrual flow, irregular cycles, breast discharge, or scanty or overabundant body hair growth is telling that she may not be ovulating. This may be due to an intrinsic malfunction of her reproductive organs or hormones or to a systemic disease causing other body chemistry problems.

Fertility Factor—2: Sperm-Mucus Interaction

Normally, the cervical mucus forms an impervious plug that keeps foreign materials, including sperm, from entering the uterus. Once each month, responding to estrogen, the cervical mucus becomes clear, thin, and stringy so sperm can swim through the cervix into the uterus.

Fertility Factor—3: Fertilization

Fertilization depends on the sperm's ability to penetrate the outer layers of the egg and transfer its genetic information.

Fertility Factor—4: Tubal Factor

Other clues uncovered during the physical examination may point to transport problems. Abdominal adhesions can prevent the egg from entering the fallopian tube as well as impede its passage through the tube. Endometriosis can cause adhesions and impair ovulation.

Depending on their size and location, fibroids and ovarian cysts can also interfere with egg transport. These conditions will usually respond to surgery.

Fertility Factor—5: Embryo Implantation

The egg or (if the egg is fertilized) the embryo has to successfully implant in the woman's uterus. Sometimes, during the physical examination one can detect obvious causes for miscarriage such as congenitally malformed reproductive organs, an abnormally shaped cervix or a cervix distorted by previous surgical procedures.

Female Hormone System

What is Ovulation?

Ovulation is a fascinating harmony performed by several different "players"—the hypothalamus, pituitary gland, and ovary. The hypothalamus maintains the hormonal "tempo" by regularly pulsing GnRH (gonadotropic-releasing hormone). These pulses stimulate the pituitary gland to produce LH (luteinizing hormone) and FSH (follicle-stimulating hormone).

The pituitary gland plays the chorus—a pattern repeated from month to month in a beautifully precise rhythm. Each month the pituitary secretes FSH to stimulate the development and growth of over one thousand eggs. This phase in the ovulation cycle is known as the follicular phase. At puberty, a woman has about half a million primitive germ cells. Only four or five hundred, however, will ever reach maturity. Due to some mysterious mechanism,

which we do not yet understand, usually each month only one of the thousand developing eggs becomes dominant and grows to maturity. This egg, or ovum, is cradled within the ovary in a tiny, fluid-filled capsule called the follicle.

During the follicular phase of the cycle, LH acts on the ovary's theca cells to initiate estrogen production by the granulosa cells. The estrogen makes the follicle even more responsive to FSH, which further stimulates follicular growth and development of the egg. As the follicle expands toward the surface of the ovary, the egg increases in size nearly forty times. The ovary tells the pituitary when it needs more or less FSH to finish the job of egg maturation by making a feedback hormone called inhibin (folliculostatin).

Shortly before ovulation, the genetic material (nucleus) in the egg divides (meiosis) to half the number of chromosomes in the cell. If the egg is fertilized, a second meiotic division leaves the ovum with twenty-three chromosomes—a perfect complement to the sperm's twenty-three. To form an egg, the female germinal cell divides twice, as does the male germinal cell. During female germ cell divisions, however, the "surviving" ovum jealously hoards the bulk of cellular material (nutrients) and casts off the excess genetic material (polar bodies). The egg or (if the egg is fertilized) the embryo survives on these nutrients until the embryo successfully implants in the woman's uterus.

Estrogen also stimulates the uterine lining (endometrium) to become thick, lush, and filled with nutrients for the embryo. The cervical mucus responds to elevated estrogen by becoming clear, watery, and stringy. Normally, impervious to sperm, at midcycle the mucus welcomes the sperm and promises easy passage toward the egg.

When a woman's estrogen level peaks at midcycle, the pituitary "knows" that the egg is ready to embark on its journey. The pituitary responds to the estrogen peak by producing a surge of LH, which releases the egg within 18 to 36 hours. The outer wall of the ovary dissolves away from the bulging follicle; and within 2 to 3 minutes, the ovum escapes into the woman's abdominal cavity. Surrounded by a sticky protective layer of cells (cumulus oophorus), the egg gently floats toward the fallopian tube. The expelled follicular fluid stimulates the fimbriated end of the fallopian tube to reach toward the ovum, grasp the ovary, and vacuum up the egg. The muscles and tiny hairs (cilia) lining the fallopian tube gently coax the egg on its 3 to 4 days journey through the narrow passage. For conception to occur during this cycle, the sperm must fertilize the egg in the fallopian tube within 12 hours of ovulation.

During the egg's journey, the ruptured follicle begins an amazing transformation into the corpus luteum. Stimulated by LH from the pituitary gland, this yellow-pigmented, glandular, ovarian structure enlarges to make up nearly a third of the ovary. During the luteal phase (latter half of the cycle), the corpus luteum produces progesterone, a hormone that prepares the uterine lining for implantation of the embryo. Progesterone also acts on your body's temperature-regulating mechanism by raising basal body temperature (BBT) approximately one-half degree. Thus, shortly after ovulation, a woman will see a rise on her BBT chart. If fertilization does not take place, the corpus luteum deteriorates. Estrogen and progesterone levels decline rapidly in the week or so prior to menstruation. Deprived of these hormones, the endometrium atrophies and menstrual flow begins. At the site of the original follicle, the corpus luteum degenerates and leaves a minute piece of scar tissue as a reminder of its brief existence. If fertilization takes place, a corpus luteum of pregnancy forms to maintain the uterine lining (endometrial bed) and support the implanted fertilized ovum (conceptus) (Fig. 24.10).

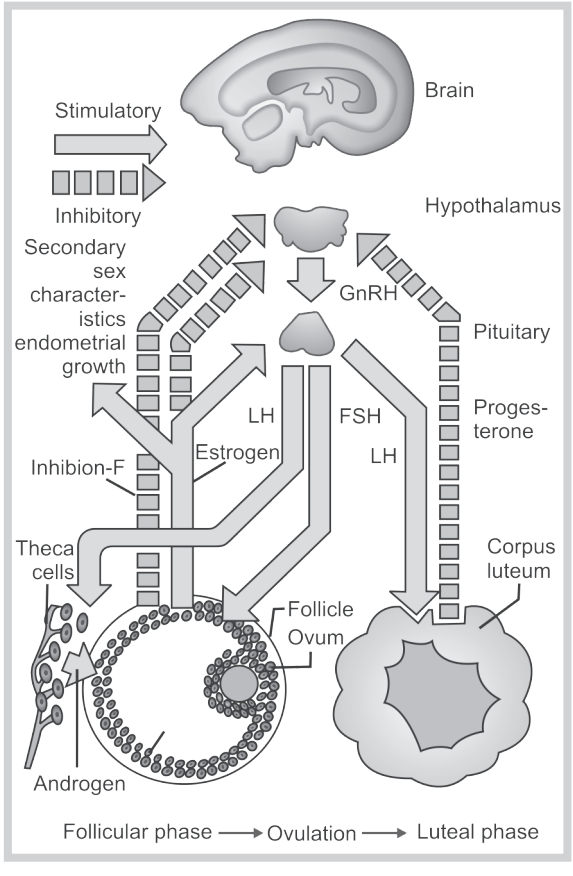


FIG. 24.10: Female hormone system

The hypothalamus, pituitary, and ovary must all work in perfect harmony. When they do not, the most obvious symptom is abnormal menses.

What Makes One have a Period?

Normally, each month estrogen and progesterone stimulate the growth of the uterine lining. When the progesterone-producing corpus luteum deteriorates toward the end of the cycle, "progesterone withdrawal bleeding" occurs: you have a period. Waves of vasoconstriction (blood vessel spasms) restrict the blood supply to the endometrium and thus provoke the onset of menses. At the conclusion of menses, clotting factors seal off exposed bleeding sites, and resumed estrogen production begins restoring the endometrium.

Clues from the Menstrual History

The Three Types of Menstrual Patterns

The Regular Menstrual Period

The critical point about this category is that the period is regular from month to month, beginning like clockwork every 25 days or every 35 days, for example. If the periods are regular, then she is probably ovulating. The consistently irregular menstrual cycle, however, where one month she begins menstruating after 25 days, the next month after 34, and the next in thirty, may indicate that she has a fertility problem. If a woman reports a regular menstrual history, one should look at other areas of the reproductive system for a breakdown in the fertility formula (Fig. 24.11).

Irregular Menstrual Periods or Amenorrhea for Six or More Months

This is the most common complaint found with fertility problems. The woman's menstrual periods occur infrequently and at unpredictable intervals. Some women, even report that at some point their periods stopped altogether. Because these women are capable of menstruating (as demonstrated by their history), there is a good chance that with the proper treatment ovulation and a regular menstrual cycle will resume.

Nonexistence of the Menstrual Period

Women who have never menstruated may have genetic abnormalities, congenitally deformed reproductive organs, delayed puberty, or a pituitary malfunction. If by the age of 16 a woman has not started menstruating, she should be concerned. It is important to diagnose the problem early and to determine if such women will respond to hormonal therapy or surgical correction.

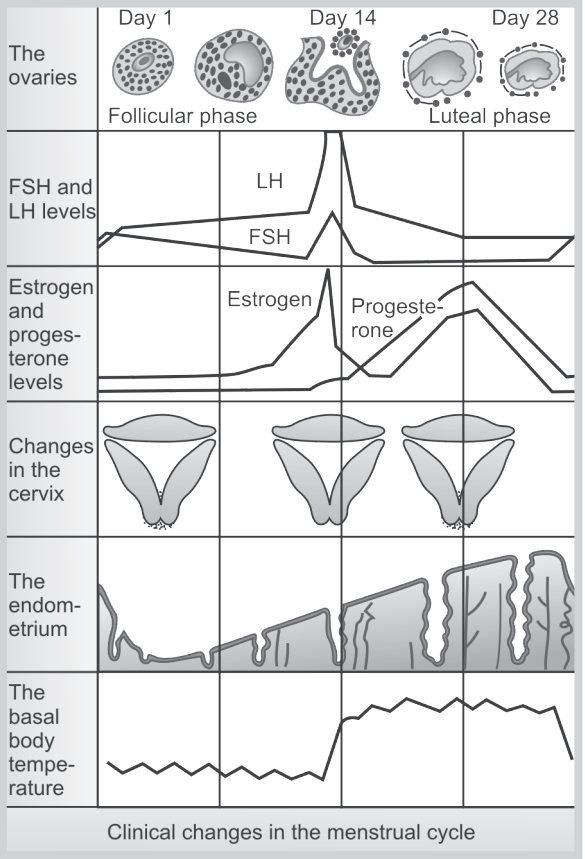


FIG. 24.11: The menstrual cycle

Clues from Physical Examination

The Physical Examination

During the physical examination, the doctor looks for evidence that the woman is ovulating, that her mucus allows sperm to reach the egg in good shape, and that the fertilized egg can successfully implant and grow in her uterus. A number of things may go wrong during this process. The sperm may not be able to journey through inhospitable cervical mucus or, having reached the egg, they may be unable to penetrate its surface. The egg may get lost in the body cavity and never find its way into the fallopian tube. Fallopian tubes, damaged by infection or trapped in adhesions, may not be capable of moving the egg toward the uterus. The growing, fertilized egg may become entangled in webs of intratubal adhesions caused by infection and develop into an ectopic pregnancy. Or the uterine lining may fail to nourish the early embryo. Once the doctor determines where these processes are breaking down, he has a good chance of restoring her fertility.

During the physical examination, the doctor would look for evidence of systemic disease: jaundiced (yellow)

skin and eyes are indicative of liver disease; tenderness in the middle of the back and water retention (edema) may indicate kidney malfunction.

The body build and secondary sex characteristics may provide additional clues to hormonal imbalance. Undersized breasts, scanty pubic hair, and underdeveloped hips all suggest a female hormone deficiency. An enlarged clitoris and abnormal hair growth such as a mostache may suggest excess male hormones. Rarer conditions such as ambiguous genitalia (not clearly male or female) and duplicate reproductive organs may point to genetic or enzyme defects that can interfere with ovulation. Although breast size, body conformation, and hair distribution are not conclusive evidence, they may corroborate suspicions created by other clues.

Based on the menstrual history and physical examination, the doctor will recommend a number of tests to confirm this diagnosis.

Diagnostic Approaches for Irregular Menstrual Periods or Amenorrhea

Several basic tests will help determine why your periods are abnormal.

Pregnancy Test

This may seem surprising, but pregnancy is the single most common reason for women reporting that their periods have stopped. Always conduct a pregnancy test to rule out the possibility of pregnancy.

Progesterone Withdrawal Test

The progesterone withdrawal test will confirm if the uterus is capable of menstruating. If it is, then the cause of menstrual irregularity lies with the hormonal systems. If the uterus cannot "bleed," then the problem lies with the uterus itself.

The doctor can bring period either by giving you oral progesterone over a 5- or 10-days period or by giving a progesterone injection. After taking the progesterone, the period should begin within 14 to 20 days.

Positive Response to Progesterone Withdrawal

If progesterone withdrawal causes the period to start up, then a number of things are clear.

First, we know that the ovaries are producing enough estrogen to build up the uterine lining. We also know that the uterus is capable of responding to estrogen and progesterone stimulation. Since the uterus is functioning normally, the fertility problem lies somewhere in the hormonal system.

Second, the failure to menstruate is because of failure to ovulate. For some reason, the pituitary is not producing the LH spike necessary to release the ovum from the follicle.

Two conditions must exist before the pituitary will release an LH surge: the follicles growing in the ovaries must release enough estrogen to signal the pituitary that it is time to release the LH surge—in other words, that at least one egg has reached maturity. And the pituitary gland must be capable of generating the LH spike.

One can suspect that the hypothalamus just isn't prodding pituitary well enough. If the follicles do not grow to maturity, there will never be enough estrogen to trigger the LH spike to release the egg and thus ovulate. A pituitary malfunction can cause the same problem.

Negative Response to Progesterone Withdrawal

Most women will "bleed" in response to progesterone withdrawal. However, if one does not, it is possible that the estrogen supply is not adequate to stimulate uterine lining growth. If the uterus is normal, taking estrogen to prime the growth of the uterine lining should guarantee that one will have a period after progesterone withdrawal. So repeat the progesterone withdrawal after estrogen stimulation.

If the estrogen/progesterone-stimulated cycle fails to produce a "bleed," it means that the uterus cannot respond to estrogen and progesterone stimulation: we can pinpoint the uterus as the problem.

Positive Withdrawal to Estrogen/Progesterone Stimulation

When one has a period after taking estrogen and progesterone, we know that the uterus is capable of menstruating. The reason the patient had not been menstruating is that her ovaries were not producing adequate amounts of estrogen. At this stage in the diagnostic procedures, we do not know for certain why the ovaries are not producing estrogen, but several possibilities exist:

1. The ovaries are not capable of producing estrogen.
2. The hypothalamus is not stimulating the pituitary to release FSH and LH, which control follicular development and estrogen production.
3. The pituitary is unable to produce adequate amounts of LH and FSH.
4. Other hormonal imbalances are tricking the pituitary into "thinking" that it is doing a good job when, in fact, it is not.

Since, estrogen stimulation is vital for the growth of the uterine lining, one should measure estrogen hormone levels to confirm this diagnosis before venturing into new diagnostic territories. In addition, measure FSH level to rule out ovarian failure (A high FSH level indicates that the ovaries have been severely damaged or have run out of eggs).

Detecting Ovarian Failure

Ovarian failure occurs when the ovaries are severely damaged or when they run out of eggs. When this happens, the pituitary gland tries to force the ovary to manufacture estrogen and to ovulate by working overtime to produce FSH. The pituitary gland's signals fall on deaf ears, though, because the damaged ovaries cannot respond to the extra FSH stimulation.

Ovarian failure may be caused by a number of conditions including infection, chemical toxins, medications, radiation exposure, tumor, surgery, immunologic dysfunction and genetic abnormalities.

Diagnosing Anovulation

Once uterine abnormalities and ovarian failure are ruled out, we confirmed that the periods are irregular because of not ovulating (anovulation). For some reason, the pituitary is not sending adequate amounts of LH and FSH to the ovaries.

Symptoms of Anovulation

Although a few anovulatory women will have normal periods, most will have a few or no periods at all (amenorrhea). Prolonged or heavy periods (menorrhagia), spotting during the middle of the cycle (metrorrhagia), and prolonged spotting may also occur. Women with anovulatory menstrual periods do not experience the typical menstrual discomforts often found in ovulatory women: breast soreness, mood changes, or cramping. The anovulatory woman's BBT chart will be flat (monophasic) and her cervical mucus will fern, indicating that progesterone (produced by the corpus luteum that forms after ovulation) never opposes the estrogen stimulation.

Tests Used to Determine the Cause of Anovulation

In the next phase of testing, the doctor will try to determine why the pituitary gland is not stimulating the ovaries to ovulate. He needs to answer a number of questions:

- Is the hypothalamus not "beating the drum" by producing regular pulses of GnRH?
- Is the pituitary gland damaged?
- Is the pituitary gland getting misleading feedback messages about ovarian function?

Several tests will give me the additional answers he needs.

Hormonal Tests for Diagnosing the Cause of Anovulation

Prolactin Pituitary Hormone

Excessive prolactin can suppress pituitary output (LH and FSH) and can act directly on the ovary to suppress follicular growth.

Thyroid Hormone

Hyper and hypothyroidism can interfere with hormonal metabolism (the rate at which hormones are used up by the body) and with the delicate hormonal balance between the pituitary and ovary. In addition, through an intriguing mechanism, hypothyroidism may contribute to excess prolactin production.

FSH and LH Pituitary Hormones

Elevated FSH almost always indicates ovarian failure. If FSH and LH are depressed, suspect one of three things: that a faulty hormonal feedback mechanism is inappropriately telling the pituitary to cut back production; that the hypothalamus is not "beating the drum" to stimulate the pituitary to function; or that a pituitary inadequacy prevents the gland from functioning normally.

Adrenal Androgens (DHEAS and Testosterone)

In the presence of excessive hair (hirsutism) or male secondary sex characteristics (enlarged clitoris or ambiguous genitalia), elevated male hormone (testosterone), elevated DHEAS, or elevated adrenal androgens may indicate a congenital enzymatic defect, polycystic ovaries, or a tumor in the pituitary gland, adrenal gland, or ovary. Testosterone or adrenal androgens can suppress ovulation as well as cause a number of other problems discussed later.

Conditions that can Interfere with Ovulation and Menstruation

- Pregnancy
- Hypothalamic malfunction:
  - Emotional stress (endorphins?)
  - Amenorrhea
  - Athletics (extreme exercise)
  - Dieting, poor nutrition, weight loss, low body fat
  - Anorexia
  - Idiopathic (drugs, toxins, medications?).
- Pituitary gland malfunction:
  - Hyperprolactinemia
  - Tumor
  - Surgery
  - Trauma
  - Empty sella syndrome
  - Sheehan's syndrome
  - Cushing's disease.
- Hormonal feedback problems affecting pituitary gland:
  - Hepatorenal disease
  - Adrenal disease
  - Cushing's syndrome

- Congenital adrenal hyperplasia
- Polycystic ovary
- Hypo/hyperthyroidism
- Obesity (excess estrogen).

➢ Ovarian abnormalities:
- Ovarian cysts
- Endometriosis
- Infection.

➢ Premature ovarian failure
➢ Incidental fertility findings:
- Asherman's syndrome (adhesions in the uterus)
- Cervical stenosis (cervix closed from surgery).

➢ Idiopathic (no identifiable cause).

Practical Evaluation of Hormonal Status

➢ The patients with a disorder of reproductive function serve as their own bioassay
➢ The history and physical examination are most important in evaluating any patient with reproductive dysfunction
➢ Evaluating the female patient with normal pubertal development as a reference is often useful in determining the cause of the reproductive dysfunction
➢ Laboratory tests are used to confirm what is suspected on the basis of the initial evaluation
➢ Immunoassays of WHO recommended standards are recommended for proper clinical correlation
➢ Measurements of basal FSH, LH, PRL and TSH are warranted in all amenorrheic patients once pregnancy has been excluded
➢ Radiographic studies of the sella turcica are indicated in amenorrheic women with low levels of circulating LH and FSH, whether prolactin is elevated or not. High sensitive immunoassays are required to ascertain the lower end values
➢ Individuals with hypothalamic or pituitary tumors and those with presumptive hypopituitarism should undergo dynamic testing of pituitary function
➢ Individuals with hirsutism should have serious etiologic factor eliminated for appropriate laboratory testing.

ALGORITHM FOR EVALUATING AMENORRHEA (FIGS 24.12 to 24.18), IMMUNOASSAYS FOR LH, FSH AND PRL

Sensitivity

The LH and FSH levels can drop to very low concentrations as low as 1 mIU/mL. Hence, assays have to be with very high sensitivity.

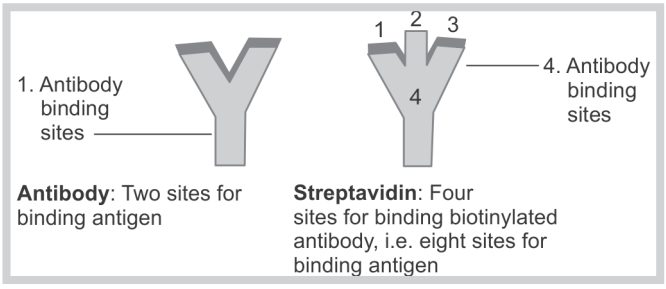


FIG. 24.12: Antibody binding sites and streptavidin

Sensitivity is of diagnostic importance in hypogonadism particularly when it is very essential to differentiate between the low and normal values of LH and FSH.

Detection Limits of Various Immunoassays

➢ 2nd Gen ELISA 2.0 mIU/mL
➢ RIA 0.2 mIU/mL
➢ Chemiluminisence 0.2 mIU/mL
➢ 3rd Gen RIAC ELISA 0.02 mIU/mL

Conditions in Which LH/FSH Levels can go Below 2.0 mIU/ mL

➢ **Hypopituitarism**
LH < 1.5 mIU/mL
FSH < 1.0 mIU/mL.
➢ **Pituitary tumor + Hypopituitarism**
- LH < 2.0 mIU/mL
- FSH < 1.5 mIU/mL.

➢ Non-functional pituitary tumor
- LH < 1.2 mIU/mL
- FSH < 1.0 mIU/mL.

➢ **Hypogonadism**
- LH < 1.5 mIU/mL
- FSH < 1.5 mIU/mL.

Streptavidin-Biotin Assay System

The strength and speed of the binding between avidin and biotin is used to provide amplification of signal and as the basis of generic signal generation reagents.

As compared to low sensitivity assays, streptavidin-biotin based assay systems offer an increase in signal ratio as well as improvement in rate of change of the measured signal, which in turn offers the immunoassay users greater accuracy from the test system in question (Fig. 24.19).

Calibrator Matrix

Calibrators should ideally be prepared by using a base material identical to that in the test samples. For clinical

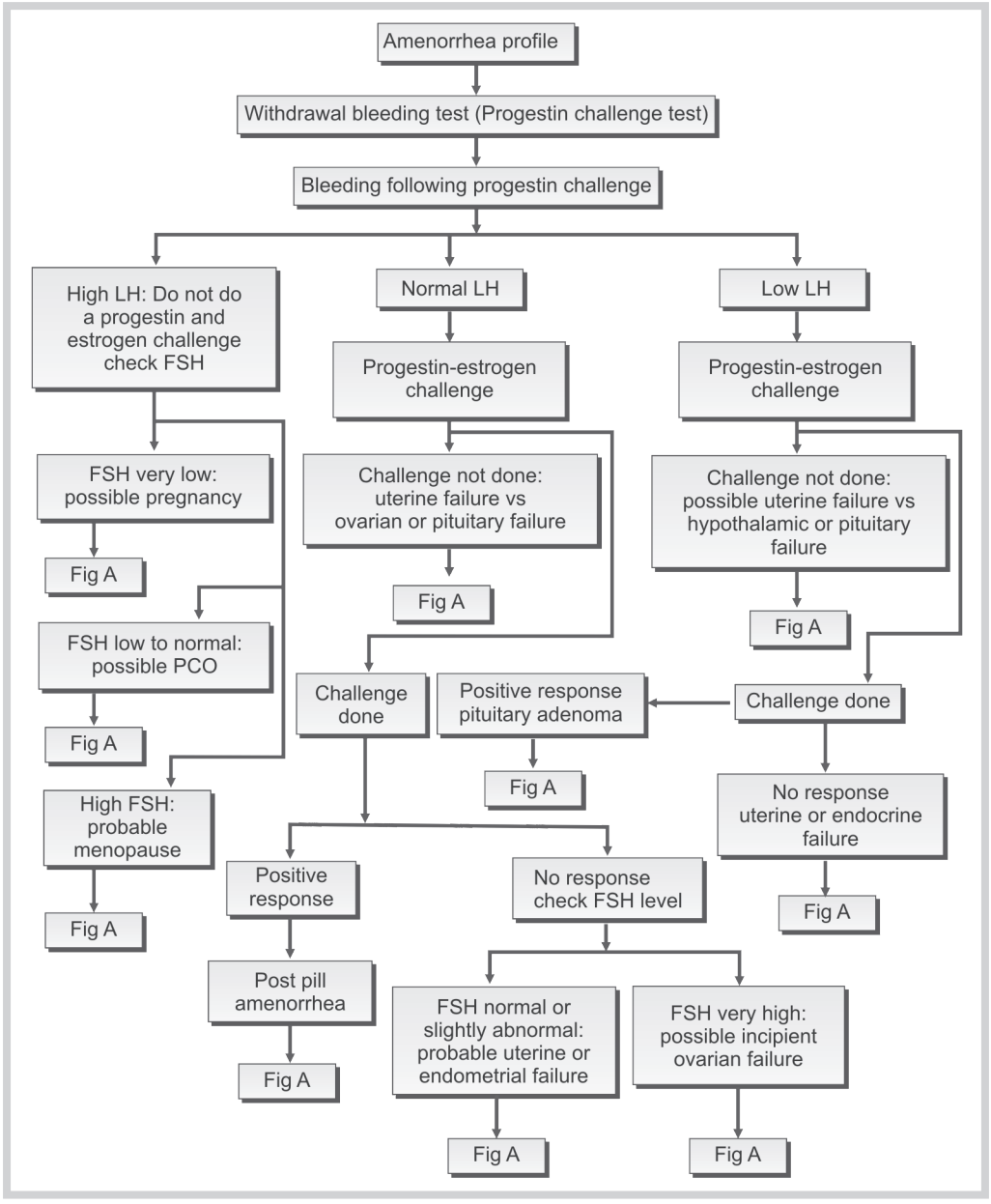


FIG. 24.13:

applications, human serum is the preferred base matrix (Fig. 24.20).

WHO Std. Reference

The World Health Organization's International Laboratory for Biological Standards is now providing qualified investigators with an International Reference Preparation of Human Pituitary Gonadotropins (LH and FSH) for Immunoassay (coded WHO 1st IRP 68/40 and WHO 2nd IRP 78/549). Data compared with these standard preparations can be reported in terms of International Units. These units differ from those obtained with use of the 2nd IRP-hMG).

Thus, the importance of knowing the "standard preparation" that is used and the "normal range" for any given laboratory is obvious. Also important is that commercially available assays may use different standards, and some of the kits do not even state what reference preparation is provided.

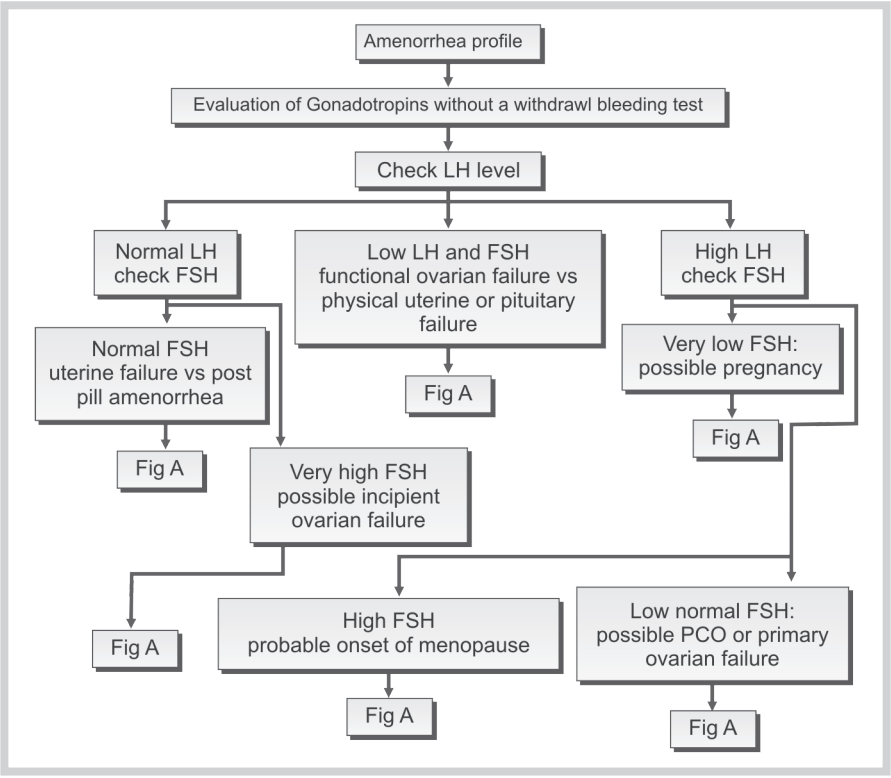


FIG. 24.14:

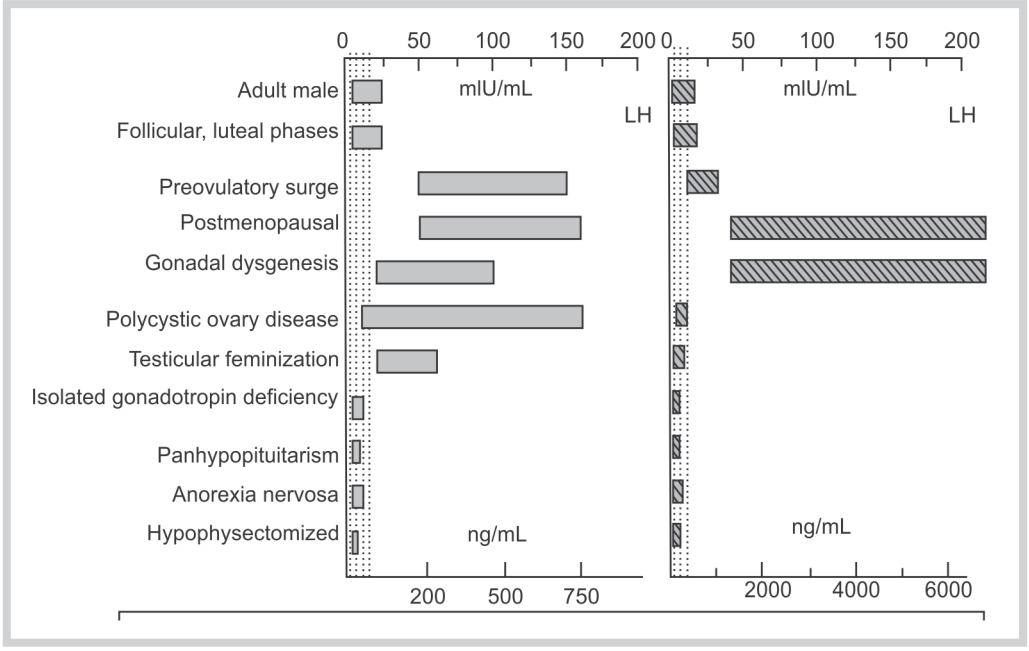


FIG. 24.15:

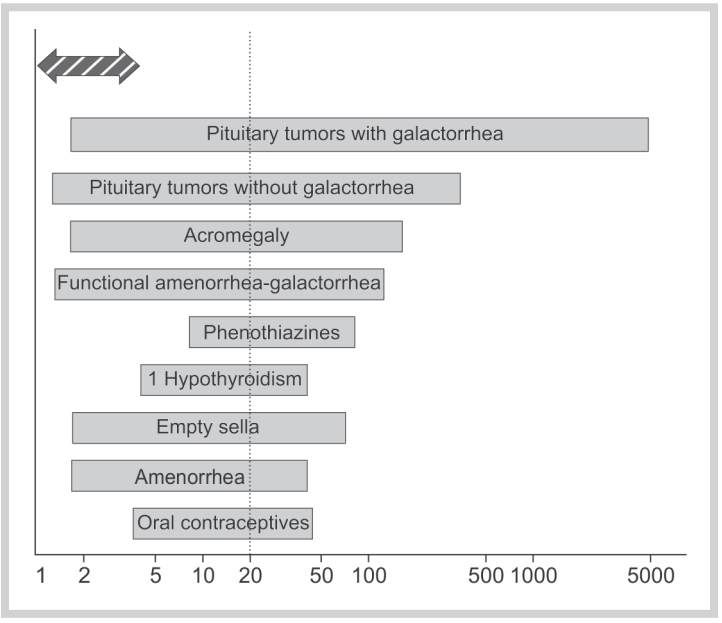


FIG. 24.16:

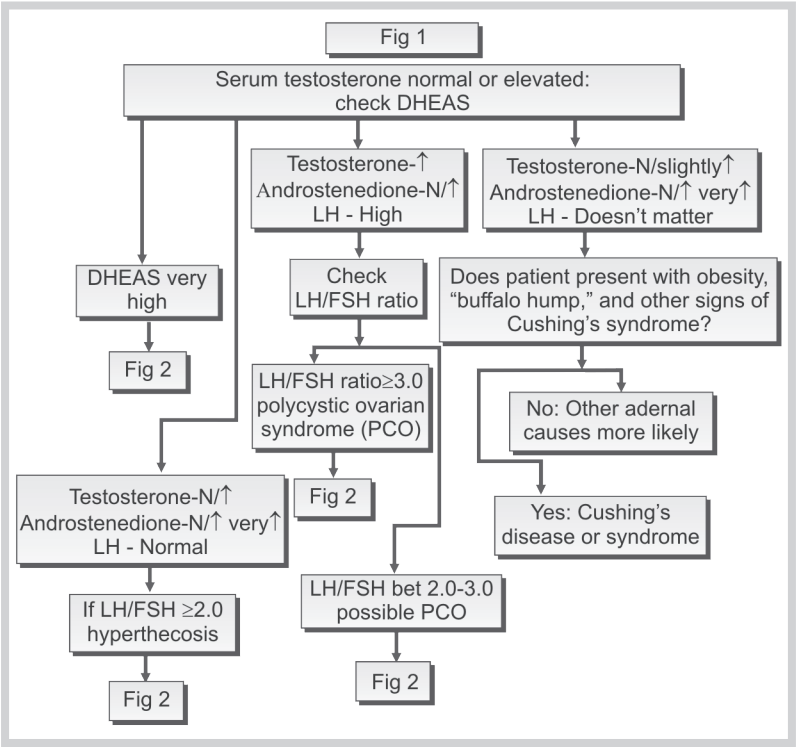


FIG. 24.17:

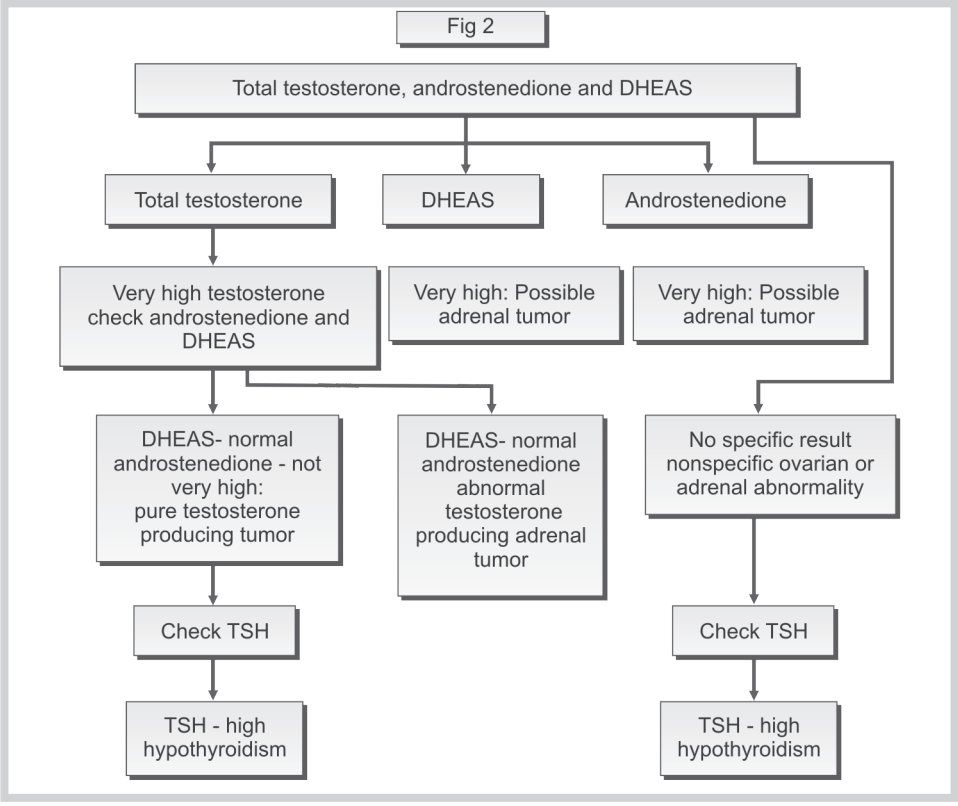


FIG. 24.18:

FIGS 24.13 TO 24.18: Algorithm for evaluating amenorrhea

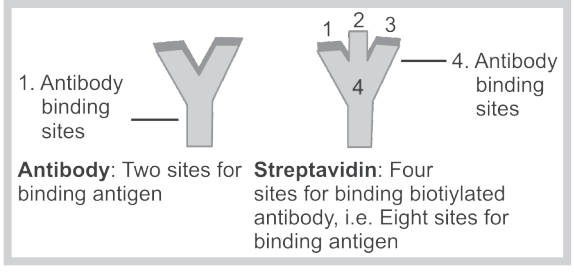


FIG. 24.19: Streptavidin-biotin assay system

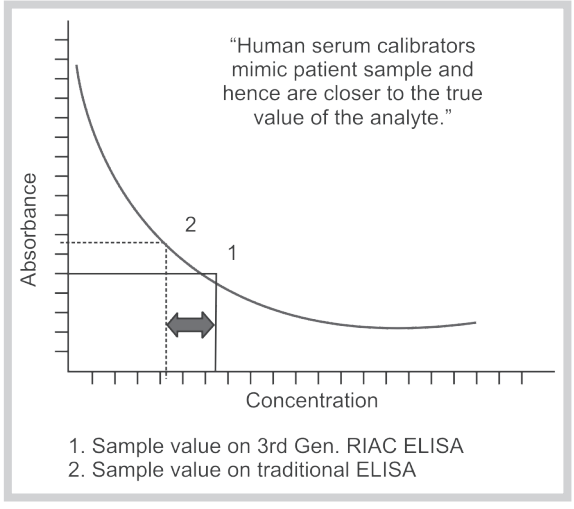


FIG. 24.20: Difference between ELISA generations

Cross Reaction

LH, FSH and PRL are pituitary hormones and are structurally similar to other pituitary hormones. Therefore, the tendency to cross react is very high when it comes to the estimation of these assays. For example, the tendency of PRL to cross react with GH is very high. Similarly, LH cross-reacts to a very high degree with hCG because of similar alpha chains. Hence, LH estimates are invalid in pregnant women or persons with hCG-secreting tumors. Immunoassays should be highly specific with minimal cross reaction.

Monoclonal Capture

Monoclonal captures are better compared to traditional polyclonal capture as it is more specific.

Assays having polyclonal capture antibodies give rise to different results. Monoclonal antibodies, which react against one highly specific antigenic determinant of a given hormone (epitype characterization), can help alleviate interlaboratory differences in results (Fig. 24.21).

Normal Ranges

	LH:mIU/mL	*FSH: mIU/mL*	*PRL:(ng/mL)*
Women:			1.2 – 15.5
Follicular phase	0.8 – 10.5	3.0 – 12.0	
Midcycle	18.4 – 61.2	8.0 – 22.0	
Luteal phase	0.8 – 10.5	2.0 – 12.0	
Postmenopausal	8.2 – 40.8	35.0 – 151.0	1.5 – 18.5
Men	0.7 - 7.4	1.0 – 14.0	1.8 – 17

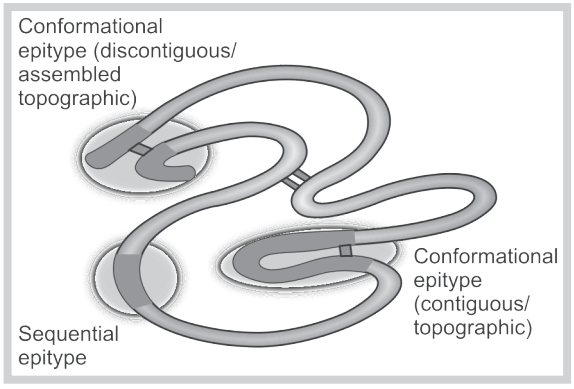


FIG. 24.21: Epitype characterization

Sample Collection and Storage

LH and FSH

Plasma, serum or urine can be used for LH and FSH measurements. Both hormones are stable for 8 days at room temperature and for 2 weeks at 4°C; for longer periods, the specimen should be stored frozen at or below –20°C. Because of episodic, circadian and cyclic variations in the secretion of gonadotrophins, a meaningful clinical evaluation of these hormones may require determinations in pooled blood specimens, multiple serial blood specimens, or timed urine specimens (Fig. 24.22).

Prolactin

Serum is the specimen of choice for PRL assays and can be stored at 4°C for 24 hours. Freezing is preferred for maintaining long-term stability. Specimens should be collected 3 to 4 hours after the patient has awakened, since PRL levels rise rapidly during sleep and peak in early morning hours. Emotional stress, exercise, ambulation, and protein ingestion also elevate PRL levels. As PRL is secreted episodically, multiple sampling techniques may be advantageous (e.g. pooling equal volumes of sera from specimens drawn at 6 to 18 min intervals).

The ranges will differ from laboratory to laboratory and depend on the reference preparation used for the standards.

The range of values is plotted on a logarithmic scale. Measured values differ significantly, depending on the laboratory and immunoassay system employed (Fig. 24.23).

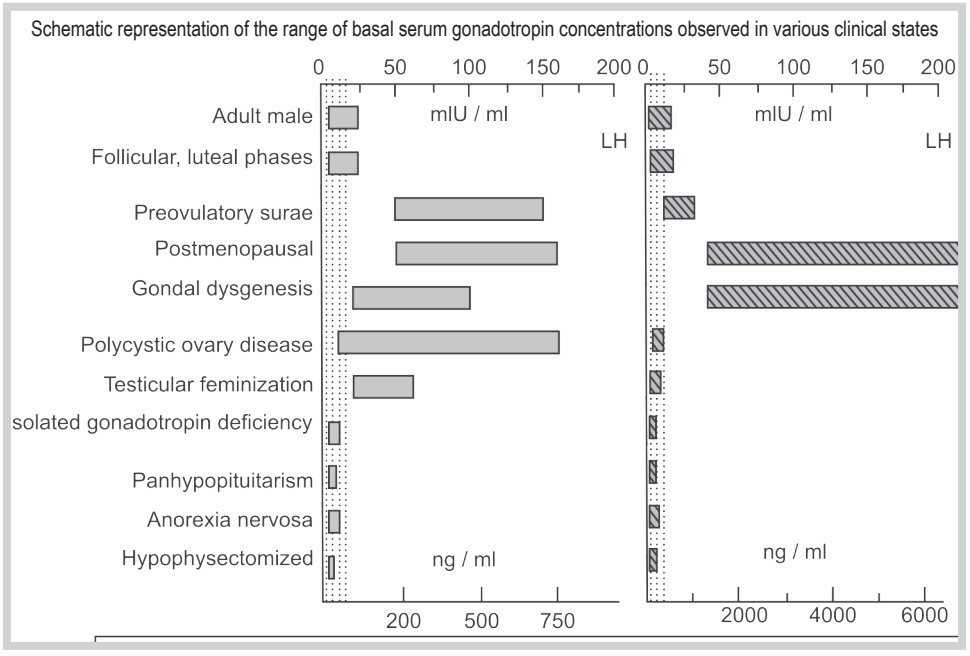


FIG. 24.22: The ranges will differ from laboratory to laboratory and depends on the reference preparation used for the standards. The dotted line represents the clinically important lower range

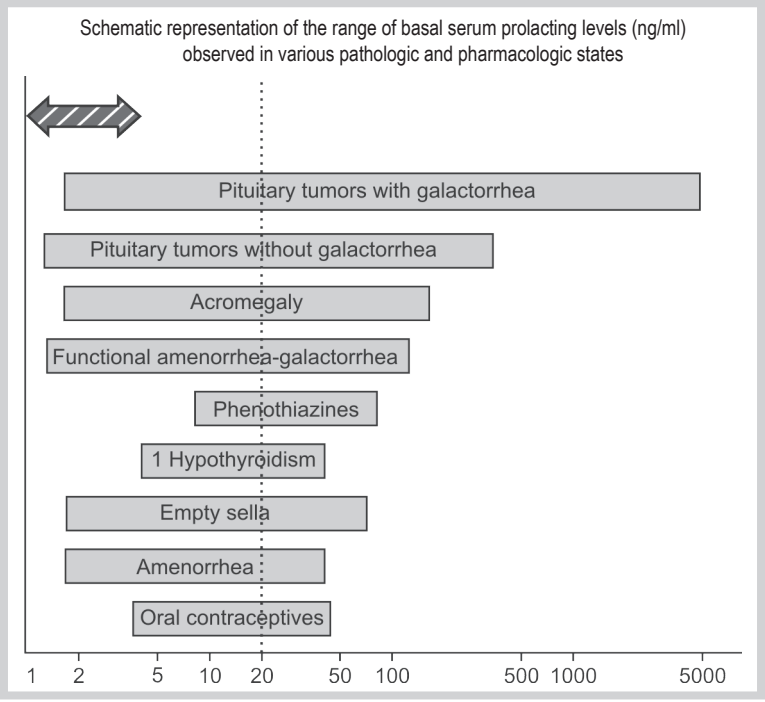


FIG. 24.23: The range of values is plotted on a logarthmic scale. Measured values differ significantly, depending on the laboratory and immunoassay system employed. The dotted line signifies the upper limit of the normal range in many laboratories. The arrow signifies the clinically important lower range

The dotted line signifies the upper limit of the normal range in many laboratories.

Hyperprolactinemia

Hyperprolactinemia is defined as consistently elevated Prolactin levels in the absence of pregnancy or postpartum lactation and is considered as pituitary disorder.

Prolactin is a pituitary hormone that plays a role in a variety of reproductive functions. It is essential for normal production of breast milk following childbirth. Also, it negatively modulates the secretion of pituitary hormones responsible for gonadal function.

Causes for Hyperprolactinemia

Common causes: Pituitary tumors, usually prolactinomas, which are under 10 mm in diameter.

Primary hypothyroidism, due to increased TRH resulting in increased TSH and Prolactin.

Ingestion of certain drugs, including phenothiazine, certain high blood pressure medicines (a-methyldopa), tranquilizers and opioids, anti nausea drugs, oral contraceptives.

Chronic kidney failure and other medical conditions.

Unexplained in about 30%.

Also associated with hypogonadotropinism and hypogonadism.

Symptoms of Hyperprolactinemia

- Amenorrhea
- Oligomenorrhea
- Corpus luteum dysfunction
- Headaches and visual difficulties
- Loss of libido and sexual profency in men
- Lowered levels of LH and FSH
- Symptoms of estrogen deficiency (such as those of menopause—hot flashes, dyspareunia), even in case of normal estrogen production
- Signs of increased levels of androgens in women.

Diagnosis

Basal Prolactin level can adequately be used to gauge pituitary tumor size and be followed over time.

Serum FSH, LH and estradiol—usually low to normal in hyperprolactinemia.

- TSH to rule out hypothyroidism.
- CT or MRI to identify microadenomas.
- Visual-field examination—in case of macroadenomas (>10 mm diameter) or any patient electing medical therapy or surveillance only.

Treatment

For patients >100 ng/mL of prolactin and normal CT/MRI or patients with only microadenomas—Bromocriptine or unmedicated surveillance.

Exogenous estrogen, in some cases, to combat low estrogen levels.

ADRENAL CORTEX

The adrenal cortex produces four major groups of hormones: (i) *glucocorticoids* (cortisol, cortisone), (ii) *androgens* (androstenedione, dehydroepiandrosterone), (iii) *Mineralocorticoids* (aldosterone, deoxycorticosterone, corticosterone), and (iv) *estrogens* and *progesterone.*

Adrenal corticosteroid production is controlled by a number of factors originating in the hypothalamic-pituitary system. The ACTH is the major tropic substance of the system. Aldosterone is under minimal control of ACTH, and its secretion is mainly influenced by volume receptors, angiotensin II and potassium concentration. The plasma cortisol, in turn, regulates ACTH secretion. The direct feedback mechanism does not seem operative for aldosterone secretion.

Cortisol-binding globulin (CBG) avidly binds cortisol and corticosterone and is the main carrier protein at normal concentrations. Estrogens increase CBG are inactive but are in equilibrium with free unbound steroid.

Actions

The mineralocorticoids increase reabsorption of sodium and chloride, increased excretion of potassium, and allow an exchange of intracellular potassium with extracellular sodium. Aldosterone is most effective in this regard. The glucocorticoids affect protein, carbohydrate, and fat metabolism, raising blood glucose, increasing gluconeogenesis and protein catabolism (with resulting osteoporosis), metabolising hepatic fat depots, decreasing tubular reabsorption of urates, increasing uropepsin secretion, and lyzing eosinophils and lymphocytes.

Clinical Disorders of Adrenal Steroids

a. *Deficiency*
   1. Acute: Addisonian crisis, Waterhouse-Friderichsen syndrome
   2. Chronic: Addison's disease.

b. *Excess*
   1. Principal glucocorticoids: Cushing's syndrome.
   2. Principal androgen excess: Adrenogenital syndrome in females, macrogenitosomia in males.
   3. Aldosterone excess: Primary hyperaldosteronism.

Methods of Evaluation of Glucocorticoids and Androgens

Evaluation of adrenocortical function may depend upon: (i) physical examination, noting particularly pigmentation of the skin and mucous membranes, pubic and axillary hair growth, blood pressure and the presence of edema, (ii) determination of serum sodium, potassium, chloride, CO_2, urea and protein, (iii) X-ray studies of the bones for osteoporosis and of the adrenal region, with or without retroperitoneal pneumography and tomography, (iv) determination of blood and urine levels of 17-ketosteroids, 17-hydroxycorticosteroids, aldosterone and specific excretory products such as androsterone and etiocholanolone, pregnanetriol, and pregnenetriolone, (v) specific function tests such as the water loading test and the response of hormone excretion levels to stimulation by exogenous ACTH, inhibition of ACTH production by corticosteroids, or inhibition of 11-β hydroxylation by metyrapone; and (vi) in the absence of interfering factors, the number of circulating eosinophils, normally between 100 and 300/ml, varying inversely with adrenocortical activity.

Urinary 17-Hydroxycorticosteroids, 17-Ketosteroid Excretion, Ketogenic Steroids, or Free Cortisol

The basal 24 hours urine excretion of 17-hydroxycorticosteroids is the most frequently used test in assessing adrenocortical activity. Paraldehyde, quinine, colchicine, iodides, sulfamerazine, and chlorpromazine interfere with the Porter-Silber steroid determination. The 17-hydroxycorticosteroids are metabolites of cortisol and cortisone. Urinary 17-ketosteroids are metabolites of: (i) adrenocortical steroids such as cortisol, (ii) adrenal androgens, and (iii) gonadal androgens.

The test, hence, reflects the activity of the adrenal cortex and the gonads in the male and the adrenal cortex in the female. There is a diurnal variation in excretion of 17-hydroxycorticosteroids and 17-ketosteroids of adrenal origin. The contribution of testosterone metabolites to the ketosteroids in the urine is minimal.

The 17-ketosteroid levels determined by the Zimmermann reaction are greatly reduced by probenecid and meprobamate administration. These drugs should be stopped for several days before urine collection.

The patient should not be receiving androgens or cortisol when specimens are collected. Testosterone propionate is excreted in the urine and is measured as 17-ketosteroids, methyltestosterone does not appear in the urine.

Method
A 24 hours urine specimen is collected in a jug containing 5 mL of 2% thymol glacial acetic acid.

Interpretation
High levels of excretion of both 17-hydroxycorticosteroids, 17-ketosteroids, ketogenic steroids, and urinary-free cortisol are found in adrenocortical carcinoma and adrenocortical hyperplasia, and of 17-ketosteroids and pregnanetriol in the adrenogenital syndrome. Low levels of excretion are found in hypopituitarism. Addison's disease, myxedema, and occasionally in anorexia nervosa.

Aldosterone

Aldosterone, Serum and Urine

Normal values

Average-Sodium diet	*Serum*	*SI units*	
Peripheral blood			
Supine	3–10 ng/dL	0.14–1.9 nmol/L	
Upright			
Adult female			
pregnant	18–100 ng/dL	0.5–2.8 nmol/L	
Nonpregnant	5–30 ng/dL	0.14–0.8 nmol/L	
Adult male	6–22 ng/dL	0.17–0.61 nmol/L	
Adrenal vein			
Child			
1 week–12 months	1–60 ng/dL	0.03–4.43 nmol/L	
Age 1–3 years	5–60 ng/dL	0.14–1.7 nmol/L	
Age 3–11 years	5–70 ng/dL	0.14–1.9 nmol/L	
Age 11–15 years	<5–50 ng/dL	<0.14–1.4 nmol/L	
Urinary aldosterone			
Norm urine	2–26 mg/24 h	5.6–73 nmol/day	
Urinary sodium	Plasma renin	µg/day	nmol/day
<30 nmol/day	5–24 AI/mL/h	35–80	97–220
20–50 nmol/day	2–7 AI/mL/h	13–33	36–91
50–100 nmol/day	1–5 AI/mL/h	5–24	14–66
100–150 nmol/day	0.5–4 AI/mL/h	3–19	8–53
150–200 nmol/day		1–16	3–44
200–250 nmol/day		1–13	3–36

This cholesterol-derived hormone is the most potent of the mineralocorticoids. Its foremost physiologic effect is that of regulating the transport of ions across cell membranes, especially those of renal tubules. This hormone causes the retention of sodium and chloride and the elimination of potassium and hydrogen. The second is the maintenance of blood pressure. Minute quantities will depress the urinary and salivary sodium to potassium ratio primarily because of diminished sodium excretion.

The three main factors that apparently affect aldosterone levels include the renin-angiotensin system, the plasma-potassium concentration and ACTH. The renin-angiotensin system appears to be the major mechanism that controls extracellular fluid by regulation of aldosterone secretion. Potassium loading results in increased aldosterone levels, whereas a potassium-deficient diet in the presence of aldosterone excess will result in a lowered aldosterone level. Increased concentrations of potassium in the blood plasma directly stimulate adrenal production of the hormone. The ACTH may affect aldosterone production in conditions of acute stress, burns, hemorrhage, and other pathologic conditions. Under physiologic conditions, ACTH seems to have little effect on aldosterone production.

Method

1. A 24 hours urine specimen is obtained.
2. Urine should ideally be refrigerated during collection.
3. Venous blood specimen is added to a heparinized or EDTA vial. Separate the cells from plasma immediately. Specimen should be obtained in the morning after the patient has been upright for at least 2 hours.
4. Specify and record the source of the specimen (e.g. peripheral venous, etc.).

Diuretic agents, progestational agents, estrogens, and liquorice should be discontinued 2 weeks prior to test. The patient's diet for 2 weeks before the test should be normal and include 3 gm of sodium per day.

Test Significance

This test is useful in detecting primary or secondary aldosteronism. Patients with primary aldosteronism characteristically have hypertension, muscular pains and cramps, weakness, tetany, paralyses and polyuria.

Clinical Relevance

1. Elevated levels occur in primary aldosteronism as in:
   a. Aldosterone producing adenoma
   b. Adrenal cortical hyperplasia
   c. Glucucorticoid remediable hyperaldosteronism.
2. Elevated levels also occur in secondary aldosteronism when aldosterone output is elevated due to external stimuli or because of greater activity in the renin-angiotensin system as in:
   a. Salt depletion
   b. Potassium loading
   c. Large doses of ACTH
   d. Cardiac failure
   e. Hepatic cirrhosis with ascites
   f. Nephrotic syndrome

g. Bartter's syndrome
h. Postsurgical syndrome
i. Hypovolemia and hemorrhage.

Interfering Factors

Values are increased in pregnancy and by posture.

Clinical Disorders of Mineralocorticoids

Primary Hyperaldosteronism

Primary hyperaldosteronism is usually due to adrenocortical adenoma. The principal manifestations of excess aldosterone secretion are hypertension and hypokalemia. Urinary aldosterone levels are high and plasma-renin activity is reduced or absent.

The most effective screening method is to determine whether hypertension is due to hyperaldosteronism or not is the serum potassium measurement. The disease must be suspected if more than 50 mEq of potassium are excreted in 24 hours and the serum potassium level is below 3 mEq/L. The patient should be on a high salt intake (2 g of salt with each meal for 4 days before electrolyte measurements and ECG are done). The electrocardiographic changes are those of prolonged hypertension and hypokalemia.

The patient with hyperaldosteronism frequently complains of severe headache. Potassium depletion causes weakness, paresthesia, flaccid paralysis, polyuria, and nocturia. Transient correction of hypokalemia by administration of spironolactone, 400 mg daily in divided doses for 3 days, is presumptive evidence of primary hyperaldosteronism. A diabetic glucose tolerance curve is present in about half of cases.

Isothenuria which does not respond to vasopressin is also due to potassium depletion.

Sodium retention causes hypernatremia and dilutional anemia due to increased plasma volume (low hematocrit). Autonomic dysfunction is manifested by a postural fall in blood pressure without changes in pulse rate.

Desoxycorticosterone acetate, 20 mg IM daily in divided doses for 3 days, causes no change in aldosterone production, if an aldosterone-producing tumor is present. The measurement of plasma aldosterone can be used. Care must be taken because of its increased response to posture, activity, and salt restriction. The plasma aldosterone does not normally increase 2-to 3-fold after 4 hours upright in patients with adenoma. While 83% of patients with the syndrome of primary aldosteronism have a solitary adenoma, the hypertension seen in patients with hyperplasia in the remaining 17% usually does not respond to subtotal or total adrenalectomy.

Secondary Hyperaldosteronism

Excessive secretion of aldosterone is seen in edematous states, such as cirrhosis with ascites, nephrosis, congestive heart failure, and toxemia of pregnancy; in non-edematous states, such as malignant hypertension; in unilateral renal arterial narrowing, and after diuretic therapy. Useful differential diagnostic aids are: (i) low serum concentration, (ii) blood volume, usually reduced in hypertensive patients, and (iii) normal or elevated plasma-renin activity.

Isolated Hypoaldosteronism

Hyperkalemia unexplained by diminished renal function can be the presenting finding in patients with reduced aldosterone levels. All other adrenal steroids are normal, and the defect resides in the defective release or production of renin.

ADRENAL MEDULLA

The adrenal medullary hormones are catecholamines: (i) epinephrine, and (ii) norepinephrine, the parent compound from which epinephrine is formed by addition of a methyl group.

Catecholamines, Plasma

Normal Values

	Normal range	*SI units*
Fractionation		
Standing		
Epinephrine	0–140 pg/mL	0–762 pmol/L
Norepinephrine	200–1700 pg/mL	1088–9256 pmol/L
Dopamine	0–30 pg/mL	0–163 pmol/L
Supine		
Epinephrine	0–110 pg/mL	0–599 pmol/L
Norepinephrine	70–750 pg/mL	381–4083 pmol/L
Dopamine	0–30 pg/mL	0–163 pmol/L
Fractionation Free		
Total	150–650 pg/mL	886–3843 pmol/L

Catecholamines, Urine

Normal Values

		SI units
Random urine		
Total catecholamines	0–18 µg/dL	0–103 nmol/dL
Daytime specimen		
Total catecholamines 24 hours urine	1.4–7.3 µg/h	8–43 nmol/h

Contd...

Contd...

Total catecholamines	0–135 µg/M^2/D^2	0–796 nmol/m^2/D^2
Panic level	>200 µg/M^2/D^2	>1180 nmol/m^2/D^2
Epinephrine		
Adult	0–15 µg	0–82 nmol/D
Children		
Age 1–4	0–6 µg/D	0–33 nmol/D
Age 4–10	0–10 µg/D	0–55 nmol/D
Age 10–15	0.5–20 µg/D	2.7–110 nmol/D
Epinephrine	> 50 µg/D	> 295 nmol/D panic level
Norepinephrine		
Adult	0–100 µg/D	0–590 nmol/D
Children		
Age 1–4	0–29 µg/D	0–170 nmol/D
Age 4–10	8–65 µg/D	47–380 nmol/D
Age 10–15	15–80 µg/D	89–470 nmol/D
Dopamine		
Age 4 years to adult	65–400 µg/D	384–2364 nmol/D
Age 4 years or less	40–260 µg/D	236–1535 nmol/D

Actions

Epinephrine is sympathomimetic, increases cardiac output and rate, systolic blood pressure, blood glucose, hepatic glycogenolysis, basal metabolic rate, sweating, and causes mydriasis and skin-vessel constriction. By contrast, norepinephrine causes bradycardia, peripheral vasoconstriction, and rise in diastolic blood pressure, and has much less prominent metabolic effects.

Clinical Disorders

Deficiency

Hypotension. Idiopathic spontaneous hypoglycemia (failure of epinephrine response to hypoglycemia).

Excess

Paroxysmal or persistent hypertension, headaches, sweating, tachycardia, elevated blood glucose.

Method of Evaluation

Chemical assay of epinephrine or norepinephrine in blood or urine, provocative and blocking tests for pheochromocytoma; glucose tolerance test; X-rays of suprarenal area.

Adrenal medullary hyperactivity, as in pheochromocytoma, produces symptoms and signs, including hypertension, through the release of large amounts of epinephrine and norepinephrine into the bloodstream. The most satisfactory single diagnostic procedure is the discovery of plasma levels of norepinephrine in excess of 0.5 µg/L. Jaundice, azotemia, and tetracycline administration also cause high levels.

When pheochromocytoma is suspected and hypertension is intermittent, or the basal blood pressure is less than 170/110, a provocative test with histamine may cause a characteristic rise in the blood pressure and in the urine and plasma catecholamines. Higher levels of basal blood pressure are best investigated by the phentolamine test.

The levels of urinary catecholamines and their metabolites (such as Vanillylmandelic acid—VMA normetanephrine and metanephrine) are greatly increased (10-100 times) in the presence of pheochromocytoma. The usual 24 hours excretion of epinephrine is up to 50 mg, of the metabolite, VMA, 2.5 µg/mg creatinine and less than 1.3 mg/24 hours for 1 ml metanephrine. The ingestion of tea, coffee, bananas, vanilla, salicylates, phenobarbital, fruit, morphine, iproniazid, and methocarbamol will invalidate the VMA measurement. Tetracyclines, vasopressors, and methyldopa can influence catecholamine determination. Monoamine oxidase inhibitors may cause a rise in metanephrine and a low VMA. The pressor amine output at rest is about half that during normal daily activity. If the urine contains increased amounts of epinephrine (40% of cases), the tumor is almost always in one of the adrenal areas or the organ of Zuckerkandl. If urine contains increased amounts of norepinephrine (60% of cases), the tumor may still be expected to be in or near one of the adrenal areas in two-third of cases; and in the remaining one-third, all possible sites must be considered.

In both, provocative and blocking tests, control blood pressure must be taken and the pressure must restabilize after venipuncture before the drug is given. Hypotensive drugs, e.g. rauwolfia, chlorothiazide, or sedatives, will confuse the results if given within 24 hours before testing. No diagnosis of pheochromocytoma should be based on these tests alone.

Histamine Provocative Test

Keep phentolamine ready for a case where there is a severe blood pressure rise in a hypertensive patient.

Method: A cold pressure test is first performed by placing the patient's forearm in a water-ice bath for 1 minute after basal blood pressure reading has been taken, and then recording postimmersion blood pressure readings at 30 seconds intervals for 3 minutes or until the basal state

is reachieved. At this time, 0.01-0.05 mg of histamine phosphate in 0.5 mL of isotonic saline is injected rapidly IV, and the readings are again followed to a basal level at 30-seconds intervals.

Interpretation: Normal subjects experience flushing, headache, and slight blood pressure fall. An elevation in blood pressure significantly greater than the cold pressure response within 2 minutes of the injection, or an increase in the basal levels of plasma catecholamines following histamine stimulation, may indicate pheochromocytoma.

Phentolamine Blocking Test

This test is used for diagnosis of pheochromocytoma in the hypertensive phase. Barbiturates interfere with the test.

Method: When the resting patient has achieved a basal blood pressure level, 5 mg of phentolamine are given rapidly IV and the blood pressure is determined at 30 seconds intervals. Maximum effect appears in 2 minutes and lasts for 3-5 minutes. If pheochromocytoma is strongly suspected, a dose of 1 mg should be administered to avoid profound and prolonged hypotension.

Interpretation: In normal individuals, phentolamine causes a slight transient fall in blood pressure. In the presence of pheochromocytoma with hypertension, a fall in systolic blood pressure of more than 35 mm Hg and a fall in diastolic blood pressure of more than 25 mm Hg appearing in 2 minutes and lasting for at least 2½ minutes is characteristic.

TESTES

From Leydig cells, the testis elaborates testosterone, androstenedione, and dehydroepiandrosterone (also from adrenal cortex). Luteinizing hormone from the pituitary stimulates the formation of testosterone, which is inactivated and conjugated in the liver and excreted as glucuronide. Testosterone production rates in men are 2-8 mg/day; in women, 0.5-2.5 mg/day.

Actions

Testosterone maintains sex organs and controls development of male sex characteristics, inhibits pituitary LH, promotes long-bone growth without closure of epiphyses, and has many anabolic effects (e.g. retention of sodium, chloride, water, decrease in gluconeogenesis). It is required for spermatogenesis. However, it cannot be taken exogenously for this purpose since it would suppress FSH and thus prevent this process.

Clinical Disorders

Deficiency

Before puberty, eunuchoidism or cryptorchism. After puberty, asthenia, loss of beard and potency, atrophy of sex organs, hot flushes, nervousness, depression and osteoporosis. Partial deficiency syndromes may occur (e.g. Klinefelter, Del Castillo).

Excess

Masculinization as with adrenocortical tumors, Leydig cell tumors, testicular teratomas and seminomas. Administered excess may depress spermatogenesis which "rebounds" to supranormal level after withdrawal.

Methods of Evaluation

Physical Examination

Noting pubic and temporal hair, prostate, and testes. The study of male hypogonadism or sperm deficiencies involves testicular biopsy, examination of the semen and spermatozoa count, and such urinary hormone determinations as indication of androgen production (17-ketosteroid excretion) or inhibition or lack of the pituitary gonadotropins (FSH determination). The absence of hyaluronidase from semen may indicate an obstructive lesion of the ducts leading from the testes, where the enzyme is produced. Urinary testosterone and plasma testosterone measurements are very helpful.

Gonadotropin Stimulation Test

Hypogonadism originating in the testis is accompanied by high excretory and serum levels of LH and FSH, whereas both are low when the defect is in the pituitary. Evidences of hypogonadism will disappear and 17-ketosteroid excretion will rise in a patient with hypopituitarism when gonadotropins are administered. This treatment is ineffective when the defect originates in the gonad.

The patient should not be receiving endocrine therapy at the time of testing.

Chorionic gonadotropin is given intramuscularly in a daily dose of 2000 IU for 3 weeks. The patient is watched for abatement of evidences of hypogonadism: in men, sperm count, testicular biopsy, 17-ketosteroids, testosterone, secondary sex characteristics, in women, vaginal smear for estrogenic effects, secondary sex characteristics.

Disappearance of the evidences of hypogonadism indicates hypogonadism secondary to pituitary failure.

Testosterone, Free, Blood

Normal values

	pg/mL	*SI Units pmol/L*	*Percentage of total testosterone*
Males values			
Adults	50–210	174–729	1.0–2.7
Children			
Cord blood	5–22	17.4–76.3	2.0–4.4
Newborn	1.5–31.0	5.2–107.5	0.9–1.7
4 weeks-	3.3–8.0	11.5–62.5	0.4–0.8
3 months			
3–5 months	0.7–14.0	2.4–48.6	0.4–1.1
5–7 months	0.4–4.8	1.4–16.6	0.4–1.0
6–9 years	0.1–3.2	0.3–11.1	0.9–1.7
10–11 years	0.6–5.7	2.1–19.8	1.0–1.9
12–14 years	1.4–156	4.9–541	1.3–3.0
15–17 years	80–159	278–552	1.8–2.7
Female values			
Adults	1.0–8.5	3.5–29.5	0.5–1.8
Children			
Cord blood	4.0–16.0	13.9–55.5	2.0–3.9
Newborn	0.5–2.5	1.7–8.7	0.8–1.5
4 weeks-3 months	0.1–1.3	0.3–4.5	0.4–1.1
3–5 months	0.3–1.1	1.0–3.8	0.5–1.0
5–7 months	0.2–0.6	0.7–2.1	0.5–0.8
6–9 years	0.1–0.9	0.3–3.1	0.9–1.4
10–11 years	1.0–5.2	3.5–18.0	1.0–1.9
12–14 years	1.0–5.2	3.5–18.0	1.0–1.9
15–17 years	1.0–5.2	3.5–18.0	1.0–1.9

Increased
Androgen resistance, hirsutism, polycystic ovary syndrome, tumor (virilizing) *See* also testosterone, total, blood.

Decreased
Hypogonadism, P-450 enzyme deficiency. (*See* also testosterone, total, blood.

Description
Free testosterone is that portion of circulating testosterone that is not bound to the sex hormone-binding globulin (SHBG) plasma protein. This test is used to differentiate true abnormal testosterone levels from those caused by abnormally low or high amounts of circulating SHBG. (See also Testosterone, Total, Blood).

Increased
Adrenal hyperplasia, adrenal tumor, arrhenoblastoma, central nervous system lesions, hirsutism (idiopathic), hyperthyroidism, ovarian tumor (virilizing), testicular feminization, testicular tumor, virilizing luteoma, and virilization. In women, idiopathic hirsutism, cystic acne, polycystic ovary syndrome, adrenogenic alopecia, abnormal menstruation, anovulation, adrenogenital syndrome with virilization, ovarian tumor, and Stein-Leventhal syndrome with virilization. Drugs include anticonvulsants, barbiturates, cimetidine, clomiphene, estrogens, gonadotropin (males), and oral contraceptives.

Decreased
Anemia, cirrhosis, cryptoorchidism, Down syndrome, gynecomastia, hypogonadism (male), hypopituitarism, impotence, Klinefelter's syndrome, male climacteric, obesity, and orchidectomy. Drugs include androgens, cyproterone, dexamethasone, diethylistilbestrol, digoxin (males), digitalis, estrogens (males), ethanol, glucose, glucocorticoids, gonadotropin-releasing hormone analogs, halothane, ketoconazole, metoprolol, metyrapone, phenothiazines, spironolactone, and tetracyline.

Description
Testosterone is the dominant androgen found in the adrenal glands, brain, ovary, pituitary, skin, kidney, and testes. It circulates both freely, and bound to plasma proteins (sex hormone-binding globulin [SHBG]). Testosterone promotes the growth and development of the male sexual organs, and increases body mass and hair replacement. This test measures total testosterone levels in clients with normal SHBG levels.

Interfering Factors
In adult males, an inverse correlation of free testosterone with age occurs. The upper limit of normal range generally decreases from the age of 20 to 60 years. The lower range of free normal does not change significantly with age.

Testosterone, Total, Blood

Normal values

	Male		Female	
	ng/dL	*SI Units nmol/L*	*ng/dL*	*SI Units mmol/L*
Adult	300–1200	10.4–41.6	30–95	1.0–3.3
Prepubertal values				
Cord blood	13–55	0.45–1.91	5–45	0.17–1.56
Premature infant	37–198	1.28–6.87	5–22	0.17–0.76
Newborn	75–400	2.6–13.9	20–64	0.69–2.22
1–5 months	1–177	0.03–6.14	1–5	0.03–0.17
6–11 months	2–7	0.07–0.24	2–5	0.07–0.17
12 months-5 years	2–25	0.07–0.87	2–10	0.07–0.35
6–9 years	3–30	0.10–1.04	2–20	0.07–0.69
Pubertal values				
Tanner stage				
1	2–23	0.07–0.80	2–10	0.07–0.35
2	5–70	0.17–23	5–30	0.17–1.04
3	15–280	0.52–9.72	10–30	0.35–1.04
4	105–545	3.64–18.91	15–40	0.52–1.39
5	265–800	9.19–27.76	10–40	0.35–1.39

STEROIDS

Estriol

Reference Values

Estriol Serum	
	Normal Values in ng/ml
Weeks of pregnancy	
14	0.2–3.0
15	0.2–3.5
16	0.3–4.2
17	0.4–5.2
18	0.4–5.8
19	0.4–6.2
20	0.4–6.8
22	0.4–9.1
24	0.4–9.1
26	1.9–9.5
28	2.2–10.1
30	2.0–10.8
32	2.5–11.3
34	2.2–12.7
36	2.5–25.0
37	3.6–25.3
38	6.6–29.7
39	6.7–25.3
40	7.2–22.9
41	8.8–31.5

Estriol Values are Increased in

Feminizing tumors, true precocious puberty, liver cirrhosis and multiple pregnancy. Drugs include oxytocin.

Estriol Values are Decreased in

Anencephaly, abortion, anemia, choriocarcinoma, diabetes mellitus, erythroblastosis, fetalis, fetal adrenal aplasia, fetal Down syndrome, fetal growth retardation, fetal encephalopathy, gynecomastia, hepatic disease, hemoglobinopathy, hydatidiform mole, intrauterine death, menopause, postmaturity, preeclampsia, and Rh-immunization, drugs include betamethasone, cascara, corticosteroids (large doses), dexamethasone, diuretics, glutethimide, estrogens, mandelamine, meprobamate, penicillins, phenazopyridine, phenophthalein, probenecid and senna.

17-β-ESTRADIOL

Reference Values

The serum or plasma 17-β-estradiol values are comprised in the following intervals:

Women	Follicular phase	30–120 pg/mL
	Ovulatory peak	150–400 pg/mL
	Luteinic phase	70–200 pg/mL
	Menopause	< 60 pg/mL
Men		< 40 pg/mL
Children		< 60 pg/mL

The measurement of estradiol is important for the evaluation of normal sexual development (menarche), causes of infertility (anovulation, amenorrhea, dysmenorrhea), and menopause, normal estradiol levels are lowest at menses and into the early follicular phase and then rise in the late follicular phase to a peak just before the LH surge, initiating ovulation. If conception occurs, estradiol levels continue to rise. At menopause, estradiol levels remain low.

Estradiol is Increased in

Adrenal tumors, cirrhosis, gynecomastia in males, hyperthyroidism, Klinefelter's syndrome, liver tumors, ovarian neoplasm, polycystic ovary syndrome.

Estradiol is Decreased in

Amenorrhea, anorexia nervosa, hypopituitarism, infertility, menopause, osteoporosis, ovarian hypofunction, pituitary disease, and polycystic ovary syndrome.

DHEAS (DEHYDROEPIANDROSTERONE SULFATE)

Reference Values

The serum or plasma dehydroepiandrosterone sulfate values are comprised in the following intervals:

	Women (µg/mL)	*Men (µg/mL)*
Newborns	0.9 – 1.8	0.9–1.8
Before puberty	0.25–1.0	0.25–1.0
Adults	0.9–3.6	0.9–3.6
After menopause	< 0.25–1.0	
Pregnancy	0.25–1.8	

Values are Increased in

Adrenal cortex adenoma and carcinoma, Cushing's disease, ectopic ACTH-producing tumors, female acne and hirsutism, oligomenorrhea in female athletes, polycystic ovarian syndrome, Stein-Levinthal syndrome, and virilizing congenital hyperplasia.

Values are Decreased in

Primary and secondary adrenal insufficiency. Low levels in amniotic fluid indicate anencephaly in the fetus.

Δ4-ANDROSTENEDIONE

Reference Values

The serum or plasma androstenedione values are comprised in the following intervals:

Women	Follicular phase	0.75–2.16 ng/mL
	Luteinic phase	0.94–2.33 ng/mL
Men		0.60–1.85 ng/mL

PROGESTERONE

Normal Values (Units—ng/mL)

Males	0.4–0.9
Females	
Follicular phase	0.40–1.7
Midluteal	4.9–18.8
Postmenopausal	Up to–1.0
On oral contraceptive pills	0.34–0.92
Pregnant females	
18–21 wk	53–76
22–25 wk	60–86
26–29 wk	71–133
30–33 wk	86–142
34–37 wk	104–175
38–41 wk	117–187

Measurement of serum progesterone have also been used to check the effectiveness of ovulation induction, to monitor progesterone replacement therapy and to detect and evaluate patients at risk for abortion during the early weeks of pregnancy, progesterone levels are increased in luteal phase of menstrual cycle, luteal cysts of ovary, ovarian tumors (e.g. arrhenoblastoma) and adrenal tumors. While decreased levels of progesterone are seen in conditions of amenorrhea, threatened abortion and fetal death, toxemia of pregnancy and gonadal agenesis.

Values are Increased in

Adrenal hyperplasia (congenital males), corpus luteum cyst, lipid ovarian tumors, molar pregnancy, ovarian chorionepithelioma, ovarian neoplasms, placental tissue (retained postparturition), precocious puberty and theca lutein cysts. Drugs include adrenocortical hormones, estrogens and progesterones.

Values are Decreased in

Adrenogenital syndrome, amenorrhea, anovular menstruation, fetal abnormality or death, luteal deficiency, menstrual abnormalities, ovarian failure, panhypopituitarism, placental failure or insufficiency, preeclampsia, Stein-Levinthal syndrome, threatened abortion, toxemia of pregnancy, Turner's syndrome, and primary/secondary hypogonadism. Drugs include ampicillin and ethinyl estradiol.

17-ALPHA-HYDROXYPROGESTERONE

Reference Values

The serum or plasma 17αOH progesterone values are comprised in the following intervals:

Women	Follicular phase	0.2–1.2 ng/mL
	Luteinic phase	1.0–4.5 ng/mL
	Menopause	0.2–0.8 ng/mL
Men		0.2–2.3 ng/mL
Children		0.2–0.9 ng/mL

Its measurement is of value in the diagnosis and measurement of congenital adrenal hyperplasia, hirsutism and infertility. Circulating 17 alpha hydroxy progesterone normally exhibits a diurnal variation similar to that of cortisol, with higher values in the morning. Serum measurement has been used in the differential diagnosis of hirsutism and infertility where 21 hydrolase deficiency is suspected. Since late-onset congenital adrenal hyperplasia can sometimes mimic the polycystic ovary syndrome, untreated congenital adrenal hyperplasia in newborn is usually associated with markedly elevated 17 alpha hydroxy progesterone levels ranging from 10 to 400 times the upper limit of the normal.

TOTAL TRI-IODOTHYRONINE (T3)

Expected Values for the T3 EIA Test System

	(in ng/dL)
Expected Ranges (±2 SD)	69–202

Interpretation of Total T3 in ng/mL

Age	*ng/mL*
Adults	0.60–2.3
Children	
Cord blood	0.15–0.75
First 72 hours	0.32–2.16
7–14 days	AVG–2.5
2–14 weeks	1.60–2.40
4–16 weeks	1.17–2.09
16–52 weeks	1.10–2.80
1–5 years	1.05–2.69
5–10 years	0.94–2.41
10–15 years	0.83–2.31

Free Tri-iodothyronine (FT3)

Interpretation

Several drugs are known to effect the binding of tri-iodothyronine to the thyroid hormone carrier proteins or its metabolism to T3 and complicate the interpretation of free T3 results.

Circulating autoantibodies to T3 and hormone-binding inhibitors may interfere.

Heparin has been reported to have in vivo and in vitro effects on free T3 concentration. Therefore, do not obtain samples in which this anticoagulant has been used.

In severe nonthyroidal illness (NTI), the assessment of thyroid status becomes very difficult. TSH measurements are recommended to identify thyroid dysfunction.

Familial dysalbuminemic conditions may yield erroneous results on direct free T3 assays.

"Not Intended for Newborn Screening."

Expected Ranges of Values

Expected Values for the Free T3 EIA Test System (in pg/mL)

	Adult	*Pregnancy*
Expected ranges (±2 SD)	1.4–4.2	1.8–4.2

T-Uptake

Interpretation

The T-uptake test is dependent upon a multiplicity of factors: thyroid gland and its regulation, thyroxine binding globulin (TBG) concentration, and the binding of the thyroid hormones to TBG. Thus, the T-uptake test alone is not sufficient to assess clinical status.

The free thyroxine index (FTI), which is the product of the T-uptake ratio and the total thyroxine concentration, has gained wide clinical acceptance as a more accurate assessment of thyroid status. The FTI value compensates for any condition or drug, such as pregnancy or estrogens, which alters the TBG and the T4 levels but does not change the thyrometabolic status. A table of interfering drugs and conditions which affect the T-uptake test has been compiled by the Journal of the American Association of Clinical Chemists.

Expected Ranges of Values

Expected Values for the T-uptake EIA Test System

Thyroid Status	*Percent T-uptake*	*T-ratio*
Euthyroid	25–35	0.83–1.17
Hypothyroid or TBG (Excess binding)	less than 25	less than 0.83
Hyperthyroid or TBG (Reduced binding)	greater than 35	greater than 1.17

Total Thyroxine (T4)

Interpretation

Total serum thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroxine-binding globulin (TBG) concentration, and the binding of thyroxine to TBG. Thus, total thyroxine concentration alone is not sufficient to assess clinical status.

Total serum thyroxine values may be elevated under conditions, such as pregnancy or administration of oral contraceptives. A T3 uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated T4 is caused by TBG variation.

A decrease in total thyroxine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphenylhydantoin or salicylates.

"Not intended for newborn screening."

Expected Range of Values

Expected Values for the T4 EIA Test System (in µg/dL)

	Male	*Female**
Expected Ranges	(±2 SD) 4.4–10.8	4.8–11.6

*Normal patients with high TBG levels were not excluded except if pregnant

Normal Values (µg/dL)

Age	
Adults	5.0–12.0
Pregnant > 14 weeks	9.1–14.0
Elderly (> 60 years)	
Female	5.5–10.5
Male	5.0–10.0
Children	
Cord blood	
First 72 hours	7.4–13.0
7–14 days	11.8–22.6
4–16 weeks	9.8–16.6
4–12 months	7.2–14.4
1–5 years	7.8–16.5
5–10 years	7.3–15.0
6.4–13.3	
10–15 years	5.6–11.7
Panic levels	
Thyroid storm possible	> 20.0
Myxedema possible	< 2.0

Free Thyroxine (FT4)

Interpretation

Total serum thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroxine-binding globulin (TBG) concentration, and the binding of thyroxine to TBG. Thus, total thyroxine concentration alone is not sufficient to assess clinical status.

Total serum thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives. A T3 uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated T4 is caused by TBG variation.

A decrease in total thyroxine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphenylhydantoin or salicylates.

"Not intended for newborn screening."

Expected Range of Values

Expected values for the free T4 EIA test system (in ng/dL)

	Adult	*Pregnancy*
Expected Ranges (±2S. D.)	0.8-2.0	0.8-2.2

Thyrotropin (TSH)

Interpretation

Serum thyrotropin concentration is dependent upon a multiplicity of factors: hypothalamus gland function, thyroid gland function, and the responsiveness of pituitary to TRH. Thus, thyrotropin concentration alone is not sufficient to assess clinical status.

Serum thyrotropin values may be elevated by pharmacological intervention. Domperiodone, amiodazon, iodide, phenobarbital, and phenytoin have been reported to increase TSH levels.

A decrease in thyrotropin values has been reported with the administration of propranolol, methimazol, dopamine and d-thyroxine.

Genetic variations or degradation of intact TSH into subunits may affect the binding characteristics of the antibodies and influence the final result. Such samples normally exhibit different results among various assay systems due to the reactivity of the antibodies involved.

"Not intended for newborn screening."

Expected Ranges of Values

Expected values for the TSH IEMA test system

	(in µIU/ mL)
Low normal range	0.39
High normal range	6.16

Normal Values (µIU/ mL)

Age	
Adults/infants/children	0.3–6.2
Adults > age 80 years	Up to 10.0
Newborn by day 3	< 20.0
Newborn by day 10	< 10.0
Newborn by day 14	< 6.2

Example of Chemiluminescence Immunoassay Method—TSH Estimation Thyroglobulin Antibodies

Interpretation

The presence of autoantibodies to Tg is confirmed when the serum level exceeds 125 lU/mL. The clinical significance of the result, coupled with antithyroid peroxidase activity, should be used in evaluating the thyroid condition. However, clinical inferences should not be solely based on this test but rather as an adjunct to the clinical manifestations of the patient and other relevant tests.

The cost benefits should be considered in the use of thyroglobulin antibodies testing when performed in concent with antithyroid peroxidase (TPO). The widespread practice of performing both tests has been questioned.

Expected Range of Values

Expected values for the anti-Tg ELISA test system

	(in IU/mL)
Upper 95% (+2*) level	124.7

Thyroid Peroxidase Antibodies

Interpretation

The presence of autoantibodies to TPO is confirmed when the serum level exceeds 40 IU/mL. The clinical significance of the result, coupled with antithyroglobulin activity, should be used in evaluating the thyroid condition. However, clinical inferences should not be solely based on this test but rather as an adjunct to the clinical manifestations of the patient and other relevant tests.

Expected Range of Values

Expected values for the anti-TPO ELISA test system

	(in IU/mL)
Upper 95% (+2a) level	39.2

Luteinizing Hormone (LH)

Interpretation

LH is suppressed by estrogen; but in women taking oral contraceptives, the level may be low or normal. Excessive dieting and weight loss may lead to low gonadotropin concentrations.

Luteinizing hormone is dependent upon diverse factors other than pituitary homeostasis. Thus, the-determination alone is not sufficient to assess clinical status.

Expected Range of Values

Expected values for the LH IEMA test system (in mIU/mL) (IRP 68/40)

Women	Follicular phase	0.8–10.5
	Midcycle	18.4–61.2
	Luteal phase	0.8–10.5
	Postmenopausal	8.2–40.8
Men		0.7–7.4

Usage

To evaluate infertility in women and men (high serum values are related to gonadal dysfunction, and low values of LH are related to dysfunction or failure of the hypothalamus or pituitary gland) to evaluate hormonal therapy for inducing ovulation and to evaluate endocrine problems related to precocious puberty in children. The results of LH assay are shown in Figure 24.24.

Values are Increased in

Amenorrhea, endocrine, problems related to precocious puberty in children, hyperpituitarism, Klinefelter's syndrome, liver disease, menopause, menstruation, ovarian or testicular failure (primary gonadal dysfunction) Stein-Levinthal syndrome (polycystic ovarian disease), tumor (pituitary, testicular), and Turner's syndrome (ovarian dysgenesis). Drugs includeanticonvulsants, clomiphene, naloxone, and spironolactone.

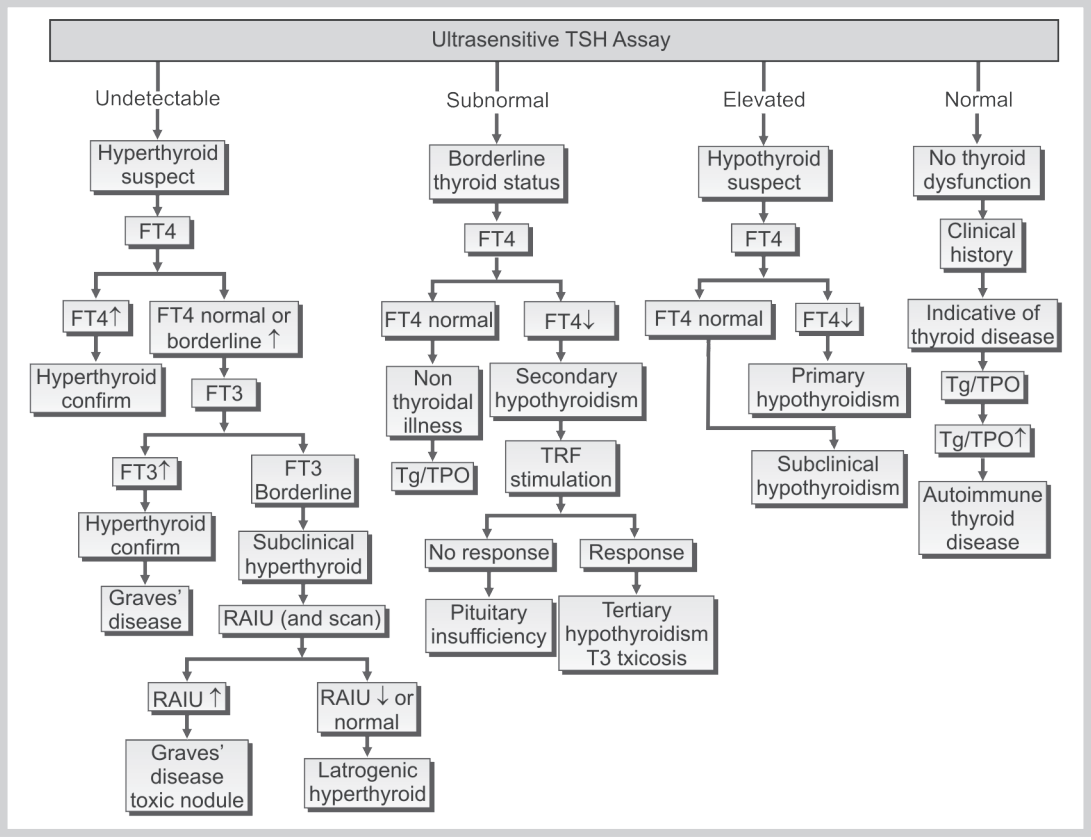


FIG. 24.24: Ultrasensitive TSH assay

Values are Decreased in

Adrenal hyperplasia or tumor, amenorrhea, (pituitary failure, secondary gonadal insufficiency), anorexia nervosa, anovulation, hypophysectomy, hypopituitarism, hypothalamic disorder, malnutrition, pituitary disorder, and testicular failure (related to pituitary failure). Drugs include digoxin, estrogen compounds, oral contraceptives, phenothiazines, progesterone, stanozlol, and testosterone administration.

Follicle Stimulating Hormone (FSH)

Interpretation

The FSH is suppressed by estrogen, but in women taking oral contraceptives, the level may be low or normal. Excessive dieting and weight loss may lead to low gonadotropin concentrations.

Follicle-stimulating hormone is dependent upon diverse factors other than pituitary homeostasis. Thus, the determination alone is not sufficient to assess clinical status.

Normal Values in mIU/mL

Sex/phase	
Males	1.0–14.0
Females:	
Follicular phase	3.0–12.0
Midcycle	8.0–22.0
Luteal phase	2.0–12.0
On oral contraceptives	Up to 3.0
Postmenopausal	35.0–151.0

Values are Increased in

Acromegaly (early), amenorrhea (primary), anorchism, castration, gonadal failure, hyperpituitarism, hypogonadism, hypothalamic tumor, hysterectomy, Klinefelter's ovarian failure, pituitary tumors, precocious puberty, premature menopause, seminiferous tubule failure, seminoma, Stein-Levinthal syndrome (polycystic ovary syndrome), testicular agenesis, testicular destruction (due to radiation or mumps

orchitis), testicular failure, testicular feminization syndrome (complete), and Turner's syndrome (primary hypogonadism).

Values are Decreased in

Adrenal hyperplasia, amenorrhea (secondary), anorexia nervosa, anovulatory menstrual cycle, delayed puberty, hypogonadotropism, hypophysectomy, hypothalamic dysfunction, neoplasm (adrenal, ovarian, testicular), panhypopituitarism, and prepubertal child. Drugs include chlorpromazine, estrogens, oral contraceptives, progesterone, and testosterone.

Prolactin Hormone (PRL)

Interpretation

Patient specimens with abnormally high prolactin levels can cause a hook effect, that is, paradoxical low absorbance results. If this is suspected, dilute the specimen 1/100 with 0 calibrator, reassay (multiply the result by 100). However, values as high as 3000 ng/mL have been found to absorb greater than the absorbance of the highest calibrator.

Patients receiving preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human antimouse antibodies (HAMA) and may show either falsely elevated or depressed values when assayed.

Pregnancy, lactation, and the administration of oral contraceptives can cause an increase in the level of prolactin.

Drugs such as morphine, reserpine and the psychotropic drugs and domperidone, etc. increase prolactin secretion.

Since prolactin hormone concentration is dependent upon diverse factors other than pituitary homeostasis, the determination alone is not sufficient to assess clinical status.

Expected Range of Values

Normal range of HPRL in ng/mL

Age/sex/phase	
Newborn	> 250
Adult male	< 20
Adult female, nonlactating	< 25
Follicular phase	< 28
Luteal phase	5–40
Postmenopausal	< 12
Pregnancy	
Trimester 1	< 80
Trimester 2	< 160
Trimester 3	< 400
Pituitary tumor	> 100

HPRL Levels are Increased in

Acromegaly, Addison's disease, amenorrhea, anorexia nervosa, breast stimulation, bronchogenic carcinoma, Chiari-Frommel syndrome, coitus, Del Castillo's syndrome, ectopic tumors, endometriosis, exercise.

Forbes-Albright syndrome, galactorrhea, hyperestrogen states, hyperpituitarism, hypothalamic disorders, hypothyroidism (primary), hysterectomy, idiopathic causes (e.g. early micro-adenoma that are undetectable by radiology), impotence, lactation, Nelson's syndrome, neurogenic causes, pituitary tumors, polycystic ovaries, pregnancy. Chronic renal failure, sleep and stress, drugs include amitryptiline, amoxapine, amphetamines, benzamides, chlorprothixine, desipramine, doxepin, droperidol, estrogens, haloperidol, imipramine, isoniazid, maprotiline, meprobamate, methyldopa, metoclopramide, nortriptyline, opiates, oral contraceptives, phenothiazines, procainamide hydrochloride, protriptyline, reserpine, thioridazine, thiothixene, thyrotropin, triavli, and trimipramine maleate and gastric intestinal prolinetic drugs.

HPRL Levels are Decreased in

Gynecomastia, hirsutism, osteoporosis, and pituitary necrosis/infarction. Drugs include apomorphine hydrochloride, clonidine, bromocripine mesylate, dihydroergotamine, mesylate, dopamine, ergonovine maleate, ergotamine tartarate, ergoloid mesylate, lergotrile, levodopa, and lisuride hydrogen maleate.

Human Chorionic Gonadotropin (hCG)

Interpretation

False positive results may occur in the presence of a wide variety of trophoblastic and nontrophoblastic tumors that secrete hCG. Therefore, the possibility of an hCG secreting neoplasia should be eliminated prior to diagnosing pregnancy.

Also, false positive results may be seen when assaying specimens from individuals taking the drugs Pergonal and Clomid. Additionally, Pergonal will often be followed with an injection of hCG.

Spontaneous microabortions and ectopic pregnancies will tend to have values which are lower than expected during a normal pregnancy, while somewhat higher values are often seen in multiple pregnancies.

Following therapeutic abortion, detectable hCG may persist for as long as 3 to 4 weeks. The disappearance rate of hCG, after spontaneous abortion, will vary depending upon the quantity of viable residual trophoblast.

Normal Values in mIU/mL

Males	< 5.0
Females:	
Nonpregnant	< 20.0
< 1 week gestation	Up to 50.0
2 weeks gestation	50–500
3 weeks gestation	100–10,000
4 weeks gestation	1000–30,000
5 weeks gestation	3,500–115,000
6-8 weeks gestation	12,000–270,000
12 weeks gestation	15,000–220,000

Values are Increased in

Choriocarcinoma, eclampsia, ectopic pregnancy, erythroblastosis fetalis, germ cell tumors, gynecomastia, hydatidiform mole, insulinoma, neoplasms (colon, lung, pancreas, stomach), ovarian cancer, pregnancy, seminoma, and testicular cancers and possibly bladder cancer.

Values are Decreased in Abortion and Ectopic Pregnancy

The hCG testing may help differentiate actual pregnancy from an ectopic pregnancy in conjunction with an ultrasound.

Avoid medications such as anticonvulsants, antiparkinsonism agents, hypnotics, tranquilizers, which may cause a false positive result.

Factors that Affect Results

- False positive results may be due to incorrect handling of the test sample, excessive production of luteinizing hormone (LH) of the pituitary gland, absence of gonadal hormones in menopausal women or hCG producing tumors
- False negative results may be due to the test being performed too early in pregnancy.

Other Data

Although not usually present in healthy males or nonpregnancy females, elevated levels of hCG may be detected in patients with certain malignant tumors.

CIA™ INSULIN (Chemiluminescence Immunoassay)

(Courtesy: Lilac Medicare)

Insulin Microplate CIA

Intended Use: Monobind insulin microplate CIA test is intended to be used for the quantitative determination of insulin levels in human serum. The test is for in vitro diagnostic use only.

Summary and Explanation of the Test

Human insulin is a peptide produced in the beta cells of the pancreas and is responsible for the metabolism and storage of carbohydrates. As a result of biofeedback the insulin levels increase with intake of sugars and decline when sugar content is low for absorption. In the diabetic population the mechanism of insulin production is impaired because of genetic predispositions (***Type I***) or because of lifestyle and/or hereditary factors (***Type II***). In such cases either the insulin production has to be boosted by medication or it has to be supplemented by oral or intravenous methods. The quantitative determination of insulin can help in dose selection the patient has to be subjected to.

On the other hand the circulatory insulin can be found at much higher levels like in patients with pancreatic tumors. These tumors secrete abnormally high levels of insulin and thus cause hypoglycemia. Accordingly, fasting hypoglycemia associated with inappropriately high concentrations of insulin strongly suggests an islet-cell tumor (insulinoma). To distinguish insulinomas from factitious hypoglycemia due to insulin administration, serum ***C***-peptide values are recommended. These insulinomas can be localized by provocative intravenous doses of tolbutamide and calcium.

Principle

Immunoenzymometric Assay

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin-coated on the well and exogenously added biotinylated monoclonal anti-insulin antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing

the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$EnzAb_{(p)} + Ag_{Ins.} + {}^{Btn}Ab_{(m)} \underset{K_a}{\overset{K_a}{\rightleftharpoons}} EnzAb_{(p)}\text{-}Ag_{TSH}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btn}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

$Ag_{INS.}$ = Native Antigen (Variable Quantity)

$EnzAb_{(p)}$ = Enzyme labeled Monoclonal Antibody (Excess Quantity)

$EnzAb_{(p)}\text{-}Ag_{INS.}\text{-}{}^{Btn}Ab_{(m)}$ = Antigen Antibodies Sandwich Complex

K_a = Rate Constant of Association

K_a = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$EnzAb_{(p)}\text{-}Ag_{INS.}\text{-}{}^{Btn}Ab_{(m)} + Streptavidin_{C.W.} \Rightarrow$$

$$\Rightarrow \text{immobilized complex}$$

$Streptavidin_{C.W.}$ = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the solid surface.

After equilibrium is attained, the antibody bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibodybound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Expected Values

Insulin values are consistently higher in plasma than in serum; thus, serum is preferred. Compared with fasting values in non-obese non-diabetic individuals, insulin levels are higher in obese non-diabetic subjects and lower in trained athletes. Although proinsulin cross reacts with most competitive insulin assays, there is virtually less than 0.01% cross reaction found with proinsulin using *Monobind Insulin Microwell CIA*.

Each laboratory is advised to establish its own ranges for normal and abnormal populations. These ranges are always dependent upon locale, population, laboratory, technique and specificity of the method.

Based on the clinical data gathered by Monobind in concordance with the published literature the following ranges have been assigned. These ranges should be used as guidelines only:

Children < 12 years	< 10 µIU/mL
Adult (Normal)	0.7 – 9.0 µU/mL
Diabetic (Type II)	0.7 – 25 µIU/mL

C-Peptide

Expected Values

C-peptide values are consistently higher in plasma than in serum; thus, serum is preferred. Compared with fasting values in non-obese non-diabetic individuals, C-peptide levels are higher in obese nondiabetic subjects and lower in trained athletes.

Anti-insulin

Clinical Relevance

Type 1 Diabetes is mainly characterized by limited or fully missing secretion of the hormone insulin. Morphological studies demonstrated a destruction of the beta cells of the so-called Langerhans' Islet Cells in Type 1 diabetics. Numerous researchers described the appearance of antibodies directed against the islet cells and insulin as the causal reason for the onset of the disease.

Anti-insulin antibodies are found in 37% of patients with newly detected Type I Diabetes, in 4% of their relatives of the first degree and in up to 1-5% of healthy controls. A positive correlation between the appearance of anti-insulin and anti-islet cell antibodies has been reported.

Anti-insulin autoantibodies may be detected several months and in some cases years before the onset of the fully clinical manifestation of the diseases. Occasionally also autoantibodies to pro-insulin may appear.

These "true" anti-insulin autoantibodies directed against endogenous insulin have to be distinguished from those autoantibodies which are developed in insulin-dependent diabetics undergoing therapy with insulin preparations of animal origin. In fact, the latter have to be referred to side effects. These side effects may occur as local reactions of the skin by development of insulin-specific autoantibodies. These autoantibodies are causing the formation of an insulin depot and they may simulate a resistance against the hormonal treatment with animal insulin.

Autoantibody specificity	*type I diabetics (%)*	*healthy controls (%)*
Anti-islet cell antibodies	32	1
Antibodies against islet cell surface antigens approx.	50	2
Anti-insulin antibodies	up to 70	0
Anti-thyroid peroxidase ab's (Anti-TPO)	18	6
Anti-single-stranded		
DNA ab's (Anti-ssDNA)	85	9

Additionally, other immunological phenomena have been reported for Type I diabetics. A lot of other autoantibody specificities have been d etected in those patients too, but these antibodies must not cause additional autoimmune phenomenon.

Indications

- Anti-insulin antibodies in Type I diabetics
- Development of anti-insulin antibodies under insulin therapy.

Normal Values

In a normal range study with serum samples from healthy blood donors, the following ranges have been established with the anti-insulin test:

Anti-insulin	*[U/mL]*
Normal	< 5
Borderline	5–10
Elevated	> 10

Positive results should be verified concerning the entire clinical status of the patient. Also, every decision for therapy should be taken individually.

CHAPTER 25

Histopathology

PREPARATION OF TISSUES

Fixation

This is the process of killing and hardening. The first phase of fixation is the rapid killing; the second phase, the hardening of tissue. After removal, the tissue should be put in the fixative immediately. The choice of a fixing agent should be determined by the purpose of which the tissue is to be stained or preserved. If several special stains may be required, small blocks of the tissue should be fixed in each of the following: 10% neutral formalin, Zenker's fluid, Bouin's fluid and absolute alcohol or Carnoy's fluid.

Blocks should be cut thin enough so that the fixing fluid will penetrate the tissue in a reasonably short time. To do this blocks should not be more than 0.5 cm thick and should be immersed in at least 20 times their volumes of fixative.

Ten percent formalin is the most widely used fixative because it is compatible with most stains. Length of fixation depends on the size of blocks.

I. *10% Formalin solution*

37–40% Formaldehyde	100 cc
Tap water	900 cc

II. *Buffered neutral formalin solution*

37–40% Formaldehyde	100 cc
Distilled water	900 cc
Sodium phosphate monobasic	4 g
Sodium phosphate dibasic	6.5 g

III. *Zenker's fluid*

Distilled water	1000 cc
Mercuric chloride	50 g
Potassium dichromate	25 g
Sodium sulfate	10 g

Add 5 cc of glacial acetic acid to 95 cc of Zenker's fluid before use.

IV. *Lugol's solution* (Weigert's Modification)

Potassium iodide	2 g
Iodine	1 g
Distilled water	100 cc

V. *Bouin's fluid*

Picric acid, saturated aqueous solution	750 cc
37–40% Formaldehyde	250 cc
Glacial acetic acid	50 cc

VI. *Carnoy's fluid*

Absolute alcohol	60 cc
Chloroform	30 cc
Acetic acid glacial	10 cc

VII. *Absolute alcohol and acetone are also used as fixatives for bacteria, glycogen, and some of the enzymes.*

Decalcification

Bone and calcified tissue should be cut into small pieces with a saw before fixation. After they are thoroughly fixed, they are placed in a gauze bag tied with a string, which has been dipped in melted paraffin. The bag is suspended in a large quantity of decalcifying solution, at least a quart for blocks of the average size. Stirring or agitation of the fluid hastens decalcification. Every trace of decalcifying solution must be removed by washing the pieces in running water for several hours before dehydration and embedding.

Nitric Acid Method

1. Decalcify sections in large quantities of 5% aqueous solution of nitric acid for 1 to 4 days. Change the solution daily. Sections of bone may be tested by bending, piercing with a sharp needle or X-ray.

2. Wash in running water for 24 hours.
3. Neutralize in 10% formalin to which an excess of calcium or magnesium carbonate has been added.
4. Wash in running water for 24 to 48 hours.
5. Dehydrate, clear and embed in either paraffin or celloidin.

The method is used only for small pieces of bone, which must be processed rapidly. Exposure of unduly long length to nitric acid impairs or destroys nuclear staining.

Formic Acid Sodium Citrate Method

1. Decalcify for 5–14 days in formic acid-sodium citrate solution. Change solution daily for best results.

   Solution A

Sodium citrate	50 g
Distilled water	250 cc

   Solution B

Formic acid (90%)	125 cc
Distilled water	125 cc

   Mix solutions A and B for use
2. Wash in running water for 4 to 8 hours.
3. Dehydrate, clear and embed.

This technique gives better staining quality than the nitric acid method.

Electrolytic Method

1. Decalcify in the electrolytic apparatus in formic acid-hydrochloric acid for 1 to 4 hours.

   Electrolytic decalcifying solution

Formic acid (90%)	100 cc
Hydrochloric acid	80 cc
Distilled water to make	1000 cc
2. Wash in running water for 24 hours.
3. Dehydrate, clear, and embed.

Processing of Tissues

Embedding in paraffin is accomplished most rapidly and gives the best results when thin sections of soft tissues are wanted. Since paraffin is not miscible with water, the tissue must be dehydrated and then cleared in a solution that is miscible with paraffin.

Automatic tissue processing units are usually employed these days. Several manufacturers provide such units (Fig. 25.1). This instrument has several beakers, each filled with a specific fluid in it. The basket containing the tissue sections with identification tags is automatically dipped and revolved in the beakers at preset timings. Usually, paraffin-embedding process is started in the afternoon and is complete by mornings on the following day. Various schedules for paraffin processing are given below.

FIG. 25.1: Automatic tissue processing unit (*Courtesy:* Yorco Sales Pvt. Ltd)

Method I

1.	Alcohol 80%	1–2 hours
2.	Alcohol 95%—2 changes	1–2 hours each
3.	Alcohol absolute—3 changes	1–2 hours each
4.	Xylene—2 changes	1–2 hours each
5.	Melted paraffin—3 changes	1–2 hours each
6.	Embed in paraffin and cool quickly.	

Method II

1.	Alcohol 80%—2 changes	1–2 hours each
2.	Alcohol 95%—2 changes	1–2 hours each
3.	Alcohol, absolute—3 changes	1–3 hours each
4.	Chloroform—2 changes	1–2 hours each
5.	Paraffin, melted—3 changes	1 hour each
6.	Embed and cool quickly.	

Method III

1.	Alcohol 80%—2 changes	1–2 hours each
2.	Alcohol 95%—2 changes	1–2 hours each
3.	Alcohol, absolute—3 changes	1–2 hours each
4.	Benzene—1 change	1–2 hours each
5.	Paraffin bath—3 changes	1 hour each
6.	Embed and cool immediately	

(In place of alcohol, one may use acetone. Xylene and benzene can be used in place of one another. Benzene, however, is carcinogenic and should be avoided).

Preparation of Sections

It is important that the knife used for cutting sections be very sharp and without nicks. A perfect edge for a microtome knife may be defined in simple terms as the junction of two smooth plane surfaces at an angle of about 14°. Knife sharpening may be done by mechanical means on commercial automatic knife sharpeners or done manually called honing and stropping. Nowadays disposable knife blades with appropriate blade holders are available.

Cutting Sections

After the paraffin block has been secured at the appropriate place in the microtome, adjustment of the block and the knife is now required. Keep a piece of cotton in a dish of tap water and an ice cube in a petridish beside the microtome (Fig. 25.2) at all times.

To facilitate sectioning, apply the wet cotton to the surface of the block after rough cutting. Then place the ice cube directly on the knife to flatten the side of the cube that is to be applied to the surface of the block. Be sure to crank the block back a fraction of a millimeter from the knife edge, so that the first section cut after the block has been soaked and chilled will not be too thick.

Collect the section ribbons in a bowl containing hot water and unfurl or straighten the sections gently with a fine tip camel's hair brush.

Attaching Sections to Slides

The glass slides on which tissue sections are to be mounted must be marked beforehand with the identifying case number. A glass marking pencil is used for this purpose.

Paraffin sections may be attached to slides in several ways. A small drop of Mayer's egg albumin is smeared over the surface of the slide with the finger and the excess rubbed off with the heel of the hand, or it can be applied with a clean foam rubber sponge. A sponge is usually preferred so that the epithelial cells from the fingers will not adhere to the slide and produce artefacts when stained.

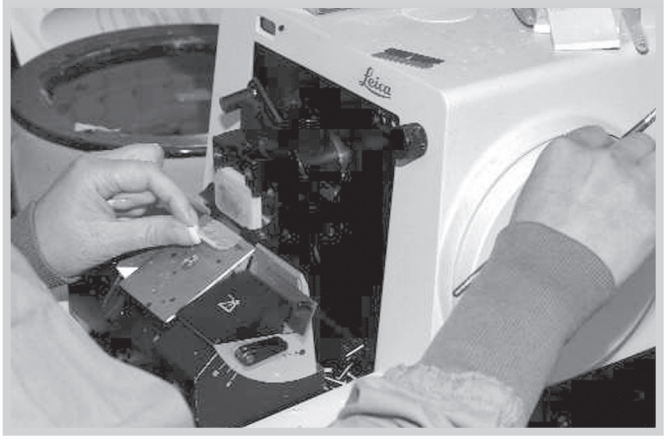


FIG. 25.2: Rotary microtome
(*Courtesy:* Yorco Sales Pvt. Ltd)

Mayer's Egg Albumin

Egg white	50 cc
Glycerin	50 cc

Mix well and filter through coarse filter paper, or through several thicknesses of gauze. Add a crystal of thymol to preserve.

Egg Adhesive from Dried Albumin

Albumin, dried	5.0 g
Sodium chloride	0.5 g
Distilled water	100.0 cc

Filter on Buchner's funnel with vacuum. To 50 cc of filtrate, and 50 cc glycerin, add a crystal of thymol to preserve.

Slides smeared by one of the above-mentioned egg adhesives are taken below the floating section and the section is placed in the center of the slide; leave it to dry to be stained subsequently.

Technique for Frozen Sections

1. Fix small blocks of tissue in 10% formalin.
2. Wash blocks in water before freezing.
3. Put a drop of water on the holder and place the block in position parallel to the knife edge.
4. Holding the block with the index finger or a glass slide, turn on the coolant gas/fluid slightly. When block is firmly fixed to holder, release more coolant gas/fluid until the block is frozen.
5. Start sectioning and continue until a complete section is obtained. Usually, the block will have thawed to about right consistency by this time. If it is frozen too hard, the sections may shatter. Allow the block to thaw slightly and try again. If it has become too soft, the sections will also shatter or fracture. The correct temperature can only be judged by experience. Sometimes rubbing the finger across the block will give it the right consistency for good sections to cut. It is best to cut slowly.
6. Lift the sections off the knife edge with the tip of the little or ring finger which has been dipped in distilled water. Place in a Petri dish of distilled water. Dry the knife between sections as water will cause the following section to be uneven or perforated.
7. Frozen sections may be stained with polychrome methylene blue, hematoxylin and eosin, or fat stains.

Staining

Staining may be done by the free flotation method or the sections may be picked up on the slide first and stained as usual.

Mounting

Fat stains must be mounted in glycerin jelly. Sections stained by other techniques may also be mounted in glycerin jelly or they may be dehydrated, cleared in xylene and mounted in DPX mountant.

Removal of Pigments and Precipitates

Mercury Precipitate

If Zenker-fixed material stored for a long-time is to be stained with alum hematoxylin, it will be found that areas where the mercuric chloride is deposited stain deep blue and distort the microscopic picture. Therefore, it is necessary to:

1. Deparaffinize the sections by putting them on a hot plate and then take through two changes of xylene, absolute alcohol and 95% alcohol.
2. Place in alcoholic iodine (1 g iodine in 100 cc of 80% alcohol) for 10 to 15 minutes.
3. Rinse in tap water.
4. Place in 5% aqueous sodium thiosulfate solution (hypo) for 5 minutes.
5. Wash in running tap water for 10 to 20 minutes and rinse well in distilled water before staining.

Formalin Produced Precipitate

Method I

1. Deparaffinize the sections through two changes each of xylene, absolute alcohol, and 95% alcohol.
2. Rinse well in distilled water.
3. Let stand in saturated aqueous picric acid solution for 1 to 3 hours.
4. Wash well in running tap water.

Note

This picric acid solution will not bleach malarial pigment.

Method II

1. Deparaffinize the sections through two changes each of xylene, absolute alcohol and 95% alcohol.
2. Rinse well in distilled water.
3. Place in bleaching solution for 5–10 minutes.

Bleaching solution

Hydrogen peroxide	25.0 cc
Acetone	25.0 cc
Ammonium hydroxide	1 drop.

4. Wash well in running tap water, and stain as desired.

Melanin Pigment

1. Deparaffinize the sections through two changes each of xylene, absolute alcohol and 95% alcohol.
2. Rinse well in distilled water.
3. Place in 0.25% aqueous potassium permanganate solution for 1 to 4 hours.
4. Wash well in water.
5. Place in a 5% aqueous oxalic acid solution or a hydrobromic acid solution (HBr 1 part, distilled water 3 parts) until sections are clear (2–5 minutes).
6. Wash in running tap water for 10 minutes, rinse in distilled water, and stain as desired.

Malarial Pigment

Method I

1. Deparaffinize the sections through two changes each of xylene, absolute alcohol, and 95% alcohol.
2. Wash well in distilled water.
3. Place in a 5% aqueous solution of ammonium sulfide for 20–24 hours.
4. Wash in running tap water for 15–20 minutes, rinse well in distilled water and stain as desired.

Method II

1. Deparaffinize the sections through two changes each of xylene, absolute alcohol, and 95% alcohol.
2. Place in a saturated alcoholic picric acid solution or 1 to 24 hours.
3. Wash well in running tap water and distilled water, and stain as desired.

Method III

1. Deparaffinize the sections through two changes each of xylene, absolute alcohol, and 95% alcohol.
2. Rinse well in distilled water.
3. Place in the following bleaching solution for 5 minutes or less.

   Bleaching solution

Acetone	50 cc
Hydrogen peroxide (3%)	50 cc
Ammonia water (28%)	10 cc

4. Wash well in running tap water and distilled water, and stain as desired.

ROUTINE STAINING PROCEDURES

Hematoxylin, a natural dye, is the most important staining reagent used in histologic work. Used alone, it has little affinity for tissue; but in combination with (mordants) aluminum, iron, chromium, copper or tungsten salts, it is a powerful nuclear stain and a chromatin stain. The active coloring agent, hematein, is formed by the oxidation of hematoxylin. This process, known as "ripening", takes several days or weeks unless it is hastened by the addition of an oxidizing

agent, such as mercuric oxide, hydrogen peroxide, potassium permanganate, sodium perborate or sodium iodate.

Most commonly used hematoxylins are used in combination with aluminum in the form of alum. Various formulations used go by the following names, e.g. Harris, Mayer, Delafield, Ehrlich, Bullard and Bohmer.

Section stained with hematoxylins may be counterstained with eosin, Congo red, eosinol, safranin or other contrasting media.

There are two methods of staining when hematoxylin is used: progressive and regressive.

Progressive: The hematoxylin contains an excess of aluminum salts or acid, thus increasing the selectivity for nuclei. After staining with hematoxylin, the slides are washed well in water and counterstained.

Regressive: This is accomplished by overstaining in a relatively neutral solution of hematoxylin, then removing the stain from the other constituents with acid alcohol or other differentiating agent.

Commonly Employed Hematoxylins

Harris's Alum Hematoxylin

Hematoxylin crystals	5.0 g
Alcohol, absolute	50.0 g
Ammonium or potassium alum	100.0 g
Distilled water	1000.0 cc
Mercuric oxide (red)	2.5 g

Dissolve the hematoxylin in the alcohol, the alum in water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as rapidly as possible. Remove from the heat and add the mercuric oxide slowly. Reheat until it becomes dark purple, remove from flame immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Addition of 2 to 4 cc of glacial acetic acid per 100 cc of solution increases the precision of the nuclear stain. Filter before use.

Mayer's Hematoxylin

Hematoxylin	1 g
Distilled water	1000 cc
Sodium iodate	0.2 g
Ammonium or potassium alum	50 g
Citric acid	1 g
Chloral hydrate	50 g

Dissolve the hematoxylin in water, using gentle heat if necessary. Then add the sodium iodate, then the alum. Shake until the alum is dissolved, then add the citric acid, and finally the chloral hydrate. The final color of the stain is reddish-violet. Stain will keep well.

Counterstains for Hematoxylin Stains

Stock 1% Aqueous Eosin Solution

Eosin Y, water soluble	10 g
Distilled water	1000 cc
Dissolve and add:	
Glacial acetic acid	2 cc

Stock 1% Alcoholic Eosin Solution

Eosin Y, water soluble	1 g
Distilled water	20 cc
Dissolve and add:	
Alcohol, 95%	80 cc

Working Eosin Solution

Eosin, 1% stock solution	1 part
Alcohol, 80%	3 parts

If deeper shade of red is desired in staining, add 0.5 cc of glacial acetic acid to each 100 cc of stain.

Routine Hematoxylin and Eosin Stain

Fixation: May be used after any fixation.

Technique:

- Paraffin or frozen
- Cut paraffin sections at 6 microns.

Solution

1. Harris's hematoxylin
2. Acid alcohol

70% alcohol	1000 cc
Hydrochloric acid, concentrated	10 cc

3. Ammonia water

Tap water	1000 cc
Strong ammonia water	2 to 3 cc

4. Saturated lithium carbonate solution

Lithium carbonate to saturate	
Distilled water	

5. Alcoholic eosin solution
6. Lugol's solution (Weigert's modification)

Potassium iodide	2 g
Iodine crystals	1 g
Distilled water	100 g

7. Alcohol iodine solution

Iodine crystals	1 g
Alcohol, 95%	100 cc

8. Sodium thiosulfate solution (Hypo)

Sodium thiosulfate	5 g
Distilled water	100 cc

Staining Procedure

1. Xylene, absolute alcohol, 95% alcohol.
2. If sections are Zenker's fixed, treat with either Lugol's solution or 1% alcoholic iodine solution 10–15 minutes. Wash with tap water. Treat with 5% sodium thiosulfate 5 minutes. Wash well with tap water.
3. Harris's hematoxylin for 15 minutes.
4. Rinse in tap water.
5. Differentiate in acid alcohol—3 to 10 quick dips. Check the differentiation with the microscope—nuclei should be distinct and the background very light or colorless.
6. Wash in tap water very briefly.
7. Dip in ammonia water (for 10–20 seconds) saturated lithium carbonate solution until sections are bright blue.
8. Wash in running tap water for 10–20 minutes.
9. Stain with eosin for 15 seconds to 2 minutes depending on the age of the eosin and the depth of counterstain required.
10. 95% alcohol.
11. Absolute alcohol—at least two changes.
12. Xylene—two changes.
13. Mount in DPX mountant.

Results

Nuclei—blue

Cytoplasm—pink.

Special Stains

Stains for Connective Tissue

a. Mallory's Phosphotungstic Acid Hematoxylin Stain

Discussed in detail later.

b. Mallory's Phosphomolybdic Acid Hematoxylin Stain

Results:

Collagen fibers—deep blue.

c. Heidenhain's Iron Hematoxylin Stain

Results:

Chromatin, nucleoli, mitochondria, and parts of striated muscle fibers are stained black. Other tissue elements are stained by contrast stain used. Demonstrates amoeba.

d. Mallory's Aniline Blue Collagen Stain

Results:

Nuclei—red

Ground substance of cartilage, mucin and amyloid—varying shades of blue

Erythrocytes and myelin—yellow

Elastic fibrils—pale pink, pale yellow or unstained.

e. Lee-Brown's Modification of Mallory's Aniline Blue Stain

Results:

Nuclei—orange

Collagen—blue

Glomerular basement membrane of kidney—deep blue.

f. Van Gieson's Stain for Collagen Fibers

Discussed in detail later.

g. Barbeito-Lopez Trichrome Stain

Results:

Nuclei—violet red.

Cytoplasm—green to pale blue

Reticulum fibers—deep blue

Collagen—brilliant green

RBCs—brilliant orange

Bacteria—violet red.

h. Gomori's One-Step Trichrome Stain

Results:

Muscle fibers—red

Collagen—green

Nuclei—blue to black

Striations of muscle are easily demonstrable.

i. Heidenhain's Aniline Blue Stain

Results:

Chromatin, osteocytes and neuroglia—red

Cytoplasm—pink to blue

Collagen and reticulum—blue

Muscle—red to yellow.

j. Gallego's Iron Fuchsin Stain

Results:

Nuclei—gray to black

Collagen and reticulum—deep blue

Muscle—greenish to orange yellow

Calcium—reddish to purple brown

Mast cell granules—deep red

Cartilage—purple to violet

Mucus—blue violet

Cytoplasm—olive to brown

Elastic fibers—purple to red.

k. Weigert's Resorcin-Fuchsin Elastic Stain

Results:

Elastic fibers—blue-black to black

Nuclei—blue to black

Collagen—pink to red

Other tissue elements—yellow.

l. Gomori's Aldehyde Fuchsin Stain
Results:
Elastic fibers and mucin—deep blue
Beta cells of pancreas—deep blue
Other—stain of counterstain.

m. Verhoeff's Elastic Stain
Discussed in detail later.

n. Wilder's Reticulum Stain
Discussed in detail later.

o. Koneff's Stain for Bone and Cartilage
Results:
Hyaline cartilage and osteoid—blue
Bone—bright red to reddish brown
Matrix of vesicular zone of epiphyseal disc and remnants of matrix—green
Proliferating cartilage—blue
Hyaline cartilage—blue.

Stains for Cytoplasmic Granules

a. Gomori's Chromaffin Stain
Results:
Chromaffin granules—purplish red
Only alpha cells of pancreatic islets, some cells of anterior pituitary and granules of neutrophils and myelocytes stain likewise.

b. Acid Fuchsin Aniline Blue Method for Pituitary Granules
Results:
Acidophil granules—orange vermillion
Basophil granules—deep cobalt blue
Connective tissue—bright blue
RBCs—intense vermillion.

c. Fontana-Masson Stain for Argentaffin Granules
Discussed in detail later.

d. Luna's Mast Cell Stain
Results:
Mast cells—deep purple-red
Other cellular elements—blue-black.
Background—yellowish orange.

Stains for Fats and Lipoids

a. Osmium Tetroxide Stain for Fat
Results:
Fat—black
Background—yellow to brown.

b. Oil Red O Fat Stain
Discussed in detail later.

c. Sudan Black B Stain for Fat
Results:
Fat—black, blue, or blue-black.
Nuclei—red.

Stains for Carbohydrates and Mucoproteins

a. Best's Carmine Stain for Glycogen
Discussed in detail later.

b. Periodic Acid-Schiff (PAS) Reaction
Discussed in detail later.

c. Mayer's Mucicarmine Stain
Discussed in detail later.

d. Alcian Blue
Results:
*A*cid mucopolysaccharides—blue
Nuclei—pink.

e. Alcian Blue-PAS Stain
Results:
*Ex*clusively acid substances (e.g. various connective tissue mucins)—blue.
Neutral polysaccharides (e.g. glycogen and Brunner-gland mucin)—magenta.
Certain substances (e.g. most epithelial mucins and cartilage ground substance) are colored by both Alcian blue and PAS, yielding varying shades of purple to very deep color. The cell bodies of fungi are red to purple, while mucoid capsules (e.g. *Cryptococcus neoformans*) are blue.
Other features appear about the same as with the ordinary PAS stain.

f. Crystal Violet Amyloid Stain
Discussed in detail later.

g. Bennhold's Congo Red Amyloid Stain
Results:
Amyloid—pale pink to red
Nuclei—blue.

h. Toluidine Blue Metachromasia Stain
Results:
Metachromatic tissue—pink.

Stains for Pigments and Minerals

a. Modification of Mallory's Reaction for Iron
Results:
Iron pigments—bright blue
Nuclei—red
Cytoplasm—pink to rose.

b. Gomori's Iron Reaction
Results:
Iron pigments—bright blue
Nuclei—red
Cytoplasm—pink to rose.

c. von Kossa's Method for Demonstrating Calcium
Discussed in detail later.

d. Svain's Bile Pigment Stain
Results:
Bile pigments—emerald green.
(Localizations cannot be considered reliable because of the diffusibility of the reactants and the final color).
Nuclei—red
Cytoplasm—pink to rose.

e. De Galantha's Method for Demonstration of Urate Crystals
Results:
Urates—black
Connective tissue—yellow.

Stains for Bacteria, Fungi and Inclusion Bodies

a. Kinyoun's Acid-Fast Stain
Results:
Acid-fast bacteria—bright red
Background—light blue.

b. Ziehl-Neelsen Stain for Acid-Fast Bacteria
Discussed in detail later.

c. Fite-Faraco Stain for Acid-Fast Bacilli
Results:
Acid-fast bacilli—red
Background—light blue.

d. Brown and Brenn Stain for Bacteria in Tissue
Results:
Gram-positive bacteria—blue
Gram-negative bacteria—red
Nuclei—red
Other tissue elements—yellow.

e. Levaditi's Method for Staining Spirochetes in Blocks
Results:
Spirochetes—intensely black
Background—yellow to light brown.

f. Warthin-Starry Method for Staining Spirochetes
Results:
Spirochetes—black
Background—pale yellow to light brown.

g. Silver Method for Spirochetes and Donovan Bodies
Results:
Spirochetes, Donovan bodies, also fungi and bacteria—black
Background—yellow to brown.

h. Gridley's Stain for Fungi
Results:
Mycelia—deep blue
Conidia—deep rose to purple
Background—yellow
Elastic tissue and mucin also stain deep blue.

i. Gomori's Methenamine-Silver Nitrate Technique (Grocott's application to Fungi)
Results:
Fungi—sharply delineated in black
Mucin—taupe or dark gray
Inner part of mycelia and hyphe—old rose
Background—pale green.

j. Phloxine Toluidine Blue Stain for Malarial Parasites
Results:
Malarial parasites—pale blue cytoplasmic structures within erythrocytes
Erythrocytes—orange to red
Cytoplasm—pale rose with deep red granules
Nuclei—blue to purple
Supporting stroma—orange red.

k. Parson's Stain for Negri Bodies
Results:
Negri bodies—bright orange-red
Nuclei—blue
Erythrocytes—copper.

l. Hematoxylin-Shorr S3 Stain for Inclusion bodies
Results:
Inclusion bodies—brilliant red
Connective tissue—light red

Elastic tissue—purplish red
Muscle—red
Keratin—orange
Erythrocytes—orange red
Nuclei—blue.

m. Giemsa's Stain for Rickettsiae

Results:
Rickettsiae—violet
Nuclei—blue
Cytoplasm and connective tissue—pink
Erythrocytes—salmon.

SOME STAINING TECHNIQUES IN DETAIL

Mallory's Phosphotungstic Acid Hematoxylin Stain: PTAH

Fixation: Zenker-fixed best. If formalin-fixed, tissue should be mordanted from 1 to 12 hours in a saturated solution of mercuric chloride or in Zenker's fluid.

Technique: Paraffin, sections cut at 6 microns.

Solutions

Phosphotungstic Acid Hematoxylin

Hematoxylin	1 g
Phosphotungstic acid	20 g
Distilled water	1000 cc

Dissolve the solid ingradients in separate portions of the water, the hematoxylin with the aid of gentle heat. When cool, combine. No preservative is necessary. Spontaneous ripening requires several weeks but the addition of 0.177 g of potassium permanganate will cause the stain to ripen at once.

Staining Procedure

1. Deparaffinize sections through 2 changes of xylene, absolute and 95% alcohol to distilled water as usual.
2. Remove mercury precipitate by placing in alcoholic iodine solution for 5 to 10 minutes.
3. Wash in tap water.
4. Clear off iodine in 5% sodium thiosulfate (hypo) solution for 5 minutes.
5. Wash in running water for 10–20 minutes.
6. Stain for 12 to 24 hours in phosphotungstic acid hematoxylin solution.
7. Differentiate in 95% alcohol—check differentiation under the microscope.
8. Absolute alcohol, 2 changes.
9. Xylene, 2 changes.
10. Mount in DPX.

Results

Nuclei—blue
Fibrin—blue
Fibroglia and microglia—blue
Collagen—yellowish to brownish red
Coarse elastic fibrils—purplish tint.

van Gieson's Stain for Collagen Fibers

Fixation: Formalin.

Technique: Paraffin, cut sections at 6 microns.

Solution

Weigert's Iron Hematoxylin

Solution A

Hematoxylin	1 g
Absolute alcohol	100 cc

Solution B

29% ferric chloride	4 cc
Distilled water	95 cc
Hydrochloric acid, concentrated	1 cc

Working Solution
Equal parts of Solutions A and B.

van Gieson's Solution

Acid fuchsin, 1% aqueous solution 2.5 cc
Picric acid, saturated aqueous solution 97.5 cc.

Staining Procedure

1. Xylene.
2. Absolute alcohol.
3. 95% alcohol.
4. Rinse in distilled water.
5. Stain in Weigert's hematoxylin solution for 10 minutes.
6. Wash in distilled water.
7. Counterstain in van Gieson's solution for 1 to 3 minutes.
8. 95% alcohol.
9. Absolute alcohol—2 changes.
10. Xylene—2 changes.
11. Mount in DPX/Add 3 drops of saturated alcoholic picric acid to each 50 cc of xylene used in clearing. Mount from acidified xylene. This intensifies the background and prevents sections from fading.

Results

Collagen—red
Muscle, cornified epithelium—yellow
Nuclei—blue to black
Running water will remove van Gieson's solution
Solution B will remove Weigert's hematoxylin.

Masson's Trichrome Stain

Fixation: Bouin's or formalin. Mordant sections of formalin-fixed material in Bouin's fluid for one hour at 56°C, or overnight at room temperature.

Technique: Paraffin, cut sections at 6 microns.

Solutions

Bouin's Solution

Picric acid saturated aqueous solution	75 cc
Formaldehyde, 37–40%	25 cc
Glacial acetic acid	5 cc

Weigert's Iron Hematoxylin

Solution A and B and working solution as in Van Gieson's stain for collagen fibers

Biebrich Scarlet-Acid Fuchsin Solution

Biebrich Scarlet, aqueous 1%	90 cc
Acid fuchsin, aqueous, 1%	10 cc
Glacial acetic acid	1 cc

Phosphomolybdic-Phosphotungstic Acid Solution

Phosphomolybdic acid	5 g
Phosphotungstic acid	5 g
Distilled water	200 cc

Aniline Blue Solution

Aniline blue	2.5 mg
Acetic acid	2 cc
Distilled water	100 cc

Light Green Solution

Light green	5 cc
Distilled water	250 cc
Glacial acetic acid	2 cc
Heat water, dissolve light green, cool, filter and add acid.	

1% Acetic Water Solution

Glacial acetic acid	1 cc
Distilled water	100 cc

Staining Procedure

1. Xylene.
2. Absolute alcohol.
3. 95% alcohol.
4. Rinse in distilled water.
5. Mordant in Bouin's fixative for 1 hour at 56°C, or overnight at room temperature.
6. Cool and wash in running water until yellow color disappears.
7. Rinse in distilled water.
8. Weigert's iron hematoxylin solution for 10 minutes. Wash in running water 10 minutes.
9. Rinse in distilled water.
10. Biebrich scarlet-acid fuchsin solution for 15 minutes. Save solution.
11. Rinse in distilled water.
12. Phosphomolybdic acid-phosphotungstic acid solution for 10 to 15 minutes before aniline blue solution. Aqueous phosphotungstic acid 5% for 15 minutes before light green counter stain. Discard solution.
13. Aniline blue solution for 5 to 10 minutes or light green solution for 1 minute. Save solution.
14. Rinse in distilled water.
15. Acetic water 1% for 3 to 5 minutes. Discard solution.
16. Alcohol, 95%.
17. Absolute alcohol—3 changes.
18. Xylene—2 changes.
19. Mount in DPX.

Results

Nuclei—black
Cytoplasm, keratin, muscle fibers, intercellular fibers—red
Collagen, mucus—blue.

Verhoff's Elastic Stain

Fixation: Formalin.

Technique: Paraffin, sections to be cut at 6 microns.

Solutions

Elastic Tissue Stain

Dissolve 1 g of hematoxylin in 22 cc of absolute alcohol in an open dish on a hot plate. Cool, filter and add 8 cc of a 10% aqueous solution of ferric chloride and 8 cc of iodine solution (2 g iodine, 4 g potassium iodide dissolved in 100 cc of distilled water). Always use freshly made solutions for better results.

Ferric Chloride Solution

Ferric chloride	2 g
Distilled water	100 cc

Van Gieson's Stain

Acid fuchsin, aqueous solution 1%	5 cc
Saturated aqueous solution picric acid	100 cc

Sodium Thiosulfate (Hypo) Solution

Sodium thiosulfate	5 g
Distilled water	100 cc

Staining Procedure

1. As usual deparaffinize and take to water.
2. Verhoff's elastic tissue stain for 15 minutes.
3. Wash in distilled water.

4. Differentiate in 2% ferric chloride—only a few minutes. Check under microscope and if differentiated too far, restain.
5. Place in 5% sodium thiosulfate for 1 minute.
6. Wash in tap water 5 minutes.
7. Counterstain in van Gieson's stain for 1 minute.
8. Absolute alcohol—2 changes.
9. Xylene—2 changes.
10. Mount in DPX.

Results

Elastic fibers—blue-black to black
Nuclei—blue to black
Collagen—red
Other tissue elements—yellow.

Wilder's Reticulin Stain

Fixation: Formalin.

Technique: Paraffin. Sections cut at 6 to 10 microns.

Solutions

Phosphomolybdic Acid Solution

Phosphomolybdic acid	10 mg
Distilled water	100 cc.

Uranium Nitrate Solution

Uranium nitrate	1 g
Distilled water	100 cc.

Ammoniacal Silver Solution

To 5 cc of 10.2% aqueous solution of silver nitrate, add 28% ammonia water, drop by drop, until the precipitate which forms is almost dissolved. Add 5 cc of 3.1% sodium hydroxide and barely dissolve the resulting precipitate with a few drops of ammonia water. Make the solution up to 50 cc with distilled water. Use at once. Glassware must be clean.

Reducing Solution

Distilled water	50 cc
Neutral formaldehyde, 40%	0.5 cc
Uranium nitrate 1% aqueous solution (Make fresh just before use)	1.5 cc.

Gold Chloride Solution

Gold chloride solution, 1%
[Break glass vial (15 grains) in graduated cylinder with 100 cc distilled water for 10% solution]
Distilled water 40 cc.

Sodium Thiosulfate (Hypo) Solution

Sodium thiosulfate	5 g
Distilled water	100 cc.

Nuclear Fast Red (Kernechtrot) Stain

Dissolve 0.1 g nuclear fast red in 100 cc of a 5% solution of aluminum sulfate with aid of heat. Cool, filter, add grain of thymol as a preservative.

Staining Procedure

1. Xylene, absolute alcohol, 95% alcohol, distilled water.
2. Remove mercury precipitates if Zenker-fixed.
3. Wash well in distilled water.
4. Phosphomolybdic acid solution for 1 minute (oxidizer).
5. Rinse well in running water or cells will hold the yellow.
6. Dip in 1% aqueous uranium nitrate for 5 seconds or less (sensitizer).
7. Wash in distilled water for 10 to 20 minutes.
8. Place in ammoniacal silver solution for 1 minute.
9. Dip very quickly in 95% alcohol and go immediately into.
10. Reducing solution for 1 minute.
11. Rinse well in distilled water.
12. Tone in gold chloride solution for 1 minute or until sections lose their yellow color and turn lavender. Too much toning will make sections red. Check individually under microscope.
13. Rinse in distilled water.
14. Place in 5% sodium thiosulfate for 1 to 5 minutes.
15. Wash well in tap water.
16. Counterstain, if desired, with alum hematoxylin and eosin, or nuclear fast red. Rinse well in distilled water.
17. Alcohol, 95%.
18. Absolute alcohol—2 changes.
19. Xylene—2 changes.
20. Mount in DPX.

Results

Reticulin fibers—black
Collagen—Rose color
Other tissue elements—depending on counter-stain used.

Fontana-Masson Stain for Argentaffin Granules

Fixation: Formalin.

Technique: Paraffin. Cut sections at 6 microns.

Solutions

Silver Nitrate Solution (Fontana)

Dissolve 10 g of silver nitrate in 100 cc of distilled water. To 65 cc of this solution, add ammonium hydroxide until a clear solution with no precipitate is obtained. Add, drop

by drop, enough of the remaining 5 cc of silver nitrate solution to cause the above solution to become slightly cloudy. Let stand overnight before using. When ready to use, dilute each 25 cc of silver solution with 75 cc of distilled water and filter.

Gold Chloride Solution

Gold chloride solution	10 cc
Distilled water	40 cc

Sodium Thiosulfate Solution

Sodium thiosulfate	5 g
Distilled water	100 cc

Nuclear Fast Red (Kernechtrot) Solution

Dissolve 0.1 g of nuclear fast red in a 5% solution of aluminum sulfate with the aid of heat. Cool, filter add a grain of thymol as a preservative.

Staining Procedure

1. Deparaffinize and hydrate to water.
2. Immerse slides in silver nitrate solution for 1 hour at 56ºC.
3. Rinse in distilled water.
4. Immerse in gold chloride solution for 10 minutes.
5. Rinse in distilled water.
6. Place in sodium thiosulfate for 5 minutes.
7. Rinse in distilled water.
8. Counterstain with eosin or nuclear fast red, if desired. Rinse in distilled water.
9. Alcohol, 95%.
10. Absolute alcohol.
11. Xylene.
12. Mount in DPX.

Results

Argentaffin granules and melanin—black.
Nuclei—pink.

Oil Red O Fat Stain

Fixation: Formalin.

Technique: Cut frozen sections at 10 to 15 microns. Collect in distilled water.

Solutions

Oil Red O solution

Oil Red O	1 to 2 g
Alcohol, 70%	50 cc
Acetone	50 cc

Glycerin Jelly

Gelatin	10 g
Distilled water	60 cc

Heat until gelatin is dissolved. Add:

Glycerin	70 cc
Phenol	1 cc

Harris's Hematoxylin for counterstain

Staining Procedure

1. Carry sections through on an angled glass rod.
2. Dip sections in 70% alcohol for only a second.
3. Place in oil red O in a tightly closed container for 5 minutes.
4. Wash quickly in 70% alcohol-avoid folds in sections.
5. Wash in water.
6. Counterstain in Harris's hematoxylin for a few minutes.
7. Wash in water.
8. Blue in ammonia water. If sections are too dark when removed from the hematoxylin, they may be differentiated in 1% acetic water for a few seconds, then blued in ammonia water.
9. Wash in water.
10. Mount in glycerin jelly.

Results

Fat—orange to bright red
Nuclei—blue.

Best's Carmine Stain for Glycogen

Fixation: Tissue must be fixed in absolute alcohol or Carnoy's fluid. Since glycogen is soluble in water go directly from fixative to the clearing agent and then to paraffin.

Technique: Paraffin. Cut sections at 6 microns.

Solutions

Carmine Stock Solution

Carmine	2 g
Potassium carbonate	1 g
Potassium chloride	5 g
Distilled water	60 cc

Boil gently and cautiously for several minutes. Cook in open dish (evaporating dish). When cool, add 20 cc of strong ammonia water. Keep in icebox.

Working Solution of Carmine

Stock carmine solution	10 cc
Ammonia water, 28%	15 cc
Methanol	15 cc

Differentiating Solution

Absolute alcohol	20 cc
Methanol	10 cc
Distilled water	25 cc

Staining Procedure

1. Xylene, absolute alcohol.
2. Dip slides in very thin solution of celloidin—dry for a few seconds.
3. Place in water to harden for a few seconds.
4. Stain in Harris's hematoxylin solution for 15 minutes.
5. Differentiate in acid alcohol. Leave nuclei a little dark because ammonia decolorizes them slightly.
6. Place in working solution of carmine for 20 to 30 minutes. Carry a control slide for checking.
7. Place for a few seconds in the differentiating solution.
8. Wash quickly in 80% alcohol.
9. Alcohol 95%.
10. Absolute alcohol—2 changes.
11. Xylene—2 changes.
12. Mount in DPX.

Results

Glycogen—pink to red.
Nuclei—blue.

Periodic Acid-Schiff (PAS) Reaction

Fixation: Formalin.

Technique: Cut paraffin sections at 6 microns.

Solutions

Coleman's Feulgen Reagent

Dissolve 1 g of basic fuchsin in 200 cc hot distilled water. Bring to boiling point. Cool and add 2 g potassium metabisulfite, and 10 cc normal hydrochloric acid. Let bleach for 24 hours, then add 0.5 g activated carbon. Shake 1 minute and filter through coarse filter paper. Repeat filtration until solution is colorless. Store in a refrigerator.

OR

Schiff's Leuko-Fuchsin Solution

Dissolve 1 g basic fuchsin in 200 cc hot distilled water. Bring to boiling point. Cool to 50°C. Filter and add 20 cc normal hydrochloric acid. Cool further and add 1 g anhydrous sodium bisulfite, or sodium metabisulphite. Keep in the dark for 48 hours until solution becomes straw colored. Store in refrigerator.

Test for Schiff's Leuko-Fuchsin Solution

Pour a few drops of Schiff's solution into 10 cc of 37–40% formaldehyde in a watch glass. If the solution turns reddish purple rapidly, it is good. If the reaction is delayed and the resultant color deep blue-purple, the solution is breaking down.

Normal Hydrochloric Acid Solution

Hydrochloric acid, Concentrated specific gravity 1.19	83.5 cc
Distilled water	916.5 cc

0.2% Light Green counterstain (Stock)

Light green crystals	0.2 g
Distilled water	100 cc
Glacial acetic acid	0.2 cc

Working Light Green Solution

Light green, stock solution	10 cc
Distilled water	50 cc

Diastase Solution

Diastase	0.5 g
Distilled water, sterile	100 cc

Store in refrigerator. Good for 1 week.

(Instead of diastase solution, one can use one's own saliva).

Staining Procedure

1. Xylene.
2. Absolute alcohol.
3. Alcohol 95%.
4. Rinse in distilled water.
   (If PAS reaction with digestion is needed, place sections in 0.5% diastase for 20 minutes. Saliva can also be used. Rinse in running tap water 10 minutes, and wash in distilled water).
5. Periodic acid solution for 5 minutes (oxidizer).
6. Rinse in distilled water.
7. Place in Coleman's Feulgen or Schiff's leuko-fuchsin for 15 minutes.
8. Place in running tap water for 10 minutes for pink color to develop.
9. Stain in Harris's hematoxylin for 6 minutes or light green counterstain for a few seconds. Light green is recommended for counterstaining sections in which fungi are to be demonstrated. Omit steps 10 through 14 if light green is used.
10. Rinse in tap water.
11. Differentiate in acid alcohol—3 to 10 quick dips.
12. Wash in tap water.
13. Dip in ammonia water to blue sections.
14. Wash in running tap water for 10 minutes.
15. Alcohol, 95%.
16. Absolute alcohol—2 changes.
17. Xylene—2 changes.
18. Mount in DPX.

Results

Glycogen, mucin, hyaluronic acid, reticulin, fibrin or thrombi, colloid droplets, hyaline of arteriosclerosis,

hyaline deposits in glomeruli, cells in renal arterioles where preserved, most basement membranes, colloid of pituitary stalks and thyroid, amyloid infiltration and other elements may show a positive reaction—rose to purplish red.

Nuclei—blue
Fungi—red
Background—pale green (with light green counterstain).

Mayer's Mucicarmine Stain

Fixation: Formalin.

Technique: Cut paraffin section at 6 microns.

Solutions

Weigert's Iron Hematoxylin

Solution A

Hematoxylin	1 g
Alcohol, 95%	100 cc

Solution B

Ferric chloride 29% aqueous solution	4 cc
Distilled water	95 cc
Hydrochloric acid, concentrated	1 cc

Working Solution
Equal parts of solutions A and B. Prepare fresh.

Metanil Yellow Solution

Metanil yellow	0.25 g
Distilled water	100 cc
Glacial acetic acid	0.25 g

Mucicarmine Stain

Carmine	1 g
Aluminum chloride, anhydrous	0.5 g
Distilled water	2 cc

Mix stain in small test tube. Heat over small flame for 2 minutes. Liquid becomes almost black and syrupy. Dilute with 100 cc of 50 % alcohol and let stand for 25 hours. Filter. Dilute 1 to 4 with tap water for use.

Staining Procedure

1. Deparaffinize sections through 2 changes of xylene, absolute and 95% alcohol to distilled water as usual. Remove mercury precipitates through iodine and hyposolutions if necessary.
2. Stain for 7 minutes in working solution of Weigert's hematoxylin.
3. Wash in tap water for 5 to 10 minutes.
4. Place in diluted mucicarmine solution for 30 to 60 minutes or longer.
   Check control slide with microscope after 30 minutes.
5. Rinse quickly in distilled water.
6. Stain in metanil yellow solution for 1 minute.
7. Rinse quickly in distilled water.
8. Rinse quickly in 95% alcohol.
9. Dehydrate in 2 changes of absolute alcohol, clear with 2 to 3 changes of xylene, and mount in DPX.

Results

Mucin—deep rose to red
Nuclei—black
Other tissue elements—yellow.

Crystal Violet Amyloid Stain

Fixation: Formalin

Technique: Cut paraffin sections at 6 microns.

Solutions

Stock Crystal Violet Solution

Crystal violet-to saturate-approximately	14 g
Alcohol, 95%	100 cc

Working Crystal Violet Solution

Crystal violet, stock solution	10 cc
Distilled water	300 cc
Hydrochloric acid, concentrated	1 cc

Abopon Mounting Medium

Abopon	50 g
Distilled water	25 cc

Dissolve with the aid of heat.

If this medium cannot be obtained, then put a drop of water on the section to be seen and examine under microscope.

Staining Procedure

1. Deparaffinize sections through 2 changes of xylene, absolute and 95% alcohol to distilled water as usual.
2. Stain in working crystal violet solution for 1 to 2 minutes. Use control slide. Check with microscope.
3. Rinse well in tap water.
4. Mount in Abopon.
5. Seal edges of coverslip with nail polish.

Results

Amyloid—purplish violet
Other tissue elements—blue.

Von Kossa's Method for Demonstrating Calcium

Fixation: Alcohol or 10% formalin. Alcohols are preferred.

Technique: Cut paraffin sections at 6 microns.

Solutions

5% Silver Nitrate Solution

Silver nitrate	5 g
Distilled water	100 cc

Solution stable only for 2 weeks.

5% Sodium Thiosulfate Solution

Sodium thiosulfate	5 g
Distilled water	100 cc

Nuclear Fast Red (Kernechtrot) Stain

Dissolve 0.1 g nuclear fast red powder in 100 cc of a 5% aqueous aluminum sulfate solution with the aid of heat. Cool and filter. Add a crystal of thymol as preservative. Keeps well at room temperature. Can be reused.

Staining Procedure

1. Deparaffinize sections through 2 changes of xylene, absolute and 95% alcohols to distilled water.
2. Place in 5% silver nitrate solution for 30 to 60 minutes exposed to direct sunlight, ultraviolet lamp, or a 100 watt desk lamp light. Use chemically clean container.
3. Rinse in distilled water.
4. Place in 5% solution thiosulfate for 2 to 3 minutes.
5. Wash well in distilled water.
6. Counterstain in nuclear fast red for 5 minutes.
7. Wash in distilled water.
8. Dehydrate with 2 changes of 95% alcohol, absolute alcohol, clear with 2 changes of xylene and mount in DPX.

Results

Calcium salts—black
Nuclei—red
Cytoplasm—pink to rose.

Ziehl-Neelsen Stain for Acid-Fast Bacteria

Fixation: Formalin.

Technique: Paraffin sections at 4 to 6 microns.

Solutions

Carbol Fuchsin solution

Acid carbolic, white fused crystals, melted	2.5 cc
Alcohol, absolute	5 cc
Basic fuchsin	0.5 g
Distilled water	50 cc

Store at room temperature. Filter before use.

1% Acid Alcohol

Hydrochloric acid, concentrated	1 cc
Alcohol, 70%	99 cc

OR

1% Sulfuric Acid Water

Sulfuric acid concentrated	1 cc
Distilled water	99 cc

Working Methylene Blue Solution

Methylene blue	0.5 g
Glacial acetic acid, concentrated	0.5 cc
Tap water	100 cc

Staining Procedure

1. Deparaffinize sections through 2 changes of xylene, and run through absolute and 95% alcohols to distilled water as usual. Remove mercury precipitates through iodine and hyposolutions, if necessary.
2. Stain sections with carbol fuchsin solution for 10 minutes. Filter solution before use.
3. Rinse well in tap water.
4. Decolorize with 1% acid alcohol or 1% sulfuric acid water, until sections are pale pink.
5. Wash thoroughly with running water for 8 minutes.
6. Counterstain by dipping one slide at a time in working methylene blue solution. Sections should be pale blue. Overstaining will mask bacilli.
7. Wash with tap water and distilled water.
8. Dehydrate with 2 changes of 95% alcohol and absolute alcohol, clear with 2 changes of xylene, and mount in DPX.

Results

Acid-fast bacilli—bright red.
Erythrocytes—yellowish-orange.
Other tissue elements—pale blue.

AUTOMATION IN HISTOPATHOLOGY

Hypercenter (Shandon)

Modular enclosed tissue processing system:

- A unique, safe, totally enclosed system that will not release vapors into the laboratory during processing
- Keyboard programing and memory with video display and simple touch button controls accept ordinary language
- Separate command, reaction and storage modules offer flexible choice of layouts
- One command module can control up to 5 reaction and storage modules independently and simultaneously
- Nine programs per reaction module—total capacity 45 programs, each of which can be recalled instantly
- Programs can be monitored at every stage.

Histocenter (Shandon)

Integrated tissue embedding center:

- Provides efficient utilization of bench space—easy to clean work surfaces all on one level
- Incorporates processed tissue storage, cassette opening and base
- Wax temperature can be preset and monitored continually on digital display—can also be switched to monitor hotplate temperature
- Wax dispenser can be foot operated leaving both hands free
- Wax flow rate easily adjusted with nozzle heated to prevent wax solidifying
- Illuminated work area and integral magnifier.

2 LE (Shandon)

Two liter automatic tissue processor

- Simple to use electronic timer and program controls provide virtually unlimited programing capacity with constant digital read out
- Capacity of 160 cassettes in double load or up to 336 specimens in divided baskets
- Antievaporation lids on all beakers reduce fume levels
- Two independent program memories can store up to 4 complete processing cycles
- Vacuum impregnation during processing cycle if optional vacuum pump utilized.

Duplex (Shandon)

One liter automatic tissue processor

- Economic versatile processing for single or double load operation
- 12 station cycle—with 9 or 10 one liter beakers and 2 or 3 thermostatically controlled wax baths
- Automatic agitation ensures efficient impregnation of tissue
- Wide range of tissue baskets—large capsule and divided types in stainless steel and plastic
- Standard 24 hours timer plus 24 hours delay.

Hypercut (Shandon)

Rotary microtome

- Exceptional strength and stability with heavy weight construction, which eliminates vibration and chatter
- Unique linear bearing action provides smooth almost effortless control.
- Sections paraffin wax embedded tissue blocks consistently and accurately from 0.5 to 30
- Unique, easy to adjust knife holder accepts wide range of knives including glass, tungsten carbide tipped, and disposable type
- Optional lightweight knife holder provides additional access to cutting area.

Autosharp 5 (Shandon)

Automatic microtome knife sharpener

- Rapid high quality consistent sharpening of microtome knives, no special training required
- Designed to sharpen tungsten carbide tipped as well as conventional steel knives
- Wide range of knife holders available for C type, D type and knives up to 250 mm long
- Easy to use damper; touch button controls and digital timer control
- Unique, hard wearing microsharp iron lapping plate with extended life
- Easy to adjust facet angle indicator.

Linistain GLX (Shandon)

Random access stainer for histology

- Special built-in fume extraction system, protects laboratory personnel from xylene fumes
- Provides consistent uniform staining of specimens on standard microscope slide
- Random access provides flexibility to use various staining routines and eliminates batching
- Allows free choice of stain source and type
- Running water wash selectable at all positions
- Proven, reliable design with a few moving parts
- Compact, narrow shape allows wise use of bench space.

Varistain 24 (Shandon)

Automatic 24 position batch stainer

- Simple-to-use electronic timer and program controls provide virtually unlimited programing capacity with constant digital read out
- Immersion times can be programed to the second with all the versatility of hand staining
- Individual immersion times can be reprogrammed to take account of changing conditions
- Two independent program memories can store up to 4 complete staining routines. Three capacities of stainless steel slide carrier available—10 slide, 40 slide and 60 slide
- Compatible with Autoslip automatic over slipper.

Varistain 12 (Shandon)

Automatic 12 position stainer

- Designed for H and E staining and hematological techniques.
- Equal or unequal times can be selected.
- 224 slides per hour output on continuous loading operation.
- Single load operation for 16 or 32 slides horizontally or 48 slides vertically.
- 60 minutes timer with additional option for 30 minutes, or short immersion timers.
- Aeration and agitation provided.

Autoslip (Shandon)

Automatic coverslipper

- Applies mountant and coverslip to standard microscope slides automatically, presenting the finished slide for examination.
- Eliminates hand coverslipping—one of the most unpleasant and hazardous operations in histology and cytology.
- Produces coverslipped slides of consistent high quality at the rate of up to 160 slides per hour.
- Coverslipped slides held in xylene saturated atmosphere to prevent drying out of specimens prior to coverslipping.
- Unique plastic slide clips enable slides to be transferred direct from Varistain 24 series of automatic stainers.

CHAPTER 26

Cytology

Cytology is that branch of diagnostic medicine, which deals with the study of individual cells and/or tissue fragments spread on laboratory slide and stained appropriately.

Stains used commonly are Papanicolaou's stain and May-Grünwald-Giemsa (MGG) stain. For the former, alcohol fixation is required; and the latter, fixation should be done by using methanol. For acid-fast bacilli staining, ethanol fixation is used. Wherever, ethanol fixation is required, the material obtained and spread on a slide should be immediately immersed into ethanol. For MGG staining, the smears are air dried and methanol fixed.

For fine needle aspirations and thick cellular discharges/fluids, no centrifugation is required. While relatively watery, thin and hypocellular fluids need centrifugation, the sediment so obtained is smeared on to the slides, stained, and examined.

The advantage of cytological techniques is the rapidity with which the diagnosis can be provided. However, this branch has not, and cannot, replace the ultimate in diagnosis, namely—histopathology.

Cytological study can be done on discharges from the body (vaginal, nipple, sinus, etc.), scrappings obtained (from buccal mucosa, or other mucosal surfaces approachable by employing fiber-optic endoscopes), or by aspirating from palpable lumps (abscesses, growths, etc.). In any case, the material obtained is smeared on slides, an easy and convenient way is to put the material between two slides and pull them apart or smearing on the slide by using a coverslip with application of gentle pressure. All liquids (relatively hypocellular, e.g. urine, body cavity fluids, etc.) have to be centrifuged, the sediment is used for smearing. If a bigger chunk of cellular material is obtained, it can be submitted for histopathological examination too. Do not forget to add EDTA to containers in which coagulable fluids are to be collected.

Cytological diagnosis is an important part of cervical (gynecological) lesions, accessible mucosal lesions and soft tissue tumors palpable superficially or else approached under fluoroscopic guidance. Using vacuum to suck and needle movement in various directions (to dislodge tissue fragments), sufficient amount of material can be obtained.

The commonly used stains are Pap's stain and MGG. Details of Pap's staining are mentioned below. MGG staining is similar to that of blood peripheral smear staining.

PAPANICOLAOU METHOD OF STAINING SMEARS (MODIFIED)

Preparation of Smears

1. Exfoliated cells degenerate rapidly; therefore, smear should be prepared and fixed immediately. If there is to be any delay, the specimen should be fixed in 95% alcohol and refrigerated until smears can be prepared. Specimens requiring centrifugation (e.g. urine and various fluids) are preserved by adding an equal volume of 50% ethyl alcohol, centrifugation is at 2000 rpm for 30 minutes.
2. Viscid secretions (e.g. vaginal, cervical and prostatic) should be smeared directly onto clean glass slides and fixed immediately.
3. Body fluids and watery exudates (e.g. urine, spinal fluid, pleural fluid, etc.) will not adhere to the glass slides unless the slide is first coated with a layer of Mayer's egg albumin (one drop per slide).
4. The sediment or centrifuged specimen is smeared onto glass slides coated with Mayer's egg albumin. Any remaining sediment should be processed as a biopsy specimen for conventional histology examination.

Fixation

1. Use equal parts of ether and 95% alcohol.
2. Smears should be fixed immediately while still wet, though partial drying along the edges may be permitted to prevent the material from becoming detached from the slide.
3. Fixation time is 30 minutes to 1 week.
4. If unstained slides are to be mailed, they must be placed in the fixative for at least 2 hours, then dried and placed face-to-face before shipment.

Solutions

1. Harris's Hematoxylin (modified)
   - Hematoxylin crystals 5 g
   - Absolute alcohol 50 cc
   - Ammonium or potassium alum 100 cc
   - Distilled water 1000 cc
   - Mercuric oxide 2.5 g

For this purpose, acetic acid should not be added.

2. Orange G6 (OG 6)
   - Orange G6, 0.5% solution in 95% alcohol 100 cc
   - Phosphotungstic acid 0.015 g

3. Eosin-Azure 50 (EA 50): These stains should be purchased readymade. These are ready for use and cannot be equalled by mixture, prepared in laboratory.

Staining Procedure

1. After fixation, transfer slides without drying directly from alcohol—ether solution to 95% alcohol, then through 80% alcohol, 70% alcohol, and water, to distilled water.
2. Stain in Harris's hematoxylin (modified), 8 minutes.
3. Rinse gently in tap water to prevent cells from being washed off.
4. Differentiate carefully the nuclear staining in 1% hydrochloric acid in 70% alcohol; the nuclei should be clear and sharp in detail; the cytoplasm should be light blue and clear.
5. Place in gently running tap water for 5 minutes to wash out the acid thoroughly and to blue the nuclei.
6. Rinse in distilled water and transfer through 70% alcohol, 80% alcohol, to 95% alcohol.
7. Stain in OG 6 for 2 minutes.
8. Rinse 5 times as follows:
   a. Twice in 95% alcohol
   b. Once in 1% acetic acid in 95% alcohol
   c. Once in 1% phoshotungstic acid in 95% alcohol
   d. Once in 95% alcohol.
9. Stain in EA 50 for 3 minutes.
10. Rinse 9 times as follows:
    a. 3 changes of 95% alcohol
    b. 2 changes of absolute alcohol
    c. 4 changes of xylene.
11. Mount in DPX.

Results

Nuclei—blue with clear sharp details.

Cytoplasm—varying shades of pink, blue, yellow, green gray. If necessary, slides may be decolorized in acid alcohol, washed thoroughly in tap water to remove all acid and restained.

FNAC (FINE-NEEDLE ASPIRATION CYTOLOGY)

Transcutaneous Aspiration of Palpable Lesions

A 20 mL plastic disposable syringe with 21 to 23-gauge fine needles of variable length, depending upon the site of tumor, are used for aspiration. The syringe is fitted with especially designed handle, which permits a single hand operation during aspiration. The skin is cleaned with antiseptic solution. No local anesthesia is required. The tumor mass is fixed with one hand and with the other hand aspiration is carried out. When the needle enters the tumor, the plunger of the syringe is retracted to create a vacuum in the barrel and the needle is moved to and fro 3 to 4 times. For adequate sampling, the needle may be moved in three to four different directions (Fig. 26.1). After completion of aspiration, the plunger is released before taking out the needle in order to equalize the pressure. The needle is disconnected and after filling the syringe with air, it is reconnected. The content of the needle is expressed on clean glass slides. Smears are made by applying a gentle pressure with the flat surface of another glass slide and allowed to air dry. Alternatively, the flat surface of a covership can be used to prepare the smears. Smears are routinely fixed in methanol for MGG staining. Whenever Pap staining is required for better nuclear clarity, wet fixation in absolute alcohol is recommended. Also, smears may be fixed appropriately for various cytochemical stains when fluid aspirated, is discharged into a clean tube and centrifuged at 1500 rpm. Smears are made from the deposits when the aspiration fluid is admixed with blood or frankly hemorrhagic, it may be collected in a heparinized container. Hematocrit method or lymphoprep can be used to separate the tumor cells from RBCs. In case, where cellularity is poor, the deposit is resuspended in 1 mL of supernatant, spun in a cytocentrifuge and processed in a similar manner.

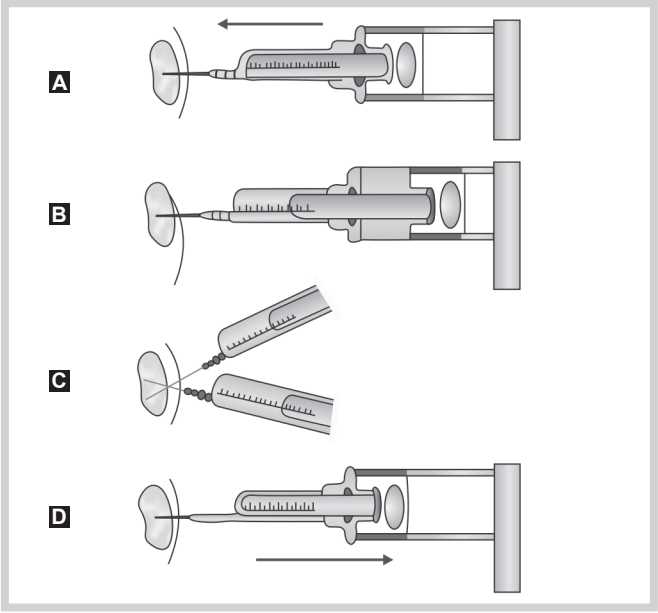


FIG. 26.1: Technique of fine-needle aspiration biopsy
A. Needle within the lymph-node
B. Retraction of the piston of the syringe in order to create negative pressure in barrel of syringe
C. Movement of needle in 2 to 3 directions/plains
D. Release of piston and withdrawal of the needle

Aspiration of Intrathoracic Masses

Lung

Aspiration cytology of intrathoracic masses is usually performed to diagnose peripheral lung lesions, which are not accessible by bronchoscope and which do not desquamate into the bronchial tree. This procedure is usually carried out around the table under television fluoroscopy. The skin is cleaned with iodine and spirit and infiltration with local anesthesia is done up to pleura. The needle length can be selected according to the depth of the lesion. The usual internal diameter of the needle is 0.6 to 0.7 mm. When the needle reaches the intrathoracic mass, the aspiration is performed as per palpable lesions. The patient is asked to hold his breath during the aspiration. The entire procedure of inserting the needle and aspirating the lesion should not take more than 20 seconds. The needle may be reinserted in a different direction if no representative material is obtained initially. After the aspiration cytology, the patient needs to be kept under observation for about 2 hours to detect rare complications like bleeding or pneumothorax if any.

Liver

An 8–16 cm needle with external diameter of 0.6 mm (22-gauge) are used for aspiration of liver (Usually, a prothrombin time is estimated before liver aspiration. If the value is very high, vitamin K is given to lower it to a satisfactory level).

No local anesthesia is necessary. Depending upon the site of lesion, the needle is introduced transcostally or subcostally. The ideal transcostal area for entry of needle is 9th intercostal space in midaxillary line. When the liver is enlarged particularly when the tumor mass is palpable, aspiration can be carried out in the subcostal region.

Prostatic: Aspiration is undertaken with the help of a needle guide and long needle (20 cm). The patient is placed in the lithotomy position. The prostate is carefully palpated through rectum before the procedure. Well lubricated gloved index finger of left hand with the needle guide is fixed over the nodule palpable through rectum. Then the needle is introduced through the needle guide and aspiration is performed as described for transcutaneous biopsy.

Ovarian tumor: Large ovarian tumors, which are palpable per abdomen can be aspirated transabdominally avoiding the loops of intestine. The lesion can also be approached through the vaginal vault with the help of a needle guide and a long needle as is described under the aspiration of prostate.

Advantages of the Procedure

1. Fine-needle aspiration biopsy is a quick, convenient, economic and almost painless procedure, which can be practiced on an outpatient basis.
2. Local anesthesia is not required.
3. Can be attempted at multiple sites and repeated if necessary.
4. Malignancy can be confirmed or excluded in potentially operable lesions suspicious of malignancy and the extent of surgery can be planned well in advance.
5. Is a good diagnostic aid prior to application of radiation in inoperable cases or where surgery is contraindicated.
6. By way of evacuation of a cyst content, it helps as a therapeutic aid in addition to providing diagnosis.
7. It helps in assessing the stage of the disease prior to surgery or radiotherapy.
8. Local recurrence or metastasis can be detected in postoperative or post-radiation follow-up cases, for further management.
9. Aspirated material can be used for immunological, cytochemical, cytogenetical and microbiological studies.

Limitations

False negative results may be obtained in the following situations:

1. If there is extensive fibrosis and sclerosis in a tumor.
2. If the tumor is highly vascular.
3. If there is tumor necrosis.

To minimize these errors, special precautions can be taken. Wherever limitations exist, suggest an excision/open biopsy.

Ultrasound-Guided Fine-Needle Aspiration Cytology

The use of ultrasound as a tool in medical diagnosis is gaining increased acceptance in most medical centers. The chief advantages of ultrasound as a diagnostic modality are three:

1. It is a noninvasive study, causing little or no discomfort to the patient and usually requiring no special preparation.
2. It does not require the use of ionizing radiation such as X-rays. Studies to date have shown no proven adverse effects from the ultrasonic beam at the conventional power levels used for diagnosis.
3. Ultrasound is capable of providing some diagnostic information, which may not be available using other noninvasive techniques.

The ultrasound beam is in many ways similar to a beam of light. It obeys the laws of optics and can (unlike X-rays) be focussed, reflected, or refracted. The beam consists of high frequency sound waves generated by vibration of a piezoelectric crystal within an ultrasound transducer. The crystal vibrates in response to an electrical signal, the frequency of vibration being a function of the shape and thickness of the crystal itself. This is exactly the same principle that governs the sound of a bell. Bells of differing shapes and sizes have different sounds. For most medical applications, the frequency used is approximately 2.5 million cycles per second (2.5 mHz). The same crystal that transmits the ultrasonic beam also functions as a listening device. For example, a pulse of ultrasound is beamed for a fraction of a second and the crystal then "listens" during a much longer interval for the echo response. Returning sound waves (echoes) strike the transducer, producing vibrations which are transmitted as electrical signals to an oscilloscope or for storage on the screen of a cathode ray tube.

What produces these echoes? The tissues of the body vary from each other in sound-transmission characteristics (acoustic impedance). When two tissues of differing acoustic impedance are apposed, the ultrasonic beam will be partially reflected at the interface between them, returning an echo signal to the transducer. The degree of difference in acoustic impedance will determine the strength of the returning echo. Thus, if soft tissue lies next to bone, which has a very high acoustic impedance, or next to air, which has a very low acoustic impedance, strong interfaces will be formed, and strong echoes will be returned. On the other hand, soft tissues (vessel walls, septa, fat, parenchyma, etc.) differ only slightly from one another in acoustic impedance and the echoes that are returned from their various interfaces are relatively weak. These echoes are recorded as spikes on an oscilloscope or stored as dots on the screen of the cathode ray tube. This latter type of storage display is called a B-scan, B because the brightness (and size) of the dots on the screen varies with the strength of the acoustic interface.

B-scanning

B-scan displays are used when performing studies of the abdomen, retroperitoneum, and pelvis. The orientation of the dots on the storage screen varies with the orientation of the transducer relative to the patient's body. As the transducer (attached to a rigid hinged arm, which holds it in any plane selected) is moved across a section of the patient's body, it sends a narrow, well-directed ultrasonic beam through the tissues. As this beam traverses the abdomen, it is partially reflected at various interfaces, owing to relative differences in acoustic impedance. These reflected echoes are recorded as dots, which build up an image of the section on a storage screen (Fig. 26.2). When a suitable picture has been made, it may be photographed on either Polaroid or X-ray film, or recorded on heat-sensitive paper.

It is important to remember that relatively inhomogeneous tissues, such as solid organs or masses, will generally have many weak echoes recorded within them, representing small vessels, ducts, and septae traversing the tissue. Relatively homogeneous tissues, such as fluid-filled organs or cystic lesions, show a few internal echoes, even

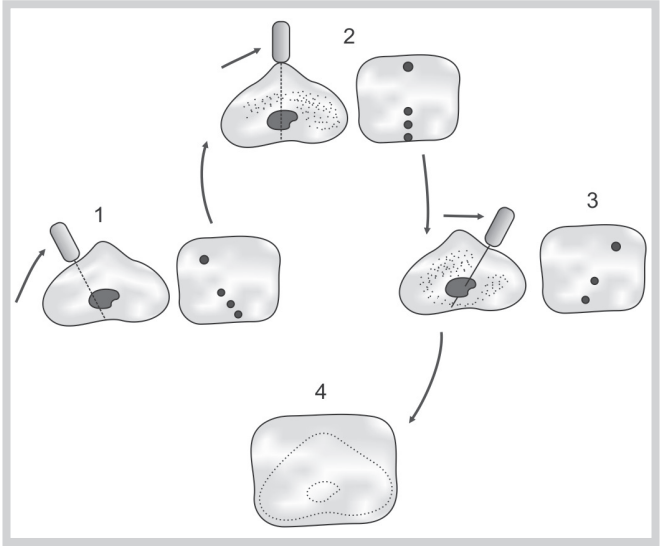


FIG. 26.2: As the transducer is moved across the body, an image of the body section traversed is built up on the storage screen

when the sensitivity of the machine is turned up very high ("high gain").

Ultrasonography-guided fine-needle aspiration cytology is being practiced in case of lesions of thyroid, and some selected cases of intrathoracic tumors and also in breast.

Advantages

1. Takes less time than to perform mammography.
2. No radiation hazards.
3. Can be used to guide the aspiration of cyst that could not be drained clinically hence avoids surgery.
4. It can guide placement of wire guide.

Disadvantages

1. Limitations in diagnostic usefulness because benign appearance overlaps, the malignant.
2. Unable to detect lesions less than 1.5 cm in diameter (breast).

Application

Differentiating cyst from solid mass.

SMEARING TECHNIQUES

One-step Smearing Technique

The standard overlap and pull-apart method of smear preparation can itself be a source of dilution. Aspirates of poorly vascularized tumors often yield semisolid, undiluted tissue fragments that can be easily expelled from the needle core as a compact drop onto a slide. If standard pull-apart smears are prepared, this material is dispersed onto two slides. A slight modification of the pull-apart technique, called the one-step technique, greatly limits what is in effect a 50% dilution of the material. The two slides are held at right angles. A small drop of aspirate material is placed near the frosted edge of a slide held by one hand at that edge. A second slide is then placed across the first, establishing a fulcrum such that the mobile slide, when carefully rotated away from the observer and downward toward the material, just covers the drop. The smearing slide is then quickly but smoothly brought toward the operator, with a smearing pressure barely greater than that caused by the capillary space dispersion of fluid between the slides. This gentle and smooth stroke yields optimal monolayer smears for semisolid or small-volume specimens. Little material is transferred to smearing slide.

Two-step Particle Concentration and Smearing Technique

For needle aspirates that are diluted by fluid, a procedure called the two-step smearing technique provides an optimal concentration of cells on the slide.

With the frosted ends away from the observer, each slide is held at the tips of its lateral border by three fingers. The thumb and index finger contact the upper outer frosted corner of each slide, and the fifth finger contacts the underside of the lower outer corner.

First, a drop of the aspirate is placed near the frosted end of a clean, grease-free slide. With the labeled ends still away from the observer, the other hand brings the smearing slide onto the first slide, at a point between the drop of aspiration and the observer. The smearing slide is then passed away from the observer and through the drop so as to collect the drop in the acute angle formed by the two slides.

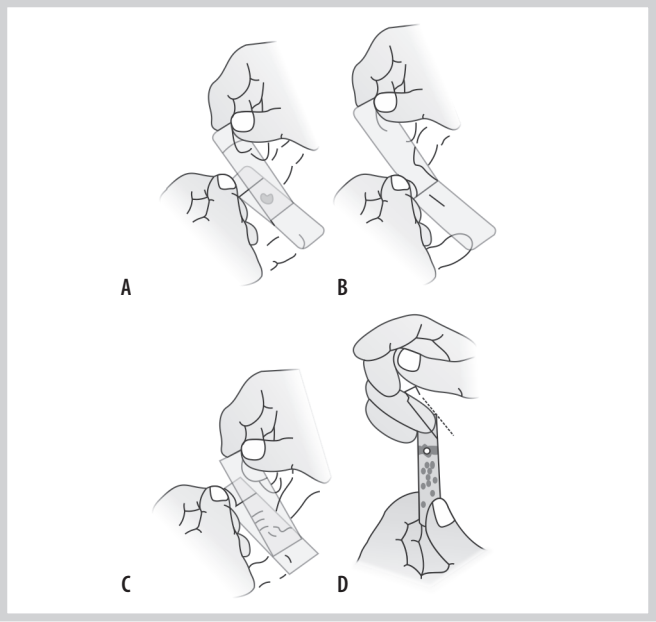
With the first slide still stationary, the mobile slide begins the concentration procedure by next moving the entrapped material away from the frosted end and toward the observer. This movement disperses much of the fluid over the central portion of the stationary slide, while most of the particles remain with the small amount of residual fluid at the line of slide intersection. At this point, the mobile slide is lifted perpendicularly upward and away from the stationary slide, and the similarities of this technique to preparing peripheral blood smears end. Next, the stationary slide is rotated vertically with its frosted end pointing downward. For 1 to 3 seconds, gravity is allowed to draw any excess fluid further away from the particles, which largely remain at the last line of slide intersection. The particles can frequently be seen by reflected light as small, slightly raised points.

At this point the basic concentration maneuvres (first step of the two-step technique) are complete, and the particles now await monolayer smearing by a slight variation of the standard pull-apart technique (the second step). By fulcrum action, the mobile slide is placed so that when slowly rotated onto the other slide it extends about 1 to 2 mm past the line of concentrated particles. As soon as the two slides come into contact, they are gently pulled apart with minimal additional pressure, yielding a well-formed monolayer (Fig. 26.3).

REQUIREMENTS FOR LABORATORY SET UP

Equipment

1. Handle constructed for disposable 20 mL or 10 mL syringe (Cameco, Enebyberg, Sweden).
2. 10 mL or 20 mL disposable plastic syringe (ASIK, Aps. Denmark).
3. Disposable needle of various sizes
   23G ×1" (0.6 × 25 mm)
   22G × 2" (0.7 × 50 mm)
   20G short and long.
4. Needle guide for transrectal biopsy (KIFA, Solna, Sweden).



FIGS. 26.3A TO D: Two-step technique. Line drawing summary. (A) Finger position. (B) Collection of aspirate, (C) Particle concentration smear, and (D) Preparation of a monolayer smear are illustrated for a right-handed operator

5. Examination table.
6. Binocular microscope.
7. Centrifuge.
8. Analytical balance.
9. Cytocentrifuge.
10. Distillation plant.
11. Refrigerator.
12. Slide cabinet.

Glassware

1. Microslide coverslips 24 × 50 mm thickness 0.17 mm.
2. Measuring cylinder.
3. Coplin jar.
4. Staining jar.
5. Conical flask.

May-Grünwald-Giemsa (MGG) Stain

Reagents

1. May-Grünwald stain
2. Giemsa's stain
3. Methanol
4. Glycerol
5. Conical flask
6. Phosphate (pH 6.8).

Preparation of Stains

i. Preparation of May-Grünwald stain: 0.3 g of powdered dye is weighed out and transferred to a conical flast of 200–250 mL capacity. A volume of 100 mL of methanol is added and the mixture is warmed to 50°C. The flask is then allowed to cool to room temperature and is shaken several times during the day. After standing for 24 hours, the solution is filtered. It is then ready for use, no ripening being required.

ii. Preparation of solution of Giemsa powder is dissolved in 54 mL of glycerol and after cooling, mixed with 84 mL of methanol GR and filtered.

Staining Techniques

Air-dried smears are fixed in a jar of methanol for 5 minutes. Fixed smears are stained as follows:

1. With May-Grünwald stain, freshly diluted with an equal part of phosphate buffer for 5 minutes.
2. With Giemsa's stain, diluted with 9 parts of phosphate buffer for 10 to 15 minutes.
3. Washed with phosphate buffer (pH 6.8).
4. Dried in air.
5. Mounted by a rectangular cover glass using DPX as mountant.

Papanicolaou's Stain with EA-36

Harris's Hematoxylin

Prepared as follows:

Hematoxylin	1 g
Absolute alcohol	10 mL
Potassium alum	20 g
Distilled water	200 mL
Mercuric oxide	0.5 g

The hematoxylin is dissolved in absolute alcohol and potassium alum in distilled water with the aid of heat. The two solutions are mixed together. The mixture is boiled, removed from the flame and mercuric oxide is added bit by bit. The flask containing the solution is then immersed into cold water bath. After cooling, it is filtered and stored in a colored bottle.

Orange G-6

Orange G-6 solution is prepared as follows:

Orange G	0.5 g
95% ethyl alcohol	100 mL
Phosphotungstic acid	0.015 g

Eosin Azure-36 (EA-36)

The stock solutions of light green SF yellowish (A), eosin yellow (B), are prepared as follows:

a. Light green—(SF)

Light green SF yellowish	0.5 g
95% ethyl alcohol	100 mL

b. Eosin yellow

Eosin yellow	0.5 g
95% ethyl alcohol	100 mL

From the stock solution, the working solution of EA-36 is prepared as follows:

Light green SF yellowish (A)	45 mL
Eosin yellow (B)	45 mL
Phosphotungstic acid	0.200 g

All these solutions are kept in the refrigerator when not in use.

Automatic Staining

Automatic stainer can process a large number of smears with excellent results.

Autostainer: Slides held in rack are automatically rotated around baths containing stains and other reagents. Time schedule for staining as mentioned previously in manual process can be obtained by calibrating a timing dial. The reagents are renewed weekly. The instrument has the advantage that it can easily be adapted for staining techniques. It uses laboratory prepared reagents and it allows for complete adaptability in staining, times. After staining, the slides are mounted with DPX.

Staining Method

Manual process

The fixed slides are transferred directly from the fixative into the following solutions:

1.	80% ethyl alcohol	10 dips
2.	70% ethyl alcohol	10 dips
3.	50% ethyl alcohol	10 dips
4.	Distilled water	3 minutes
5.	Harris's hematoxylin	1 minute
6.	Running tap water	1 minute
7.	Hydrochloric acid (0.5%)	5 dips
8.	Running tap water	1 minute
9.	Dilute solution of lithium carbonate	1 minute
10.	Running tap water	1 minute
11.	50% ethyl alcohol	10 dips
12.	70% ethyl alcohol	10 dips
13.	80% ethyl alcohol	10 dips
14.	95% ethyl alcohol	10 dips
15.	Orange G-6	1 minute
16.	95% ethyl alcohol	10 dips
17.	95% ethyl alcohol	10 dips
18.	EA-36	4 minutes
19.	95% ethyl alcohol	10 dips
20.	95% ethyl alcohol	10 dips
21.	Absolute alcohol	4 minutes
22.	Xylene	5 minutes

Slides are then mounted with DPX.

Results

Nucleus—blue
Cytoplasm of superficial cell— pink
Cytoplasm of intermediate cell—bluish green
Red blood cells—orange.

Papanicolaou's staining schedule for automated stainer (total staining time about 30 minutes)

Step No.	Time in minutes
1. H_2O	1
2. Harris's hematoxylin	3
3. H_2O	1
4. 0.1% HCl in 70% Ethanol	¼
5. H_2O	¼
6. 1% NH_4OH in 70% Ethanol	1
7. 95% Ethanol	1
8. 95% Ethanol	1
9. 95% Ethanol	1
10. 95% Ethanol	1
11. OG-Modified	2
12. 95% Ethanol	1
13. 95% Ethanol	1
15. EA-modified	3
16. 100% Ethanol	1
17. 100% Ethanol	1
18. 100% Ethanol	1
19. 100% Ethanol	1
20. Xylol	1
21. Xylol	1

Papanicolaou's staining procedure for manual set-up (total staining time under 7 minutes)

		Regressive method	*Progressive method*
1.	H_2O	30 dips	30 (seconds)
2.	Harris's Hematoxylin	60 dips	30
3.	H_2O	10 dips	10

Contd...

Contd...

4.	H_2O	10 dips	10
5.	0.1 % HCl in 70% Ethanol	5 dips	Not Necessary
6.	H_2O	10 dips	10
7.	1% NH_4OH in 70% alcohol	30 dips	30
8.	95 % Ethanol	10 dips	10
9.	95% Ethanol	10 dips	10
10.	95% Ethanol	10 dips	10
11.	OG-Modified	30 dips	30
12.	95% Ethanol	10 dips	10
13.	95% Ethanol	10 dips	10
14.	95% Ethanol	10 dips	10
15.	EA-Modified	60 dips	60
16.	100% Ethanol	10 dips	10
17.	100% Ethanol	10 dips	10
18.	100% Ethanol	10 dips	10
19.	100% Ethanol	10 dips	10
20.	Xylol	10 dips	30
21.	Xylol	10 dips	10
22.	Xylol	10 dips	10

Combined Alcian Blue—PAS Technique for Acid and Neutral Mucins

Acid mucin and neutral mucin are clearly separated by this technique. It is also useful as a routine demonstration technique for the presence of any mucin. The acid mucins are first stained with alcian blue and are not available for PAS reaction. Only the neutral mucin is stained by PAS reaction which follows. In this way, a good color distinction can be made between acid and neutral mucins.

Preparation of Stains

a. Alcian blue : 1 g
   3% acetic acid : 100 mL
b. Schiff's reagent.

Method

1. Wash the fixed smears in distilled water.
2. Stain smear with alcian blue solutions for 5 minutes.
3. Wash in water.
4. Treat with 1% periodic acid for 5 minutes.
5. Rinse in distilled water.
6. Place in Schiff's reagent for 10 minutes.
7. Wash in running water for 10 minutes.
8. Stain nuclei with hematoxylin.
9. Wash in water.
10. Dehydrate, clear and mount.

Results

Acid mucin—blue
Neutral mucin—magenta
Nuclei—pale blue.

Naphthol ASBI Phosphate Method for Acid Phosphatase

Acid phosphatase is demonstrated by an Azodye coupling technique, which depends upon the hydrolysis of a substrate containing Alpha-naphthoxl phosphate. As hydrolysis occurs, the liberated naphthol couples with a diazotized amine and forms an insoluble colored precipitate.

Burstone (1958), recommended naphthol ASBI phosphate as substrate—the primary reaction product, produced by the enzyme hydrolyzing this substrate is extremely insoluble.

Preparation of Solutions

a. Substrate solutions

Naphthol ASBI phosphate	10 mg
Dimethyl formamide	1 mL

b. Buffer solution

Sodium acetate	1.17 g
Sodium barbitone	2.94 g
Distilled water	100 mL

c. Sodium nitrite solution

Sodium nitrite	400 mg
Distilled water	10 mL

d. Pararosanilin hydrochloride stock solution

Pararosanilin hydrochloride	1 g
Distilled water	20 mL
Concentrated HCl	5 mL

Heat gently, cool to room temperature and filter

e. Distilled water preparation of incubating solution

Solution (A)	0.5 mL
Solution (B)	2.5 mL

Preparation of incubating solution

Solution (A)	0.5 mL
Solution (B)	2.5 mL
Solution (C)	0.8 mL
Solution (D)	0.8 mL
Solution (E)	6 mL

A volume of 0.4 mL of solution C and solution D mixed together and allowed to stand for 2 minutes before adding to incubating solution.

The pH should be between 4.7 and 5.0 it is adjusted with 0.1 N NaOH.

Method

1. Incubate smears at 37°C for 60 minutes.
2. Wash in water.
3. Counterstain in 2% methyl green (chloroform extracted).
4. Wash in running water.
5. Dehydrate clear and mount.

Results

Acid phosphatase activity	:	Red
Nuclei	:	Green

Alkaline Phosphatase: Azo Dye Coupling Method Using Alpha Naphthyl Phosphate

Fixation

Formol calcium at 4ºC.
Formol vapor.

Preparation of Incubating Medium

Sodium naphthyl phosphate	10 mg
0.2M Tris buffer (Stock solution A) pH	10 mL
Diazonium salt (fast red TR)	10 mg

The final pH of the incubating medium should be between 9.0 and 9.4. The sodium naphthyl phosphate is dissolved in the buffer, the diazonium salt is added and the solution well mixed. The solution is then filtered and used immediately.

Method

a. After fixation, incubate the smears at room temperature for 10–60 minutes.
b. Wash in distilled water.
c. Counterstain in 2% methyl green (chloroform extracted).
d. Wash in running tap water.
e. Mount in glycerin jelly.

Results

Alkaline phosphatase activity	:	Reddish brown
Nuclei	:	Green.

α-Naphthyl Acetate Method for Nonspecific Esterase

This method employs α-naphthyl acetate as the substrate, the enzyme releases α-naphthol during the hydrolysis of the substrate. The α-naphthol is then coupled with a suitable diazonium salt to produce an insoluble azo dye at the site of enzyme activity.

Preparation of Solutions

a. Substrate solutions

α-naphthyl acetate	50 mg
Acetone	5 mL

b. Buffer solution

Sodium dihydrogen orthophosphate	2.75 g
Distilled water	100 mL

c. Sodium nitrite solution

Sodium nitrite	400 mg
Distilled water	10 mL

d. Pararosanilin HCl - Stock solution

Pararosanilin hydrochloride	2 g
2 N-hydrochloric acid	50 mL

e. Distilled water.

Preparation of Incubating Medium

Solution	(A)	0.25 mL
Solution	(B)	7.25 mL
Solution	(C)	0.8 mL
Solution	(D)	0.8 mL

A volume of 0.4 mL of solution C and D is mixed together and allowed to stand before adding to incubating medium.

The pH should be 5.8 to 6.1.

Method

a. Incubate smears at 37°C for 20 minutes.
b. Wash in water.
c. Counterstain in 2% methyl green (chloroform extracted).
d. Wash well in water.
e. Dehydrate, clear and mount.

Results

Esterase	:	Reddish brown
Nuclei	:	Green.

Diaminobenzidine Method for Peroxidase

Preparation of Incubating Solution

3:3 - diaminobenzidine tetra-hydrochloride	5 mg
Tris buffer (pH 7.6)	10 mL
1% hydrogen peroxide	0.1 mL

Method

a. Rinse fixed smears in distilled water.
b. Transfer to incubating solution for 5 minutes at room temperature.
c. Rinse in 3 changes of distilled water.
d. Dehydrate clear and mount in DPX.

Result

Peroxidase—fine brownish granules.

Peroxidase Stain

Fixative—95% ethanol : 90 mL
40% Formaldehyde (HCHO): 10 mL

Preparation of Solutions

To 30 mL of 30% ethanol, add 0.9 g of Benzidine, mix well and then add 3 mL of zinc sulfate (3.8%). A precipitate is formed then add 3 mg of sodium acetate and 4.5 mL of IN-NaOH (4 g in 100 mL). The pH should be 6. Filter and store at room temperature.

Method

a. Fix the smear with fixative for one minute.
b. Wash gently with tap water and soak out the excess water.
c. Take 5 mL of staining solution and 3 drops of 3% hydrogen peroxide, mix well and cover the slide for 30 sec at 20°C.
d. Wash the slide in running tap water for 10 seconds.
e. Counterstain with dituted Giemsa's stain for 2 minutes.
f. Wash with tap water then dried and mounted in DPX.

Results

Peroxidase positive seen as green granules.

Oil Red O Method for Lipids

It is a useful preliminary method to indicate two major lipid classes. For detailed morphology oil red O method is used with Mayer's hemalum.

Preparation of Solution

The working solution is prepared an hour in advance by mixing three parts of a stock solution of oil red O (saturated in 99% isopropanol) with two parts of distilled water and filtering just before use.

Method

Fix the smears in formalin vapor for 5 minutes and wash in running tap water for 10 minutes.

a. Rinse in 60% isopropanol.
b. Stain for 15 minutes in Oil Red O.
c. Differentiate in 60% isopropanol until a delipidized control section appears colorless.
d. Wash in water and counterstain nuclei with Mayer's hemalum for 3 minutes.
e. Wash well in water.
f. Rinse in distilled water and mount in glycerin jelly.

Results

Unsaturated hydrophobic lipids and mineral
Oil stain : Red
Phospholipids stain : Pink

IMMUNOPEROXIDASE STAINING FOR CYTO AND HISTOPATHOLOGY

Introduction

This is an immunohistochemical technique, aimed at the specific histological localization of particular tissue antigens by immunological method. These techniques are being increasingly used for diagnostic histology. The immunoperoxidase test which makes use of specific antibodies conjugated with horse radish peroxidase or alkaline phosphatase enzymes are nowadays commonly used for histochemical detection of various antigenic markers.

Direct method: The primary antibody conjugated with enzyme, is used to react with the antigenic sites. The combined antibody-antigen complex with enzyme is developed with specific substrate. Tissues/smears are examined under light microscope to detect the substrate color at the antigenic site.

Indirect method: It is more sensitive and commonly used. The specific primary antibody is applied directly to the tissues/smears. This is followed by the second antibody (antispecies specific IgG) conjugated with enzyme. The color of the reaction is developed by using specific substrate and examined under light microscope.

Material

Peroxidase conjugated with antirabbit immunoglobulins (Igs), PBS (pH 7.2/0.2M), DAB (3.3 Diamino benzidine-4 HCl), normal swine serum, hydrogen peroxidase, antigen specific antibody raised in rabbit.

Indirect Method

1. Dewaxed paraffin sections are hydrated in usual manner. Where prefixed smears are used, these are washed with buffered distilled water.

2. Endogenous peroxidase activity is blocked with a fresh 3% solution of hydrogen peroxide in distilled water for 10–30 minutes or with acid alcohol for 15 minutes. For Cryostat, use acid alcohol or phenylhydrazine (5×10^3 M) for 15–30 minutes.
3. Wash twice with phosphate buffer saline *(pH 7.2, 0.2M).
4. Expose sections/smears to normal swine serum diluted 1:5 with buffer at 22°C, 5–10 minutes. Excess NSS/NGS is removed, without washing prior to stage 4.
5. Sections/smears are treated with optimally diluted primary rabbit antiserum at 22°C, 15–30 minutes or 24–48 hours at 4°C with highly diluted antiserum.
6. Treat sections/smears with horseradish peroxidase labeled swine/antirabbit IgG 1:20-1:100 for 15–30 minutes at 22°C.
7. Wash twice with phosphate buffer saline* (pH 7.2). The end product is revealed with a freshly made solution of 0.05%, 3, 3-diaminobenzidine tetrahydrochloride (DAB) in 0.01% H_2O_2 in wash buffer.

Sections/smears are counterstained with a weak hematoxylin, dehydrated, cleared in xylene and mounted in DPX for the DAB or PDP reactions (brown to dark brown). Aqueous mountants, e.g. glycerin gelatine, are used for the carbazole reaction (red).

Solution A

(Sodium dihydrogen orthophosphate)
NaH_2PO_4. $2H_2O$—31.2 g for 1 liter.

Solution B

Disodium hydrogen phosphate
Na_2HPO_4. $2H_2O$—31.6 g for 1 liter.

Working solution:
Solution A 70 mL + Solution B 180 mL. Make the volume up to 1 liter and dissolve 5.7 g NaCl in a liter and filter before use.

Immunofluorescence

Principle

It is a histochemical or cytochemical technique for in situ detection and localization of specific intracellular antigens. Specific antibodies conjugated with fluorescent dyes, such as fluorescein or rhodamine, are used to trace the specific antigenic areas on the tissue smear or section. This can be visualized under the fluorescent microscope, as bright purple green/red color fluorescence.

Direct Immunofluorescence
In this method, conjugated antiserum is added directly to the tissue sections or viable cell suspension.

Indirect Immunofluorescence
It is more sensitive and commonly used. The unlabeled, specific antibody is applied directly to the tissue smears/sections, followed by a second antibody treatment, i.e. antispecies specific Ig conjugated with fluorescein or rhodamine and examined under UV-microscope. Due to the use of second antibody, the sensitivity and specificity of the reaction is highly improved.

Material

FITC/antihuman Igs conjugate, phosphate buffer saline (PBS), specific antibody, glycerol buffer, fluorescent microscope. Smear or section of tissue.

Method

1. Reasonably diluted antibody put on the antigen slide fixed in methanol for half an hour at room temperature in moisture chamber.
2. Wash the slide twice in PBS pH 7.2. All washes are carried out on a magnetic stirrer.
3. Incubate slides for 30 minutes with 1:20 diluted FITC (Fluoroscein isothiocynate) conjugated with Igs in PBS/pH 7.2* containing 0.01%. Evans blue as counterstain at room temperature in a moisture chamber.
4. Wash the slide twice in PBS pH 7.2.
5. Mount the slide with 90% glycerol buffer pH 8.6.
6. Examine the slide under UV-microscope. The antigen positive areas of the cell will show purple green fluorescence, whereas the negative area would appear brick red.

AUTOMATION IN CYTOLOGY

A programable cytocentrifuge from WESCOR is available, which can be used to prepare slide from any body fluid. With the help of cytocentrifuge sample cells, one can safely and quickly deposit a monolayer of cells on to a microscope slide for staining or any other processing.

This can be used on any of the body fluids, such as CSF, urine, synovial fluid, aspirates, washes, etc. and can be programed as per the requirements.

* PBS (Phosphate buffer saline) pH 7.2/0.2 M
* PBS preparation described earlier.

CHAPTER 27

Microbiology and Bacteriology

In the following pages maximum stress is laid on diagnostic bacteriology.

CLASSIFICATION

Protophyta

Schizomycetes (Bacteria and related forms)

Actinomycetales

These members form elongated cells and have a tendency to branch, produces spores, not all are pathogenic to man

- Actinomycetaceae
- Mycobacteriaceae
- Nitrobacteriaceae.

Eubacteriales

This represents the true bacteria forms, classifiable as bacilli, cocci, or vibrios. Their staining reaction can either be gram-positive or gram-negative. Some are motile and possess peritrichous flagella. They multiply by binary fission. Widely distributed they can be saprophytes, parasites and many are pathogenic to human beings.

- Pseudomonadaceae
- Enterobacteriaceae
- Corynebacteriaceae
- Lactobacillaceae
- Micrococcaceae
- Spirillaceae
- Bacteroidaceae
- Bacillaceae
- Neisseriaceae
- Brucellaceae.

Spirochetales

These are slender, spiral shaped cells, aflagellate but move by flexing or whirling and spinning. Stainable by special stains only, they are free-living and include saprophytic and parasitic forms.

- Treponemataceae.

Microtatobiotes (The smallest living things)

Rickettsiales: Most of these are intracellular pathogens, and filtrable forms and need special methods of culture.

Virales

Thallophyta

These are the Molds and Yeasts.

Bacterial Cell Constituents

Like other living cells, all bacteria possess the cell membrane, cytoplasm and a nucleus. Special characteristics are seen in certain strains.

Capsule

This is a protective outer covering layer possessed by some bacteria.

Flagella

These assist in locomotion, their arrangement may vary.

Spores

Under unfavorable conditions for growth sporing occurs. Spores are non-reproductive. Upon return of favorable environment they are transformed into the reproducible vegetative form. Spores are spherical and have a distinctive placement within the cell. They may be central subterminal or terminal. Knowing their location assists in identification of species.

Inclusion Granules

Some of the bacteria show inclusion granules. Volutin granules are metachromatic granules and may appear as aggregates of substances concerned with cell metabolism;

when stained with toluidine blue, they stain a red violet color in contrast to blue staining of the cytoplasm. These are considered to be made of polymerized inorganic phosphate. Lipid granules may be seen in bacteria and stained with Sudan black. Polysaccharide granules stainable by iodine (like glycogen or starch) can be seen in cytoplasm of some bacteria.

Shape of Bacteria

1. Cocci

Spherical

a. Cocci in cluster—Staphylococci
b. Cocci in chain—Streptococci
c. Cocci in pair—Diplococci
d. Cocci in groups of four—Tetrad
e. Cocci in groups of eight—Sarcino.

2. Bacilli

These are cylindrical or rod-shaped organisms. They can be of the following types:

a. Length of the cell equalling its breadth, called coccobacilli, e.g. *Brucella*
b. Chinese letter arrangement as seen in corynebacteria
c. Vibrio are comma shaped, curved, rods and are named so on account of their vibratory movement
d. Spirochetes are relatively longer, thinner, flexible and coil shaped
e. Actinomycetes are the branching filamentous bacteria
f. Mycoplasma lack cell wall and hence have no definite morphology. They may be round or oval bodies with interconnecting filaments.

Bacterial Reproduction

Bacterial reproduction occurs by a simple process of binary fission.

Bacterial Physiology

Bacterial physiology and biochemistry are studied by observing cultures grown in the laboratory on artificially prepared nutrient media. Various external factors influencing bacterial growth are—food, moisture, hydrogen ion concentration, oxygen, carbon dioxide, temperature and light.

1. Food

Bacterial growth is to large extent dependent on an adequate supply of suitable food material, the specific nutrient requirements vary from species to species. The important nutrient requirements are carbon, nitrogen, inorganic salts and for certain species, accessory growth factors of bacterial vitamins.

2. Moisture

For bacterial growth moisture is essential. Drying in the air damages bacteria.

3. Hydrogen-ion-concentration or pH

Most of the microbes growth better at a slightly alkaline pH (pH 7.2–7.6). Some acidophilic bacteria flourish in acidic pH. Those needing strong alkaline medium are termed basophilic.

4. Oxygen needs

Most bacteria can grow in the presence of oxygen and air and also in its absence. Those which grow in the presence of oxygen are called aerobes, while those which grow in its absence are termed anaerobes. Those which can grow under both the conditions are called facultative anaerobes, whereas bacteria that can grow in complete absence of oxygen are named obligatory anaerobes.

5. Carbon dioxide

All bacteria need the presence of small amounts of CO_2 for growth, an amount provided by atmosphere or by the metabolic reactions occurring in the bacteria itself. However, some bacteria need a higher concentration of CO_2 (5–10%).

6. Temperature

For bacteria, there is a range of temperature at which growth can occur. So there is a maximum, a minimum and the intermediate optimum temperature (at which the growth is most rapid). In the laboratory, this optimum temperature is maintained in an incubator thermostatically controlled. Majority of bacteria grow between 25 and 40°C and are termed mesophilic. 30°C is optimal for free living and 37°C is optimal for parasites in man or animals. Bacteria that grow best between 60 and 70°C are called thermophilic, while those growing best between 15 and 20°C are labeled as psychrophilic.

7. Light

Darkness is a favorable condition for growth and viability of bacteria. Direct sunlight is injurious to bacterial growth. Some bacteria can produce pigmentation on exposure to light and are called as photochromogens.

8. Symbiosis or mutual beneficial coexistence

A living organism multiplying in a human body is called as a parasite and the person harboring is the host. When both the parasite and the host derive benefit from each other—it is termed symbiosis. Certain intestinal bacteria provide

vitamins to their host without causing any pathogenic effects—a symbiotic relationship.

Products of Bacterial Growth

While thriving in a host or on an artificial culture medium, some bacteria produce substances that exert injurious effects in the host—these are called 'toxins'. In addition, certain enzymes may be harmful to the host. Some bacteria produce pigments (harmless, help in bacterial identification).

1. Bacterial toxins

These injurious products of bacteria are of two types: (i) exotoxins (extracellular) and (ii) endotoxins (intracellular). Toxins diffuse readily from the living bacteria into the surrounding medium. They can be obtained from the medium after removal of the bacteria. This can be done by centrifugation or by filtering through a Seitz filter. The toxins remain in the supernatant fluid in the case of centrifugation and in the filtrate in the case of filtration. Certain gram-positive bacteria secrete *exotoxins*, for example, *Corynebacterium diphtheriae*. Exotoxins are antigenic and are rapidly destroyed by heat.

Endotoxins: These are toxins intimately associated with the cell wall of the most gram-negative bacteria. They are released after death and disintegration of the bacteria. The majority of pathogenic bacteria produce endotoxins only. As mentioned in the previous paragraph for exotoxins—the endotoxins would be present in the residues and not in the supernatant (centrifugation) or in the filtrate (filtration).

2. Bacterial enzymes

a. Proteolytic enzymes: An enzyme responsible for decomposition of dead animal and vegetable matter in nature.
b. Coagulase: This is often demonstrated during the study of biochemical properties of some pathogenic bacteria.
c. Amylase: This enzyme is capable of splitting starch and is not much used in the study of bacteria.
d. Lactic acid fermentation.

3. Bacterial pigments

Many bacteria have the capacity to produce pigments, e.g. *Staphylococcus aureus*—golden yellow pigment and *Pseudomonas pyocyaneus*—green pigment. Certain pigments are restricted to the bacterial colonies while others can diffuse to surrounding medium.

Koch's Postulates

The etiologic relationship between pathogen and a disease is established by fulfilling Koch's postulates, viz.

1. The pathogen must be constantly found in the body of host either alive or dead.
2. It must regularly be isolated and it must be grown in pure culture in vitro.
3. When such a pure culture is inoculated into a susceptible animal species, the typical disease must result.
4. From such experimentally induced disease, the pathogen must be again isolated.

Morphology and Staining Reactions

Bacterial identification is aided by their staining reactions. Simple stains are used to show the presence of organisms and the nature of the cellular contents in exudates.

1. Loeffler's Methylene Blue

Saturated solution of methylene blue in alcohol 30 mL. Potassium hydroxide 0.01% in distilled water—100 mL.

Method

Stain for 3 minutes after making and fixing the smear. This stain does not readily overstain.

2. Dilute Carbol fuchsin

This is made by diluting Ziehl-Neelsen's carbol fuchsin stain ten times its volume in water. The smears are stained for 10–25 seconds and are washed well with water (Overstaining must be avoided here).

The two most frequently used differential stains are the Gram and Ziehl-Neelsen techniques.

Gram's Stain

This is the most widely used but not a fully understood technique. Various theories put forward are:

a. It has been shown that gram-positive organisms contain a substance known as magnesium ribonucleate, which gram-negative organisms lack. If this substance is removed from gram-positive bacteria, they will react as gram-negative organisms.
b. When iodine is applied for staining with crystal violet or another stain of that group a compound is formed which is insoluble in water, but soluble in alcohol or acetone. It is said that the more permeable the organism (i.e. the more easily water and other fluids can pass through the cell wall), the more likely it is to be gram-negative, since the acetone or alcohol has easier access to the compound which it will dissolve.
c. It is also thought that the pH of the organism has at least some influence of the reaction. Gram-positive bacteria have a more acid cytoplasm and this is increased by the addition of iodine. According to this

school of thought it is the acidity of the cytoplasm which helps the organism to retain the stain.

Method

1. Make a thin smear of the material or culture let dry at room temperature. Heating, should be avoided as this interferes with the staining reaction.
2. Pass the slide through a flame once or twice or until it feels comfortably warm on the back of the hand.
3. Place the slide on the rack and flood with the crystal violet or gentian violet stain—stain for 1 minute.
4. Wash off the stain with Gram's or Lugol's iodine and leave the slide covered with iodine for 1 minute.
5. Rinse in water.
6. Pour on acetone or alcohol till no more blue color comes from the slide (Acetone does this more quickly than alcohol so care should be taken not to use acetone for a longer period). (Serous and mucoid material are more difficult to decolorize than saline suspensions and require a longer exposure to the decolorizing agent).
7. Rinse in water again.
8. Stain with one of the following counterstains: Safranin, Neutral red, or 1:10 Carbol fuchsin.
9. Rinse in water and allow it to dry by standing it vertically, or by blotting it with filter paper.

Results

Because the gram-positive organisms retain the crystal violet after decolorization, they appear dark blue in color. The gram-negative organisms are decolorized and take up the counterstain and therefore, appear pink in color.

Reagents

1. Crystal violet—0.5% solution in distilled water.
2. Iodine-(Lugol's)—10 g iodine, 20 g potassium iodide in 1000 mL of distilled water. Dissolve the potassium iodide in 250 mL water and then add 10 g of iodine. When dissolved make up to 1000 mL with distilled water (This solution is three times stronger than Gram's iodine and is preferable).
3. Acetone.
4. Counterstain.
   a. 1 g Neutral red
      2 mL 1% Acetic acid
      Distilled water to make 1000 mL
   b. Safranin
      1.7 g safranin
      50 mL alcohol
      Distilled water to make 500 mL
   c. Dilute carbolfuchsin
      1:10 dilution of strong carbol fuchsin.

Ziehl-Neelsen Stain

This stain is another method of categorizing certain bacteria, depending on their ability to resist decolorization by acid and alcohol. A very strong stain is used, basic fuchsin in a phenol solution and heat is applied in order that the stain can penetrate the waxy covering certain bacteria.

Method

a. Make a smear of the material and allow to dry at room temperature.
b. Flood the whole slide with strong carbol fuchsin and heat gently underneath the slide until steam is seen rising from the slide (Do not overheat, avoid boiling of the stain).
c. Rinse in water and flood the slide with 25% sulfuric acid. Leave this until the smear is pale pink in color.
d. Rinse in water and pour on alcohol for a few minutes.
e. Counterstain with malachite green, methylene blue or picric acid.
f. Dry by standing the slide vertically—do not blot dry as the tubercle organisms may get attached to the paper and later may get transferred to another slide.

Results

The tubercle bacillus resists decolorizing by acid and alcohol (i.e. it is both acid and alcohol fast) it will remain bright red while all other organisms and material will take on the color of the counterstain.

Troubleshooting (AFB-Staining)

Problem: False positive results

Possible causes	*Solutions*
1. Sputum collected without washing the mouth or in an unclean container	Patient should wash their mouth thoroughly while procuring sputum to minimize specimen contamination with food particles, mouthwash or oral drugs. Patient should be asked to collect sputum in a clean container free from waxes, inorganic materials and artefacts Artefacts may be mistaken for acid-fast bacilli
2. Oil immersion lens is not cleaned during observation of slides	Oil immersion lens should be cleaned after every observation to avoid contaminating other slides

3. Contaminated water with acid-fast bacteria used for washing of slides during staining procedure	Use clean, non-contaminated water for washing of slides during staining
4. Carbol fuchsin held on the slide for long time with improper heating	Allow the stain to stand for exactly 5 minutes with the application of heat. While heating, ensure that the stain is not boiled. Heat only till steam starts rising from the slide. Leave the slide to cool for 2 minutes before decolorizing
5. Less decolorization done for thick smear preparation	The number of times for decolorization is to be increased for thick smears. Decolorization is to be carried out till the pink color disappears and the smear appears colorless

Problem: False negative results

Possible causes	*Solutions*
1. Sputum collected inadequately, i.e. only the saliva	Thick yellowish green mucoid sputum collected from an early morning deep productive cough should be used as a specimen
2. Failure to select suitable sputum portion for smear preparation	Select a suitable portion, i.e. thick yellowish green mucoid portion of the sputum preparation
3. Longer time duration given for counter-stain, i.e. more than 30 seconds	Allow the counterstain B to stain for 15–20 seconds before washing.
4. Inadequate examination of the smear	Smear should be examined thoroughly from one edge to the other covering 100 fields or more

Modified Ziehl-Neelsen's Stain

Used for leprosy where the bacteria are less acid fast. The method is as mentioned above except that 5% Sulfuric acid is used instead of 25%.

Reagents

Carbol fuchsin:
Basic fuchsin 10 g
Alcohol—100 mL
5% aqueous phenol—1000 mL.

Decolorizing agents:
25% sulfuric acid, or
5% sulfuric acid (for *M. leprae*) or
Acid-alcohol 3% HCI in alcohol.

Counterstains:
Loeffler's methylene blue or

Malachite green—0.05% aqueous solution or

Methylene blue—0.1% aqueous solution or
Picric acid-saturated aqueous solution.

Special Stains

Used to stain flagella, capsules, spores and granules.

Stains for Diphtheria Bacillus

Ponder's Stain

Toluidine blue 0.02 g
Glacial acetic acid 1 mL
Absolute alcohol 2 mL
Distilled water to make 100 mL.

Method
Spread the stain on the film for 1 minute and wash in tap water.

Result
Dark blue granules in pale blue bacillus.

Albert's Stain

Solution I	Toluidine blue	0.15 g
	Malachite green	0.2 g
	Glacial acetic acid	1 mL
	95% alcohol	2 mL
	Distilled water	100 mL

Dissolve the dyes in alcohol and add to the water and acetic acid. Let stand for one day and filter.

Solution II	Iodine	2 g
	Potassium iodide	3 g
	Distilled water	300 mL

Method
Apply solution I for 3 to 5 minutes, wash in tap water, blot and dry. Apply solution II for one minute, wash, blot and dry.

Result
The granules stain bluish black, the cytoplasm green and other organism light green.

Modified Neisser's Method

Neisser's methylene blue

Methylene blue	1 g
Ethyl alcohol (95%)	50 mL
Glacial acetic acid	50 mL
Distilled water	1000 mL.

Method

a. Stain with Neisser's methylene blue for 3 minutes.
b. Wash off with iodine solution used in Gram's method and leave some solution on the slide for 1 minute.

c. Wash in water and counterstain with neutral red solution used in Gram's method for 3 minutes.
d. Wash in water and dry.

Result
The bacilli show deep blue granules, the remainder of the organism assumes a pink color.

Staining of Capsules

Hiss's Method

a. Saturated alcoholic solution of basic fuchsin or gentian violet 1 part to distilled water 19 parts
b. 20% aqueous copper sulfate solution.

Method
Place a few drops of solution (a) on slide. Heat to steaming and leave on slide 30 seconds.
Wash off with solution (b).

Result
Capsule appears as faint blue halo around dark purple cell.

India Ink Method
The capsule is seen as a clear halo around the micro-organism against the black background. This method may be used for demonstrating cryptococci.

Staining of Spores

Modified Ziehl-Neelsen Method

1. Ziehl-Neelsen carbol fuchsin
2. Sulfuric acid 0.5% or methylated spirit
3. Loeffler's methylene blue.

Method
1. Stain with carbol fuchsin for 5–10 minutes, heating until steam rises.
2. Wash in tap water.
3. Decolorize with 0.5% sulfuric acid or methylated spirit. If the acid is stronger than 1%, spores of many bacilli are decolorized.
4. Wash in tap water. Now the smear is examined and if both bacilli and spores are red, it is decolorized again. If the spores alone are stained, it is counterstained. Let the counterstain to act for 2 minutes. Wash in water, blot and dry.

Result
The spores are stained bright red and the bacilli blue.

Staining of Spirochetes

Fontana's Method

a. Fixative	Acetic acid	1 mL
	Formalin	2 mL
	Distilled water	100 mL
b. Mordant	Phenol	1 g
	Tannic acid	5 g
	Distilled water	100 g

c. Ammoniated silver nitrate
Add 10% ammonia to 0.5% solution of silver nitrate in distilled water until the precipitate formed just dissolves. Now add more silver nitrate solution drop by drop until the precipitate returns and does not redissolve.

Method
1. Treat the film 3 times, 30 seconds each time, with the fixative.
2. Wash off the fixative with absolute alcohol to act for 3 minutes.
3. Drain off the excess of alcohol and carefully burn off the remainder until the film is dry.
4. Pour on the mordant, heating till steam rises and allow to act for 30 seconds.
5. Wash well in distilled water and again dry the slide.
6. Treat with ammoniated silver nitrate, heating till steam rises, for half minute, when the film becomes brown in color.
7. Wash well in distilled water and dry.

Result
The spirochetes are stained brownish black on a brownish yellow background.

Staining of Fungi

Lactophenol Cotton Blue

Phenol crystals	20 g
Lactic acid	20 mL
Glycerol	40 mL
Cotton blue/methylene blue	0.05 g
Distilled water	20 mL

Dissolve the phenol crystals in the liquids by gently heating and then add the dye.

Take a portion from the fungal growth and place it on a drop of lactophenol cotton blue on a slide. Then place a cover slip over the drop and press gently. Blot to remove excess stain. Seal with varnish or nail polish.

Staining of Flagella

Loeffler's Method

Loeffler's Flagella Mordant	
Tannic acid 20% aqueous	100 mL
Ferrous sulfate crystals	20 g
Loeffler's Flagella Stain	
10% alcoholic solution of	
Basic fuchsin	10 mL
Distilled water	40 mL

Method
Flood the smear with the mordant for 5 minutes. Wash with distilled water. Add heated Loeffler's flagella stain and allow to act for 3 minutes. Wash with distilled water and dry (The slides should be very clean).

Result
Organisms stain red and flagella pink.

Negative Staining

Negative Staining is a technique by which organisms remain unstained against a dark background.

India Ink Method
A small quantity of India ink 10% nigrosin is mixed with the material on a slide. A smear is made by means of another slide and the preparation is allowed to dry. The smear is examined and the spirochetes are seen as clear transparent objects against a dark brown background. Capsules may also be demonstrated by this method.

Motility of Bacteria

Hanging Drop Method

This method is used to observe the morphology but also demonstrates the motility of organisms. A special slide with a concave center is used or else a ring of plasticine can be placed on the slide. A drop of the culture of bacterial suspension is placed on a coverslip. Vaseline is placed near the concave area of the slide approximately the corners of the coverslip. The slide is placed over the coverslip so that the drop of culture is directly under the concave area and the Vaseline adheres to the coverslip.

The slide is then quickly inverted and placed under the microscope. Motile organisms will be seen darting through the medium in which they are suspended. Motility should be differentiated from Brownian movement which is caused by bombardment of the molecules of the fluid. In motility, the organisms move in a definite direction, whereas in Brownian movement they show no direction.

CULTURE

Four factors are to be taken into account
1. Media providing optimum growth
2. Temperature
3. Atmosphere
4. Cultural characteristics, e.g. size, shape and pigmentation of colonies.

Media

Media can be (a) basic (b) enrichment (c) selective, and (d) indicator media.

1. Basic Media
These contain the necessary constituents for growth—meat extract, peptone and salt, and these are nutrient broth (liquid) or nutrient agar (solid). Many organisms would grow on these types of media and need no other factors.

2. Enrichment Media
These are used for organisms, which need an additional source of nutrition. This can be done by adding blood or serum to the nutrient agar or broth. An enrichment medium used for growth of the *Mycobacterium tuberculosis* contains eggs.

3. Differential and Selective Media
These media by virtue of their chemical composition inhibit the growth of some organisms while at the same time support the growth of others. Examples: eosin methylene blue agar and MacConkey agar contain lactose and dye or an indicator in the decolorized state. Bacteria, which ferment lactose with the production of acid will produce red color or colonies with metallic sheen differentiates the lactose fermenting coliform bacilli from colonies of lactose non-fermenting organisms. Some media, which are used are also highly selective in their action on other organisms. Such media as SS agar, deoxycholate citrate agar and bismuth sulfite agar will inhibit the growth of the majority of coliform bacilli along with many strains of proteus and will permit the successful isolation of enteric pathogens. Tellurite glycerin agar and mannitol salt agar are selective media for the isolation of coagulase positive *Staphylococcus* from material containing other organisms. Phenyl-ethyl-alcohol agar is a selective medium for the isolation of gram-positive cocci in specimens or cultures contaminated with gram-negative organisms particularly proteus. Infusion agar containing potassium tellurite and blood/serum inhibits the growth of normal throat commensals and encourages the growth of *C. diphtheriae*. Some medias make use of the selective antimicrobial activity of some antibiotics and are useful for isolating certain pathogenic organisms from material containing mixed flora. Sabouraud dextrose agar containing cycloheximide and chloramphenicol will support the growth of dermatophytes and most fungi, while markedly inhibiting the growth of many saprophytic fungi and bacteria.

4. Indicator Media
These are largely used for biochemical reactions. The most common example is sugar media containing various carbohydrates such as glucose, lactose, maltose, etc. Christensen's urea medium is used mainly in the iden-

tification of *Proteus,* which has the ability to hydrolyze the urea, and consequently because of the presence of phenolphthalein in the medium, a change of color is produced.

Temperature

Most bacteria, pathogenic in humans, give optimum growth when incubated at body temperature, i.e. 37°C. Some saprophytes, however, grow best at lower temperatures, even as low as 4ºC (cryophilic) and others at high temperatures. The latter are known as thermophilic bacteria and are used in testing effectiveness of sterilization techniques.

Atmosphere

Most organisms need oxygen for growth and are incubated in normal atmospheric conditions. Some pathogens, e.g. tetanus bacilli, will grow only in the absence of oxygen. This is achieved by using McIntosh and Fildes' jar, a thick metal or glass jar with a metal lid which can be clamped down tightly by bolts. On this lid are 2 holes-one an air inlet and the other an outlet. There are also 2 electric terminals. On the underside of the lid is a piece of asbestos saturated with palladium and covered by wire gauze. This is connected to the terminals, and acts as a catalyst in combining any oxygen still present after evacuation of the jar with the hydrogen, which is passed into the jar.

The method is given below.

1. Keep the plates upside down in the jar.
2. Place in the jar an indicator—equal parts of 10% NaOH, 6% glucose and 0.5% methylene blue, boiled until the solution becomes colorless. It should remain colorless throughout incubation. If it turns to its original blue color during incubation, complete anaerobiosis (oxygenless state) has not been achieved.
3. Tightly clamp down the lid.
4. Open the air outlet valve and close the air inlet valve.
5. Attach the apparatus to an exhaust pump, and slowly evacuate the jar (If a glass jar is used, it should be evacuated while enclosed in a padded box to avoid danger of explosion).
6. Allow hydrogen obtained from hydrogen cylinders or Kipp's apparatus in through the inlet valve after closing the outlet valve.
7. Attach the terminals to the main current and leave for 20 minutes. This heats the palladiumized asbestos to assist the combination of hydrogen with any remaining oxygen.
8. Allow a little more hydrogen in via the inlet valve.
9. Put the jar in the incubator overnight. The present day McIntosh-Filde's jars have room temperature catalysts and need no electrical charge. They are left at room temperature for 15–30 minutes before allowing more hydrogen into the jar. *There are other, less complicated methods of achieving anaerobiosis (i.e. an oxygenless state), e.g.*
   a. Boil a tube of nutrient broth and layer over it sterile Vaseline. The boiling removes the oxygen and the Vaseline prevents more entering as the broth cools. The tube is inoculated using a sterile Pasteur pipette.
   b. A sterile iron nail placed in glucose broth which has been treated as in method (1), will maintain anaerobic conditions for some time.
   c. Robertson's cooked meat medium and Brewer's thioglycollate broth are frequently used in the culture of anaerobic organisms.

Some organisms are not anaerobic, but do grow better when the amount of oxygen has been reduced. One simple technique is to place the plates in a tin or wide mouthed bottle with a tight fitting lid. A candle is lit inside the container and the lid replaced firmly. The candle flame will use off the oxygen and give an atmosphere of 5–10% CO_2. The container is placed in the incubator.

Cultural Characteristics

Bacteria grown artificially (in vitro) on agar plates are described as colonies. These colonies vary in size, shape, pigment production, and hemolysis on blood agar depending on the type of media.

Colonies are described as:

1. Shape
Circular, regular, radiating or rhizard.

2. Surface
Smooth, rough, fine, granular shiny, dull, etc.

3. Size
Usually colonies are 2–3 mm in diameter, smaller ones may be less than 1 mm.

4. Contiguity
Colonies may be discrete or swarming.

5. Consistency
May be mucoid, tenacious dry or adherent to the medium.

6. Pigmentation
Some organisms produce pigmented colonies (Staphylococci, *Pseudomonas*).

7. Opacity
On nutrient agar they may be transparent, translucent or opaque.

8. Elevation
Colonies may be raised, low convex, umbilicated or dome shaped.

9. Media Changes
Colonial growth may bring about color changes in the media themselves, e.g. hemolysis on blood agar by hemolytic streptococci. With *Pseudomonas,* the green pigment produced may diffuse into the medium.

Biochemical Reactions

Organisms that are alike in microscopic and cultural characteristic are often differentiated by their reactions in various biochemical tests.

1. Sugar Fermentation
Specific carbohydrate fermentation is a property of some organisms when grown in sugar media. Sugars most frequently employed are glucose, sucrose, lactose, mannite, maltose and dulcite. Usually, these are incorporated into peptone water, but for the more delicate organisms, Hiss's serum water must be used. Meningococci and gonococci will only react in solid serum-sugar media. Each sugar medium has a colored stopper and a set 'color scheme' may be established for the following sugars.

Glucose (green), Lactose (red), Sucrose (blue), Mannite (mauve), Maltose (blue and white), Dulcite (pink).

The organism ferments sugar and produces acid and, in certain groups, gas. Acid production is indicated by a color change of the medium, due to inclusion of a pH indicator. Gas production is shown by placing a small Durham's tube upside down in the medium during its production. Before inoculating the medium the tube should be completely filled with the medium. If gas is produced, small bubbles of gas will be seen in the inverted tube.

2. Other Biochemical Tests
Organisms may further be identified biochemically by their production of indole, change in pH (as shown by the methyl red test), by their utilization of citrate and by another test called the Voges-Proskauer reaction. These 4 tests are especially useful in the differentiation of intestinal pathogens.

Serology

Bacteriologic diagnosis can also be confirmed by estimating antibodies to specific antigens of the bacteria. Examples: VDRL and Kahn tests for syphilis. ASO for β-hemolytic streptococci and Widal for typhoid.

Preparation of Culture Media

Anything used for preparing culture media should be free from living organisms. All media prepared should be sterilized according to instructions for each type of media. pH adjustment should be correct for all media. Since, most organisms grow at a slightly alkaline pH, it should therefore be adjusted between pH 7.2–7.6. Time can be saved by using dehydrated culture media: as per the manufacturer's instructions, weigh the dehydrated medium, add the requisite amount of boiled distilled water, mix the two and sterilize the solution. Given below are methods for preparation of culture media:

Peptone Water

This medium is used for the testing of indole production, for the preparation of sugar media, and when made highly alkaline (pH 8.0–8.4) is used for the cultivation of *Vibrio cholerae*.

Peptone	10 g
Sodium chloride	5 g
Distilled water	1000 mL

Dissolve by steaming. Adjust the pH to 7.5. Filter through paper. Distribute in tubes or bottles. Sterilize at 15 lb pressure for 20 minutes. The commercially available peptone water consists of water-soluble products obtained from lean meat or other protein materials by digestion mainly with a proteolytic enzyme like pepsin, trypsin or papain. The important constituents are peptones, proteoses, amino acids and inorganic salts.

Nutrient Broth

Peptone	10 g
Sodium chloride	5 g
Meat extract	10 g
Distilled water	1000 mL.

Mix the ingredients and allow to dissolve (can be accomplished by steaming it). Adjust pH to 7.6. Phosphates may precipitate out and should be extricated by filtration. Distribute the medium in large bottles and then sterilize at 15 lb for 20 minutes. When 1% glucose is added to this nutrient broth it becomes glucose broth.

Nutrient Agar

Agar-agar is a long chain polysaccharide substance from certain seaweeds. It forms a firm gel in watery solution at concentrations of about 2%. Agar alone has no nutritive properties. It melts at about 95°C and solidifies only when cooled.

To the nutrient broth add 2% of agar—it then becomes nutrient agar. After addition of 2% agar, autoclave at

15 lb for 20 minutes. Clear with white of egg. Autoclave and filter. Distribute into flasks and sterilize at 15 lb for 20 minutes.

Blood Agar

Melt the nutrient agar and cool to 50°C. Aseptically add 5–10% sterile defibrinated sheep (ideally) blood. Mix and pour into petri dishes or tubes which are sloped. Bank blood or rabbit blood may be used.

Chocolate Agar

Add blood to nutrient agar as for blood agar. Mix well and raise the temperature to 80°C keeping well mixed. Leave at 80°C for 10 minutes. Pour into petri dishes or tubes as needed.

Sugar Media

Sugar media are used to study the biochemical reactions of bacteria. To sterilized peptone water add 1% of the required sugar and 1% Andrade's indicator. Distribute into sterile tubes containing inverted Durham's fermentation tubes. Indicator is used to study the acid formation by bacteria. If acid is produced media becomes reddish pink. Instead of Andrades indicator the following indicators can also be used.

Neutral red 0.25% to 1% solution—if acid is produced—pink color.

Phenol red 0.01%—if acid is produced—yellow color.

The sugar media are sterilized by fractional sterilization or tyndallization. The sugar may be caramelized or charred at a temperature higher than 100°C, so it is steamed on three consecutive days in Arnold's steam sterilizer.

Preparation of Andrade's Indicator

Dissolve 0.5 gram of acid fuchsin in 100 mL of distilled water. Add 16 mL of normal sodium hydroxide (NaOH) and leave overnight. The color should change from pink to brownish red and then to yellow.

Hiss's Serum Water Sugars

This is used for biochemical reaction of *Neisseriae. Corynebacterium* and other organisms requiring serum for growth.

Ox serum	1 part
Distilled water	3 part

Adjust reaction to pH 7.5 and Andrades indicator 1% and sugar 1%. Sterilize as for peptone water/sugar media.

Loeffler's Serum Slopes

(Used for cultivating diphtheria bacilli)

Ox serum	3 parts
Glucose broth 1%	1 part

Blood is collected from ox under sterile conditions and serum separated aseptically. Inspissate the medium at 75°C for 1 hour.

Media for Identification of Fungi

Sabouraud's Glucose Agar

Glucose	40 grams
Peptone	10 grams
Agar	20 grams
Water to	1000 mL

Dissolve peptone in water and adjust the pH to 5.4. Add agar and melt it at 15 lb. for 20 minutes. Then add glucose and sterilize by fractional sterilization.

READY TO POUR, STERILIZED POUCHED MEDIA FOR MICROBIOLOGICAL APPLICATIONS INSTAPREP

(*Courtesy:* Tulip Group of Companies)

Summary

Cultivation and isolation of bacteria from pathological samples is many a times key to the identification of the underlying infections. With ever-increasing strains of resistant microorganisms, susceptibility testing to antimicrobial agents complements selecting appropriate drugs/drug regimens to treat infections.

Availability of microbiology testing and such procedures being available in routine laboratories has been limited due to the availability of dehydrated media, which can be put to use only after substantial procedural and preparatory requirements. INSTAPREP media are ready to use/ready to pour and fill this long felt need using a unique proprietary technology for routine microbiological testing.

Reagent

MICROXPRESS INSTAPREP are reagents for laboratory use only.

INSTAPREP is a ready to pour sterilized pouched media for microbiological applications such as cultivation/isolation/selective growth/susceptibility tests.

Nutrient Agar

Nutrient agar is used as a general culture medium. It can be used for maintaining microorganisms for prolonged survival of cultures. It can be used for cultivation of non-fastidious organisms. Addition of sheep blood/or serum makes it suitable for cultivation of related fastidious organisms. The poured medium is light straw colored, slightly opalescent with a pH at 7.4 ± 0.2.

MacConkey Agar

MacConkey agar is the standard medium for the cultivation of enterobacteria. It is a selective and differential medium. It contains a bile salt to inhibit nonintestinal bacteria with neutral red to distinguish the lactose fermenting coliforms from the lactose non-fermenting *Salmonella* and *Shigella* species. The poured medium is a distinct clear reddish brown color with a pH at 7.4 ± 0.2.

Cysteine lactose electrolyte deficient (CLED) Agar with Andrade's Indicator

CLED agar with Andrade's indicator is a medium of choice, recommended for use in urinary bacteriology as it promotes the growth of all urinary pathogens. Additionally since, it is an electrolyte deficient medium swarming due to *Proteus* species is prevented and direct colony count is facilitated. For direct colony count, the medium is inoculated by proper dilution of the sample. Additionally CLED agar helps identify the organism directly from the first isolate based on colony morphology and color within 24 hours. The poured medium is slightly opalescent greenish/gray with a pH at 7.5 ± 0.2.

Sabouraud Dextrose Agar

Sabouraud dextrose agar is the standard agar for the cultivation and growth of fungi; particularly those associated with skin infections. The poured medium is light straw colored slightly opalescent with a pH at 5.6 ± 0.2.

Mueller Hinton Agar

Mueller Hinton agar is the standard agar recommended for susceptibility tests using antibiotic sensitivity disks. Mueller Hinton agar is recommended by NCCLS and WHO Committee on standardization of susceptibility testing for determining the susceptibility of microorganisms because of its reproducibility. The poured medium is light amber colored to slightly opalescent with a pH of 7.3 ± 0.1.

Principle

INSTAPREP ready to pour media are presterilized media with standard proven formulations. The pouched media only need to be kept in boiling (100°C) water for 10 minutes and they become ready to pour into sterile plates. A result of Tulip's long research the INSTAPREP pouched media accord flexibility to the laboratories, thereby avoiding laborious preparatory steps and wastage. INSTAPREP media also help laboratories to set up cultures on a random basis and not to be restricted to batching of cultures. As compared to prepoured plates and dehydrated media, variability, contamination and wastage is also avoided.

Storage and Stability

a. Store the pouches at room temperature (25 and 30°C).
b. Stability of the unopened pouch is as per the expiry date mentioned on the label.

Additional Material Required

Water bath (250 mL beaker) at 100°C, vertical laminar air flow/biosafety hood with Bunsen Burner, forceps/tongs, sterile petridishes (disposable/glass), scissors, disinfectant (70°C alcohol), absorbent sterile gauze, plastic/glass/wire rod for hanging pouches in water bath.

Procedures

1. Retrieve the required number of pouches from the carton.
2. Gently squeeze the gelled media to the bottom of the (Dip side) pouch, up to 'SQZ' mark.
3. Hang the pouches vertically'using a hanging rod in a boiling water bath (at 100°C) with the 'DIP side into the water and the water level up to the 'MAX' mark, for 10 minutes. Ensure that the heat source is not directly applied to the pouch. Retrieve the pouches after 10 minutes. (In case rod hanger is not used for the pouches, remove the pouches using forceps/tongs). After retrieving the pouch it should be dried diligently with gauze and then disinfected. Any residual water from the water bath should not be allowed to drip on to the poured plate to avoid contamination.
4. Wipe dry the pouch corner at the 'CUT' mark and disinfect with 70% isopropyl alcohol (IPA).
5. Cut the pouch across the 'CUT' mark with disinfected scissors.
6. Pinch open the opening at the 'CUT' mark, squeeze and pour out media aseptically into a sterile 9 mm (diameter) petri dish, taking care not to splash or form air bubbles while pouring.
7. Cover the petri dish and allow the poured media to set.
8. The poured plate is now ready to use.
9. The samples should be collected and processed aseptically before plating.

Interpretation of Results

1. Gram stain, biochemical or serological studies should be performed for the characterization and identification of the growth.

2. For CLED and MacConkey agar the microorganisms can be identified as shown in Table 27.1.
3. For susceptibility tests on Mueller Hinton (MH) agar the size of the zone of inhibition corresponds to the sensitivity of the microorganism to the particular antibiotic.

Remarks

1. The temperature of water bath must be at 100°C to liquefy the media. Cooler waterbaths will provide lumpy, uneven media.
2. Since, all agar-based media solidify rapidly, it is important that the minimum time be lost between retrieval of the pouch from boiling water bath and pouring aseptically into the sterile plates. This will produce evenly surfaced medium.
3. The poured plates on solidification can be used for plating specimens or for antibiotic susceptibility tests (Mueller Hinton Agar).
4. Good laboratory practices and hazard precautions must be observed at all times.
5. With CLED medium, use 10 µL urine inoculum and observe the growth at 24 hours. The colony count multiplied by 100 will correspond to CFU/mL of organism in the urine sample.
6. 15 mL media is sufficient for the standard 90 mm petri dishes. In case smaller petri dishes are being used more number of plates can be poured with a single pouch, proportionately.
7. The identity of the medium is imprinted on the sealing band as a product code, whereas the lot number is mentioned on the carton label.

TABLE 27.1: Colony characteristics on CLED and MacConkey Agar

Medium	*Morphology*	*E. coli*	*Proteus*	*Klebsielia*	*Candida*	*Pseudomonas*	*Salmonella*	*Shigella*	*Streptococci*	*Staphyloccocci*
CLED	Colonies	Pink regular	Green irregular	Dull large mucous	White regular	Colorless Convex	-	-	Pink regular	Pink regular
	Medium	Pink /Red	Blue /Green	Green /Gray	Pink	Blue /Green	-	-	Pink/Red	Pink/Red
Mac-Conkey Agar	Colonies	Red convex	Transparent irregular	Pink large mucous	- -	Transparent irregular	Transparent irregular	Transparent irregular		
	Medium	Red	Colorless	Pink/Red	-	Colorless	Original Red/Brown	Original Red/Brown	-	-

Troubleshooting

Problem: Smooth gelled media not formed

Possible causes	*Solutions*
1. Water bath not maintained at 100°C	Water bath should be maintained at 100°C
2. Time period at 100°C is less than 10 minutes	Ensure pouched media is kept for 10 minutes in boiling water bath
3. Maximum time lost between retrieval of the pouch from the boiling water and pouring aseptically into sterile plates	Minimum time should be lost between retrieval of the pouch from boiling water bath and pouring aseptically into sterile plates, i.e. not more than 5 minutes
4. Vibration/Shaking of surface during gelling of the media	Pour the plates on a firm surface not prone to vibration or shaking

Media for Growth of Anaerobes

Thioglycollate Medium

To nutrient broth add

0.1% sodium thioglycollate.
0.05% powdered agar.
1% glucose.
1/500,000 methylene blue.

Sterilize at 10 lb for 15 minutes. The sodium thioglycollate maintains anaerobic conditions present after autoclaving assisted by glucose and the agar, which prevents convection currents in the medium; methylene blue acts as an indicator.

Robertson's Cooked Meat Medium

Mince 500 grams of fat free ox heart (fresh) and place in 500 mL of boiling distilled water and allow it to boil for some time. Drain off the liquid through a muslin filter and while still hot, press the minced meat in a cloth and dry partially by spreading it on a cloth or filter paper. In this condition, it can be introduced into the bottle. Place about 2.9 g of dried meat in a bottle and cover with 10 mL of nutrient broth or the infusion broth filtered from the meat to which is added 0.25% sodium chloride, 0.5% peptone and pH is adjusted to 7.7. Put the caps on the bottles and autoclave at 15 lb for 20 minutes. The material to be inoculated is introduced towards the bottom of the tube in contact with the meat.

MacConkey's Medium

(For enteric gram-negative bacilli)

Peptone	20 g
Sodium taurocholate	5 g
Distilled water	1000 mL
Sodium chloride	5 g

Dissolve this in a steamer and adjust the reaction to pH 7.5. After this, add 2% agar and melt in the autoclave at 15 lb for 20 minutes. Clear with white of eggs. Than add lactose 10 g and 1% solution of neutral red, 7–10 mL to the media. Distribute in 200 mL flasks and sterilize by steaming on 3 consecutive days in the Arnold's steam sterilizer. Organisms which produce acid from lactose e.g. *E. coli* form rose pink colored colonies are called lactose fermenters. Non-lactose fermenters, such as *Salmonella typhi,* produce colorless colonies. Sodium taurocholate is a bile salt, which inhibits the growth of gram-positive bacteria and promotes the growth of enteric gram-negative bacteria.

Lowenstein Jensen Medium

(For cultivation and differentiation of human and bovine types of tubercle bacilli).

Mineral Salt Solution

Potassium dihydrogen phosphate	0.4%
Magnesium sulfate	0.04%
Magnesium citrate	0.1%
Asparagine	0.6%
Glycerol	2% (in distilled water)

Heat to dissolve. Boil the solution by placing a steamer for 2 hours and allow it to cool overnight. 600 mL is a convenient quantity to prepare. Asparagine gives the source of nitrogen and glycerol the source of carbon. To each 600 mL of mineral salt solution add 30 grams of potato starch. A luxuriant growth can be obtained even without the addition of potato starch. Eggs are washed with soap water and immersed in 5% carbolic acid for half an hour. After this wash the eggs in distilled water and break them and filter. For 600 mL of mineral salt solution, 1000 mL of egg fluid is required. Add 40 mL of 1% malachite green solution to this solution. Inspissate the media at 80–85°C for 50 minutes on three consecutive days.

Method of Inoculation

Streak Plate Method

The streak plate if properly prepared offers a most practical way of obtaining discrete colonies and pure culture. But is of no use if plate is not inoculated properly. The plates may be prepared in advance in any desired quantity and stored in the refrigerator until inoculation. The agar surface should be free of water of condensation before the streaking is done.

Method I

This method is designed for culture from broth, agar plates or slopes.

1. Place one loopful of the inoculum near the periphery of the plate.
2. With a loop spread the inoculum over the upper portion of the plate.
3. Stab the loop into the agar several times and continue streaking overlapping the previous streak.
4. Stab the loop as before and continue streaking.
5. Flame the loop allow it to cool and overlap the last streak and complete streaking.
6. Lift the loop and streak the center of the plate with zigzag motions.
7. Discrete colonies should be found in the central portion of the plate while additional importance on hemolytic activity may be gained from the effect of a reduced oxygen tension on the organism stabbed into the media.

Method II

This method of streaking may be used either for culture from solid media or for heavy broth cultures.

1. Select the wanted colony from a crowded plate or pick up growth from a slant with a needle. Streak this carefully on a restricted area of the plate. Flame the needle and put it away.
2. With the sterile cool loop make one light sweep through the needle inoculated area and streak with

close parallel strokes the top one-fourth of the plate surface. Flame the loop and allow it to cool.
3. Turn the plate and streak the remainder of the plate with the same loop. Avoiding any areas previously streaked.
4. Discrete colonies should appear in the areas which was last streaked.

Method III

This method constitutes a simple technique and is used for liquid culture largely but it may also be used for culture from solid media.

1. Place a loopful of inoculum near the periphery of the plate and cover approximately 1/4 of the plate with close parallel streaks. Flame the loop and allow it to cool.
2. Make one light sweep through the lower portion of this streaked area. Turn the plate at right angles and streak approximately 1/2 of the remaining portion without overlapping previous streaks.
3. Turn the plate and streak the remainder of the plate avoiding previously streaked area.
4. The appearance of this plate will resemble that obtained in method II.

Precautionary Measures to be Undertaken in a Microbiology Laboratory

The infected material obtained from patients is to be treated with utmost care. In fact, all individuals working in such a laboratory should wear laboratory coats, wear gloves, use nose and mouth masks and be immunized regularly for prophylaxis against communicable diseases. Outlined below are the rules, which must be observed.

1. Have an air pressure flow (air curtain) at the entry and exit doors of the laboratory. Being expensive, if this cannot be installed, the other precautions mentioned below are to be rigidly followed.
2. Change clothing before entering and leaving the laboratory. If this is not possible, use a laboratory apron to be worn over the outdoor clothing.
3. After working with cultures, and always before one leaves the laboratory, hands should be well washed, first in some disinfectant solution and then with soap and water.
4. Do not eat or drink in the laboratory.
5. The pipettes graduated or Pasteur, should be operated with rubber teats, and should always be plugged at the mouthpiece with cotton wool.
6. Do not use tongue to moisten gummed labels, envelopes, etc.
7. Discarded cultures and contaminated material should be placed in disinfectant for 24 hours or autoclaved before the containers are washed.
8. Wrapping from contaminated material should also be placed in disinfectant and not in the waste paper basket.
9. A bowl of strong disinfectant should be readily available on the working bench, so that supernatant fluids, etc. may be poured there rather than into the sink.
10. Wire loops should be sterilized before and after use and the mouths of tubes and bottles should be passed through the flame on opening and closing.
11. While working, there should not be any current of breeze, hence fans should be off and windows shut.

Specimen Collection

As far as possible obtain specimens before the commencement of therapy. This is important especially for CSF cultures. Often a purulent CSF will reveal no bacterial pathogens on smear or culture when an antibiotic has been given within the previous 24 hours. A patient with enteric fever may show a negative stool culture if the specimen has been collected while the patient was receiving suppressive antibiotics. Another important factor for the successful isolation of organisms is the stage of the disease at which the specimen is collected for culture, enteric pathogens are present in much greater numbers during the acute or diarrheal stage of intestinal infections and they are more likely to be isolated at that time. Specimens should be inoculated as soon as possible. If it is not possible then refrigerate the specimens at 4–6°C. Swabs from wounds, urogenital tract, throat, rectum and samples of feces or sputum can be refrigerated for 2–3 hours after procuring them without appreciable loss of pathogens. Urine specimens may be refrigerated for 12 hours without affecting the bacterial flora. On the other hand, cloudy CSF from a patient with purulent meningitis should be examined immediately. Gastric washing, for culture of *Mycobacterium tuberculosis* should be processed soon after delivery as the Mycobacteria die quickly in gastric washing. Specimens submitted for isolation of viruses should be frozen immediately. Specimens of hair scrapings may be submitted for isolation of fungi may be kept at room temperature before inoculation. Sputum, bronchial secretions, bone marrow and purulent material from patients suspected of having a systemic fungal infection should be inoculated to appropriate media as soon as possible.

All receptacles (containers) for collection of specimens must be sterile otherwise contaminants from the container will also be grown.

Urine

Organisms found in a normal urine are staphylococci (coagulase negative), diphtheroid bacilli and coliform bacteria. The important pathogens are *Escherichia coli, Proteus, Citrobacter, Pseudomonas, Klebsiella, Moraxella, Acinetobacter, Staphylococcus, Streptococcus faecalis, Salmonellae, Mycobacterium tuberculosis,* etc. For non-tuberculosis patients a mid-stream fresh urine specimen is good enough. Urine samples are streaked on blood agar and MacConkey agar plates. At the same time, microscopic examination of the urine should also be carried out. Catheterization is indicated only when a mid-stream specimen (MSS) cannot be obtained, if done—all aseptic precautions must be undertaken. Before collecting the specimen, the area is washed well with soapy water and dried and then the MSS collected. A Gram's stain should be done on the centrifuged sediment.

If tuberculous nephritis is suspected, a 24-hour specimen, or preferably 5 consecutive early morning specimens are sent to the laboratory. The specimens are centrifuged and the deposits pooled. A Ziehl-Neelsen stain is done and the deposits concentrated before culturing for tubercle bacilli.

EASYBACT

(*Courtesy:* Tulip Group of Companies)

Chromogenic, differential, semiquantitative bacteriuria collection and screening system.

Summary

Urinary tract infection is one of the most common infections encountered in clinical practice. Cultivation and isolation of bacteria from pathological urine samples is many a times the key to identification of underlying pathogens. With ever-increasing strains of resistant micro-organisms, susceptibility testing to antimicrobial agents for selecting appropriate drugs/drug regimens is routinely indicated. EASYBACT a ready to use CLED agar slants, fills this long felt need using a unique proprietary technology for routine bacteriuria screening.

Reagent

EASYBACT is a ready to use, precalibrated CLED medium with a pH indicator, for easy isolation, identification and enumeration of bacteriuria in urine within 18 to 24 hours. Colonies obtained from this medium can be further processed for antibiotic sensitivity testing directly using the plate method. The standard medium is slightly opalescent greenish/gray in color. EASYBACT is suitable for urinary bacteriology and supports the isolation and growth of most common urinary tract pathogens such as *E. coli, Klebsiella, Proteus, Candida, Pseudomonas,* Streptococci and Staphylococci.

Principle

As the urine sample is voided/placed in the EASYBACT vial and subsequently emptied, bacteria if present get seeded onto the medium and start growing. EASYBACT has been calibrated to yield colony counts similar to the standard plate methods. EASYBACT has a double indicator system that allows differentiation of various urinary pathogens by differential color formation within the colony as well as the medium. Study of colony morphology allows further identification. Since swarming of *Proteus* species is prevented on this media, this media is convenient for colony count.

Storage and Stability

(a) Store the EASYBACT slants at 2–8°C, away from light. Do not freeze, (b) Stability of the unused slants is as per the expiry date mentioned on the vial/carton labels, (c) Avoid jerks and vibrations while storage, shipping and incubation, (d) Upon opening, the medium must be put into use immediately.

Additional Materials Required

Incubator (37°C), blotting/filter paper, activated 2% glutaraldehyde solution.

Specimen Collection and Preparation

As pathogens accumulate in the patient's bladder overnight, first morning voided urine samples provide the best yield. Aseptically collect midstream clean catch urine or first morning catheterization/suprapubic taps in sterile containers. Fresh urine specimen is recommend for testing. Samples may be tested up to 3 hours when stored at 2–8°C. If the patients can be explained clearly, EASYBACT vial may be used to directly collect the midstream clean catch samples (Refer Notes for collection of midstream clean catch urine).

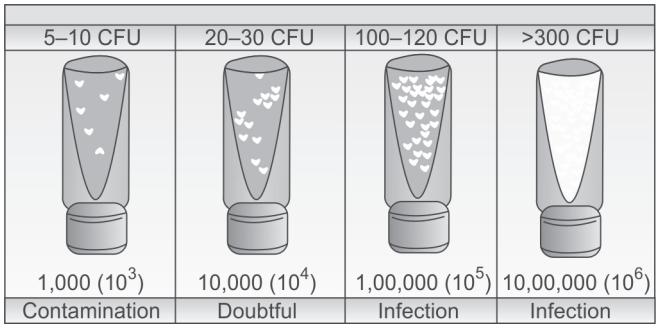
Test Procedure

1. Retrieve the required number of EASYBACT vials from the carton.
2. Bring the slants to room temperature (25–30°C) prior to testing.

3. Label the EASYBACT vials appropriately with the patient's ID.
4. Open the EASYBACT vial observing aseptic conditions.
5. Directly collect clean catch midstream urine into the EASYBACT vial or add urine samples from catheterization/suprapubic taps into the EASYBACT vial, right up to the brim.
6. Empty the urine sample from the EASYBACT vial immediately, observing aseptic conditions.
7. In case small amount of urine is retained in the vial, drain the excess urine by gently tapping the mouth of the EASYBACT vial on to a fresh clean blotting/filter paper. This is done to ensure no excess urine remains on the slant/in the bottle.
8. Recap the vial immediately.
9. Incubate the vial in an incubator, preset at 37°C for 18 to 24 hours, in an inverted position with the cap facing downwards.
10. Read the results at the end of the incubation period.

Interpretation of Results

Density Count: Read the density of the colonies as per the chart given below:



Identification of Bacteria

The pathogenic organisms can be identified as per the color chart provided with the EASYBACT kit and the table mentioned below.

Remarks

1. Discolored, dislodged, or contaminated medium should not be used.
2. Ensure that clean catch midstream urine samples are used so that surrounding external microbial flora do not give discrepant results.
3. Observe aseptic conditions while performing the test to avoid contamination with non-pathogenic bacteria.
4. Urine specimens should be collected and cultured immediately. Prolonged storage of urine samples at room temperature (25–30°C) may result in multiplication of contaminating organisms and raise the bacterial count leading to discrepancies.
5. Cultures of three consecutive first morning urine specimens are universally recommended since the reliability of the test increases to approximately 100%.
6. Whenever possible indicate if the patient is on antibiotic therapy, since urine samples of patients on antibiotic therapy may not show bacterial growth or may have low colony count.
7. Treat the specimens and used slants by immersing in 2% activated glutaraldehyde for at least 2 hours before incineration and disposal.
8. For confirmation and identification of the isolated organism, it is recommended to perform gram stain, biochemical or serological studies.
9. Good laboratory practices and hazard precautions must be observed at all times.
10. When performed correctly, EASYBACT results correlate with standard plate method using calibrated loop.
11. Optionally calibrated loops can also be used for inoculating the sample.

Notes

Procedure for collection of clean catch midstream urine samples. The objective is to collect a specimen, which will reflect as much as possible only the urine present in the urinary bladder.

Thus, a clean midstream void is recommended. Instruct the patient as follows:

1. Wash and clean the private parts with a dilute soap. Remove all traces of soap by washing with large quantity of water. Wipe dry.
2. Void out, into the toilet, the first stream of urine. This will flush out dead epithelial cells of the urinary

	E. coli	*Proteus*	*Klebsiella*	*Candida*	*Pseudomonas*	*Streptococci*	*Staphylococci*
Colonies	Pink	Green	Dullarge	White	Colorless	Pink	Pink
	Regular	Irregular	Mucous	Regular	Convex	Regular	Regular
Medium	Pink/ Red	Blue/ Green	Green/ Gray	Pink	Blue/ Green	Pink/ Red	Pink/ Red

bladder, microparticulates and normal microbial flora, which may have collected in the urine. Then hold the remaining urine in the bladder.

3. Next, void the second stream of urine aseptically into the EASYBACT vial right up to the brim. Again hold the remaining urine in the bladder.
4. Lastly, void out the remaining third stream, into the toilet.
5. This procedure ensures that the urine is voided as three discreet segments (First stream to flush out contaminants, second stream as the clean midstream for test).
6. Follow instructions as mentioned in the test procedure for performance of the test.

Troubleshooting

Problem: Twisting and swirling the media

Possible causes	*Solutions*
1. Slant shaken vigorously	This does not hamper the testing procedure. However, and vibrations should be avoided during storage, shipping and incubation

Problem: Discoloration

Possible causes	*Solutions*
1. Slants stored at higher temperatures, i.e. 25°C	Ensure that slants are stored at 2–8°C to avoid discoloration
2. Contaminated slants	If the slants are found contaminated because of various reasons, discard the slants and use fresh slants to perform the test

Problem: Discrepancy in results

Possible causes	*Solutions*
1. Contamination with non-pathogenic bacteria	Ensure that clean catch midstream urine samples are used so that surrounding external microbial flora do not give discrepant results Observe aseptic conditions while performing the test to avoid contamination with non-pathogenic bacteria
2. Urine samples stored for a long period	Urine samples should be collected and cultured immediately. Prolonged storage of urine samples at room temperature may result in multiplication of contaminating organisms and raise the bacterial count leading to discrepancies Culturing of first morning urine samples is recommended since the reliability of the test increases to approximately 100%
3. Patients on antibiotic therapy	Samples of patients on antibiotic therapy may not show bacterial growth or may have a low colony count Note the history of the patient before diagnosing for infection

Feces

Most of the organisms, which make up the intestinal flora in man belong to the family enterobacteriaceae. These may include the intestinal commensals (the coliform bacilli and *Proteus* species) as well as the enteric pathogens of *Salmonella* and *Shigella*, intestinal streptococci, clostridia and various yeasts including *Candida albicans* may be present. The cholera vibrio may also be isolated.

Samples of feces should be sent to the laboratory in disposable containers, e.g. cartons of waxed cardboard, which are incinerated after use. Since feces contain innummerable bacteria, and since selective media for enteric (intestinal) pathogens are almost invariably used, aseptic precautions are rather futile and unnecessary. It should be remembered, however, that where blood and mucus are present in the stool, this part should be especially selected for culture, since the pathogens are most likely to be found there.

The feces are inoculated into a solid bile salt medium such as MacConkey agar or deoxycholate-citrate-agar (DCA) and also onto Wilson and Blair's bismuth sulfite medium. Liquid media such as Selenite F which will inhibit the growth of the coliform bacilli or brilliant green broth, which will enhance the growth of the pathogens: are also inoculated. These are all incubated at 37°C over-night. A Gram stain of the feces is of little value except in cases of fungi and staphylococci infections.

Sputum

The commonly isolated organisms from sputum are pneumococci, beta-hemolytic streptococci and

Mycobacterium tuberculosis. If the culture for organisms other than *M. tuberculosis* is needed, all possible care has to be taken while doing so. Rinse the mouth with an antiseptic or clean water to avoid contamination from the oral cavity. The specimen should be collected in a wide mouthed sterile jar with a screw-cap lid. If the sample has to be concentrated when culturing for *M. tuberculosis*, there is less need for a sterile jar, in fact, disposable waxed cartons are preferable since they and their contents can safely be incinerated.

For routine cultures, a loopful of the sputum is inoculated onto one/two blood agar plates. When two are inoculated one is incubated in a 5–10% CO_2 atmosphere. A smear is made from specimen and stained by Gram's stain. If the need be Ziehl-Neelsen staining can also be done.

Throat and Nasal Smears

Organisms commonly isolated from a normal throat are Alpha-hemolytic streptococci, *Neisseria catarrhalis*, staphylococci, non-hemolytic streptococci, pneumococci and *Coliform bacilli*. The pathogens usually encountered are beta-hemolytic streptococci, *Corynebacterium diphtheriae, Bordetella pertussis*, meningococci, *Staphylococcus aureus*, *Haemophilus influenzae* and *Candida albicans*.

The sterile swabs should be first moistened with normal saline (sterile) and then rubbed over the infected area.

A blood agar plate is inoculated. A Gram's stain is rarely necessary except in cases where diphtheria or Vincent's angina are suspected. If diphtheria is suspected, the smears should by stained by Albert's, Ponder's or Neisser's techniques.

Pus Swabs

The infected area is carefully swabbed with spirit before the swab is taken, or the pus aspirated. Carry out a Gram's stain, streak on blood agar, MacConkey agar and then place into nutrient broth or, if anaerobes are suspected, into thioglycollate broth or Robertson's cooked meat medium. In cases of suspected gangrene or tetanus: two blood agar plates should be inoculated, one for aerobic culture and the other for incubation in an anaerobic atmosphere.

Blood Cultures

Bacteremia is an important part of any systemic infection and hence, blood culture acquires similar significance. Blood culture permits the prompt commencement of specific treatment against the offending organism and may prove to be lifesaving.

Bacteremia occurs transiently in pneumococcal pneumonia, bacterial meningitis, urinary tract infections, enteric fever and generalized *Salmonella* infections. A mild transitory bacteremia is a frequent finding in many infectious diseases but a persistent bacteremia points towards a more serious infection. When the classic syndrome of a septicemia is due to pyogenic organism, chills, fever prostration is found, one rarely finds difficulty in isolation of the causative organism. In some diseases the chances of isolating bacteria from blood culture depends on the stage of the disease at which the culture is done. For example, bacteria can be cultured during the early course of the disease, thus the cultivation of the bacteria from the blood is important since, it may be the only reliable means of making an early diagnosis available for the physician. A diagnosis of bacteremia can only be made by growing the pathogenic agents on suitable culture media. Perfect aseptic conditions must be observed while collecting blood for blood culture. Enriched aerobic and anaerobic culture media must be utilized in order to provide optimal conditions for bacterial growth. Commonly isolated pathogens from blood cultures are:

- α and β hemolytic streptococci
- Staphylococci, pathogenic and saprophytic
- *Coliform bacilli* and related organisms
- Pneumococci
- *Haemophilus influenzae*
- Enterococci
- *Clostridium perfringens*
- *Pseudomonas* species
- *Bacteroides* species
- *Neisseria meningitidis*
- *Salmonella* species
- *Pasteurella tularensis*
- *Leptospira* species
- Pathogenic yeasts and molds.

Keep all the required things—the culture media (nutrient or glucose broth), spirit lamp, pen for labeling, etc.—by the bedside of the patient. Three bottles of broth should be incubated, one for aerobic cultivation, one for anaerobic and other for incubation in 5–10% CO_2. The site selected for venipuncture is well swabbed with cotton moistened in spirit and with tincture of iodine. The needle is fitted onto the syringe without touching the needle or the nozzle of the syringe. Blood is withdrawn—about 15 mL. After withdrawing needle from arm, the needle is passed through the flame. Remove protective covering from culture bottle. The top of the screw-capped bottle is flamed and the needle inserted into the bottle through the rubber washer and 5 mL of blood is placed in each bottle. The bottle is again flamed and protective covering replaced on the bottle-top. The same procedure is carried out for each of the three bottles.

Once collected the cultures should be incubated at 37°C immediately and left overnight in the appropriate

atmospheres. Subcultures are made every two days, either until growth is found or until 2 weeks have elapsed. Only after 2 weeks have elapsed can a report of 'No growth' be sent.

If the patient has already been on penicillin or sulfonamide therapy, penicillinase or paraminobenzoic acid may be added respectively. These substances will counteract the effect of any of the drugs present in the serum. Counteracting agents for other antibiotics are not yet known.

CSF, Pleural Fluid and Other Body Fluids

The aspirated material should be sent to the laboratory immediately in sterile tubes or bottles.

They are cultured on blood agar, aerobically, anaerobically and in 5–10% CO_2. Gram and Ziehl-Neelsen stains are done on smears prepared from centrifuged sediments.

Broth cultures should also be setup. The plates and subcultures of the broth should be incubated for 48 hours before reporting as negative (i.e. No growth). Organisms isolated from CSF are: *Haemophilus influenzae, Pneumococcus, Neisseria meningitidis, Mycobacterium tuberculosis, Staphylococcus, Streptococcus,* Coliform bacilli, *Pseudomonas* and Viruses.

Ear Discharge Cultures

Organisms isolated from the ear are:

Nonpathogenic

- Coagulase negative staphylococci and diphtheroids.

Pathogenic

- *Pseudomonas, Staphylococcus, Proteus,* pneumococci, α and β hemolytic streptococci and *Coliform bacilli*.

Material from ear is taken by swab sticks. Do a Gram stain and culture to a nutrient broth, blood agar and MacConkeys agar.

Eye Cultures

Organisms isolated from the infections of the eyes are *Staphylococcus aureus*, *Neisseria gonorrhoeae,* pneumococci, α and β hemolytic streptococci and *Haemophilus*. Purulent material may be obtained from the conjunctiva with the help of a cotton swab stick. This should be inoculated to blood agar and chocolate agar and to thioglycollate media. Chocolate agar should be incubated in a 10% CO_2 jar.

GENERAL INSTRUCTIONS FOR MICROBIOLOGY

Storage of Organisms

It is unwise to maintain bacteria and fungi for long periods, in case they become contaminated. Therefore, organisms should be subcultured and checked for purity every 3 months or so to yield good results.

Precautions before and during Testing

- Before beginning practical work, hands should be washed with soap and warm water and so also after completing all testing procedures
- Hand to mouth operations such, as chewing, sucking, or mouth pipetting should be avoided
- Disposable plastic gloves should be worn during handling of infectious material.

Specimen Collection and Preparation

Collect specimen prior to use of antimicrobial agent. Wherever possible, indicate clearly that the patient is on antitubercular drugs.

- *CSF:* Collect as much as possible in a syringe, clean skin with alcohol before aspirating specimen
- *Body fluids:* Disinfect the site and collect specimen with aseptic precautions
- *Sputum:* Collect 5 to 10 mL in a sterile container from an early morning specimen of deep productive cough. For induced specimen, use sterile saline. Have patients rinse mouth with water to minimize specimen contamination with food particles, mouth wash or oral drugs
- *Urine:* As organisms accumulate in the bladder overnight, first morning void provides best yield. Collect midstream clean catch urine, first morning catheterization/suprapubic taps in sterile containers.

Inoculation of Samples

- The work surface should be swabbed with a suitable disinfectant before commencing the testing procedure as well as on completion of the testing procedure
- Sterilize inoculating loops by flaming in a Bunsen burner flame to avoid contamination. Improper decontamination procedure may lead to erroneous results
- Treat the unused specimen and contaminated containers by immersing in 2% activated glutaraldehyde for at least 2 hours before incineration and disposal.

GRAM-POSITIVE COCCI

Staphylococci

Morphology

Gram-positive cocci, mainly arranged in cluster, but also found in pairs or singly.

Culture

Colonies measure 2–4 mm in diameter, and are thick opaque, shiny discs. Each species produces a characteristic pigment, e.g.

Staphylococcus aureus	Golden yellow
Staphylococcus albus	White
Staphylococcus citrous	Lemon yellow
Staphylococcus roseus	Pink

The organism grows well on basic media, can be grown on nutrient agar, milk agar and also on MacConkey agar, although on MacConkey the colonies are much smaller and appear deep pink in color.

Coagulase Test

This test is employed to differentiate pathogens from non-pathogens. It depends on the production of an enzyme coagulase, which coagulates blood plasma. This test may be done in 2 ways.

1. Slide Test

A suspension of staphylococcal colonies is made in a loopful of saline on a slide, and a loopful of undiluted plasma is added.

Positive reactions are seen as 'clumping' of the suspension in a few seconds. In negative reactions, the suspension remains uniform.

2. Tube Test

The organism should preferably be cultured in nutrient broth overnight, but if urgent results are needed, a suspension made in broth can be used.

About 1 mL of the culture (or broth suspension) is placed in a sterile tube and an equal quantity of plasma (1/10 dilution) is added. The tube is incubated for at least 4, but not more than 6 hours. If the tube is incubated longer, another enzyme fibrinolysin produced by the bacteria will dissolve the clot formed in a positive reaction, thus giving a false negative reaction.

The tube test is more reliable as it has less false positives. Negative control organisms should always be set up, whichever method is used.

Staphylococci liquefy gel and hemolyze blood.

Pathogenicity

Staphylococcus aureus is usually the pathogenic variety causing inflammatory lesions with pus formation, e.g. boils, furuncles, blepharitis, pemphigus. It may also produce food poisoning due to enterotoxin.

Streptococci

Morphology

Streptococci are gram-positive cocci, arranged mainly in chains of varying length. They may be roughly divided into three groups by their effect on blood agar.

Alpha-hemolysis

α-hemolysis—a green coloration of the medium around the colonies, e.g. *Streptococcus viridans.*

Beta-hemolysis

β-hemolysis—a clear zone of hemolysis around the colonies, e.g. *Streptococcus pyogenes.*

Gamma-hemolysis

Gamma-hemolysis—no obvious alteration of medium around the colonies, e.g. *Streptococcus faecalis.*

Culture

In culture, they are all small (1 mm diameter), discrete, shiny, regular and semitransparent colonies, *Strep. pyogenes* and *Strep. viridans* require blood or serum for growth, but *Strep. faecalis* will grow easily both on nutrient agar and MacConkey's agar. Another means of differentiating *Strep. faecalis* from the other two is by heating a broth culture or suspension of the organism to 60°C for 30 minutes. Only *Strep. faecalis* will withstand this heating and grow subsequently if cultured. Such means of differentiating are important, as *Strep. faecalis* may appear alpha or beta hemolytic and be mistaken for one of the other streptococci.

However, the most reliable way of differentiating the β-hemolytic streptococci is by serological testing. Lancefield divided this group (of which there are many types) into several serological types, designated A-Q by precipitation reactions between the antigenic extract of the organism and antisera.

Pathogenicity

Streptococcus pyogenes

This frequently is a cause of sore throat, tonsillitis and scarlet fever. It may also give rise to cellulitis and also be responsible for infection of burns and septicemia.

Streptococcus viridans

This is usually a commensal in the upper respiratory tract but is sometimes the cause of subacute bacterial endocarditis, in which the organism is isolated from blood cultures (up to 3 successive cultures may be needed for isolating the organism).

Streptococcus faecalis

This is a commensal of the intestine, but is known to cause urinary tract infections or may be a secondary invader in such causes.

Pneumococci

Morphology

Diplococcus pneumoniae are oval shaped, gram-positive diplococci, the more virulent strains being capsulated. The diplococci are placed end to end, and in culture, may be arranged in short chains (usually).

Culture

After overnight incubation, the colonies appear similar to *Strep. viridans*, and show the same alpha hemolysis, but on further incubation the center and edge of the colony become raised, with concentric rings.

The organism is aerobic and needs the enrichment of blood/serum for growth. Pneumococci may be differentiated from *Strep. viridans* by their solubility in bile salts (e.g. sodium taurocholate). When the bile salt is added to a broth culture of the organism, lysis can be observed within 15 minutes. The test may be carried out by adding 0.1 mL of a 10% solution of sodium deoxycholate to 5 mL of broth culture.

Other Differences between Pneumococci and Streptococcus viridans

	Pneumococci	***Strep. viridans***
1.	Soluble in bile	Not soluble
2.	Sensitive to optochin	Not sensitive
3.	Ferments inulin	Does not ferment inulin
4.	Draughtsman colonies	Small convex colonies.

Pathogenicity

Pneumococci are frequently the cause of pneumonia, empyema, and meningitis. They also cause subacute-bacterial endocarditis and sometimes septicemia.

GRAM-NEGATIVE COCCI

Neisseria

Bacteria belonging to this group are gram-negative diplococci, arranged side-by-side and tending to be rather kidney shaped. Clinically three of them are important. viz. *N. catarrhalis*—a commensal in throat and upper respiratory tract: *N. meningitidis* causing meningitis; and *N. gonorrhoeae* causing gonorrhea.

N. catarrhalis

Mistakenly it was thought to cause catarrh but is no longer incriminated as the causative organism.

Morphology

In morphology, it is typical of the group, gram-negative diplococcus (sometimes also in tetrads) with long axis, parallel (i.e. side-by-side).

Culture

In culture, it differs from *N. meningitidis* and *N. gonorrhoeae* is that it will grow at room temperature and needs no enrichment medium. Colonies are 1–2 mm in diameter, very dry and difficult to emulsify in saline. They have a wrinkled, shriveled up appearance.

N. meningitidis

Morphology

The morphology is typical of *Neisseria* group. They may be present intracellularly within neutrophils of CSF in a case of meningitis.

Culture

It is best to isolate them on chocolate agar in 5–10% CO_2 atmosphere. The colonies are approximately 1 mm in diameter, shiny, smooth and easily emulsified in saline. The organism cannot grow at room temperature.

N. meningitidis and *N. gonorrhoea* can be distinguished from others by morphological and cultural characteristics alone but oxidase reaction can also be used for differentiating them. In this test, a weak solution of tetramethyl para phenylenediamine is poured over the culture plate. *Neisseria* colonies turn purple in color, other colonies retain their normal colors.

(If subcultures are to be made, the colonies must be picked off from the medium, and subcultured onto another plate as soon as possible after the solution has been added).

Biochemical (sugar) reactions are best done on solid sugar media although the reason for this is still obscure. *N. meningitidis* ferments glucose and maltose.

N. gonorrhoeae

Morphology

The morphology is similar to *N. meningitidis*. Intracellular forms may be seen in polymorphs in the exudate from the gonorrheal sore.

Culture

Serum, blood or chocolate agar is used for the isolation of the organism. The colonies of which are very smooth, regular, shiny in appearance at first, but later become crenated at the edge, with a raised opaque center. The colonies give a

positive oxidase reaction. The organisms ferment glucose only, with best results obtained using solid sugar media.

Comparison of meningococci and gonococci

	Meningococci	*Gonococci*
1.	Ferments maltose and glucose	Ferments glucose only
2.	pH of the medium should be between 7.0 and 7.4	pH of the medium should be 7.5
3.	Causes meningitis, conjunctivitis, endocarditis	Causes gonorrhea in the genitalia; conjunctivitis and arthritis

N. gonorrhoeae is very susceptible to drying and hence, material obtained should be processed immediately. One may even take the plates to be streaked at the patient's bedside or else the swab may be placed in Stuart's transport medium and sent to the laboratory in this. If the samples have to be sent by post then the latter method is most suitable and ideal.

Veillonella

These are minute, gram-negative, spherical diplococci found in the digestive tract and vagina. They are generally regarded as non-pathogenic but it is possible that at times they may be associated with disease states. They are anaerobic and produce acid and gas in glucose.

ANAEROBIC SPORE BEARING BACILLI

Clostridia

Clostridia comprise the gram-positive spore bearing anaerobic bacilli. some of them decompose protein and may form exotoxins. Majority of them are saprophytes in oil and a few are pathogenic to man.

Pathogenic Clostridia

Tetanus—*Clostridium tetani*

Gas gangrene—*Clostridium welchii/perfringens, Clostridium novyi* and *Clostridium septicum*

Botulism—*Clostridium botulinum.*

Clostridium tetani

Clostridium tetani is causative organism of tetanus in man and animals. Tetanus is usually the result of contamination of a wound with *Cl. tetani* spores. The source of the infection may be soil, dirty clothing or dust spore of *Cl. tetani*.

Morphology

It is a gram-positive bacillus 2.5 × 0.4–0.5 μm and possesses a spherical terminal spore. The terminal spore gives it a drumstick appearance. *Clostridium tetani* is motile organism.

Culture

Clostridium tetani is an obligatory anaerobe. It is readily grown on cooked-meat medium. On blood agar, under anaerobic conditions, *Cl. tetani* grows and produces hemolysis. In cooked meat medium, there is slight digestion and blackening of meat.

Clostridium tetani does not ferment any sugar. These bacilli, however, slowly liquefy gelatin. Coagulated serum is rendered more transparent and softened but is not liquefied. *Cl. tetani* is a proteolytic organism.

Clostridium tetani toxins are extremely harmful to man as well as animals. Man is, however, more susceptible to toxins.

Laboratory Diagnosis

The material is obtained from the wound.

a. *Microscopic diagnosis:* Material obtained from the wound is smeared on to a slide and stained by Gram's method. The presence of drumstick bacilli is suggestive of *Cl. tetani,* but it is not conclusive as other organisms having terminal spores as *Cl. tetani* may be present. Culture, therefore, becomes mandatory.
b. *Cultural techniques:* The most ideal medium for this purpose is the cooked-meat medium. Strict anaerobic conditions are a must. The cooked-meat medium is heated to 75–80°C for about 20–30 minutes to destroy the vegetative organisms. After inoculation, incubate for 4 to 5 days. Make smears from culture, stain and examine for typical gram-positive bacilli with spherical terminal spores.
c. *Animal inoculation:* Toxin formation may be studied by culturing the organism in a suitable liquid medium and injecting the bacteria free filtrate into a susceptible animal (white mouse), which will develop the signs and symptoms of tetanus. It is always necessary to use two animals, one with the toxin alone and the other which has been given a prophylactic dose of antitoxin.

Toxin Produced

Tetanospasmin—is neurotoxin and tetanolysin—lytic for RBC's.

Gas Gangrene Causing Organisms

Gas gangrene is a massive necrosis of tissues with gas formation and associated with an extreme toxemia. The main factor in gas gangrene is infection by anaerobic bacteria, which leads to the death of tissues and gas formation. The gas is produced by organisms by fermenting

tissue carbohydrates, *Clostridium welchii/perfringens, septicum* and *novyi;* all belong to this group.

Clostridium welchii (or C. perfringens)

Morphology

It is a long rod-shaped gram-positive bacillus forming a large oval and central or subterminal spore. It is non-motile.

Culture

Cl. welchii grows on all the usual laboratory media but needs strict anaerobic atmosphere and rather prolonged incubation, may need as long as 15 days. Best growth is obtained by using glucose agar or glucose blood agar. On cooked-meat medium, it has a luxuriant growth, produces gas and a sour odor, the meat being turned pink in color. On blood agar, marked hemolysis is produced.

The organism is mainly saccharolytic with little or no proteolytic action. It ferments glucose, maltose, lactose and saccharose with formation of acid and gas. It liquefies gelatin. On coagulated serum medium, there is no digestion or liquefaction of proteins. In litmus milk, *Cl. welchii* produces acid, gas and clotting. The formation of gas is so abundant that its action on this medium is referred to as stormy fermentation.

Toxins produced

Alpha, beta, epsilon, iota, theta, gamma, delta, etc.

Clostridium septicum

Morphology

This is a moderately large bacillus, with rounded ends and is motile. In the tissues, it develops into large, swollen, gram-positive citron bodies. Spores are readily formed and are oval, central or subterminal and bulging.

Culture

It is an obligatory anaerobe, which grows on ordinary media. Glucose promotes the growth of the organism. In litmus milk medium, slight acid is formed, and the milk is slowly clotted, but often the change is minimal. Gelatin is liquefied. In cooked-meat medium the meat is reddened but not digested. *Cl. septicum* may be associated with gas gangrene in man.

Clostridium novyi

Morphology

This resembles *Cl. welchii* morphologically, but is somewhat larger and more pleomorphic. The spores are oval, central or subterminal.

Culture

It is an obligatory anaerobe and grows well on ordinary media.

In litmus milk, late clotting may occur. It liquefies gelatin.

Clostridium novyi is associated with a markedly toxic form of gas gangrene in man.

Diagnosis of Gangrene

The bacteriological diagnosis of gas gangrene is usually combined with a general bacteriological examination of the infected wound with which this condition is associated. Specimens of the exudate should be taken from deeper parts of the wound and where the infection is most pronounced. These may be obtained in capillary tubes, but sterile swabs rubbed over the wound surface and soaked in the exudate, serve well for the purpose. At least 2 swabs should be taken from the wound, one for direct smear and another for culture. If there are necrotic tissue fragments in the wound, small pieces should be placed in a sterile screw capped bottle and used for microscopic examination and culture.

Microscopy

Gram's staining of the films should be done. If gas gangrene is present, gram-positive bacilli predominate and show subterminal spores.

Culture

The material is inoculated into cooked-meat medium. In this, all organisms will grow. The mixed growth is heated to 75–80°C for half an hour and then plated on blood or glucose agar and incubated anaerobically. The single colonies are stained and studied by biochemical reactions.

Clostridium botulinum

This is the causative organism of botulism, a kind of food poisoning due to eating tinned meat or improperly cooked meat which had been previously infected with the bacilli. The organism is widely distributed in soil and may sometimes be found in the intestinal tracts of certain domestic animals.

Morphology

It is a fairly large bacillus, 4–6 μm long and about 1 μm thick, arranged either singly or in short chains. It forms an oval terminal spore, which is thicker than the bacillus. It has slight motility.

Culture

It is a strict anaerobe. It grows well at low temperature of 20°C, although the optimum temperature is 35°C. The organism grows on ordinary media, abundant growth is produced in cooked-meat medium.

The organism is both proteolytic and saccharolytic, the former property predominating.

In cooked-meat medium, vigorous growth takes place with the blackening of the meat. Gelatin is readily liquefied.

Laboratory Diagnosis

Botulism is a food poisoning, the suspect food should therefore be examined bacteriologically. It may occasionally be possible to demonstrate the presence of toxin in the patient's blood or in the postmortem material, e.g. blood, liver—by direct animal inoculation. Gram stained film of the food may first be examined for spore forming gram-positive bacilli.

A portion of the suspected food stuff may be given to a susceptible animal, which feeding on it, may show signs and symptoms of botulism. The food can be injected into the peritoneal cavity of a mouse after suspending it in saline and obtaining supernatant fluid. After death, postmortem is carried out and the infected lesions are cultured and *Cl. botulinum* is obtained in pure culture in case of botulism.

AEROBIC SPORE FORMING BACILLI

These organisms are gram-positive spore forming bacilli, which may be arranged in chains. The majority of them are non-pathogens barring *Bacillus anthracis,* which causes anthrax.

Bacillus anthracis

This is the causative organism of anthrax in cattle, sheep and other animals. It is infective for man, generally by spores entering through injured skin or by inhalation of the spores from infected animals.

Morphology

The organism is an aerobic, large, non-motile bacillus with a cylindrical spore. The bacteria may often be arranged in chains and may often be surrounded by a capsule.

Culture

On agar, the colonies are white, granular and circular with wavy margins like "medusa head".

Gelatin stab shows an "inverted fir tree appearance".

Diseases Caused

1. Malignant pustule
2. Woolsorter's disease.

Laboratory Diagnosis

1. Smear from exudate to demonstrate the bacilli
2. Cultures
3. Guinea pig or mouse inoculation
4. Ascoli's precipitin test.

Bacillus subtilis

This organism is commonly found in air and soil. This species of often found as a contaminant in the laboratory. Morphologically, it is a long slender, motile bacillus, which may be found in chains. The colonies are large, flat and dull with a typical ground glass appearance.

Bacillus cereus

This species is a common contaminant in the laboratory. Morphologically it is a large, motile bacillus, which usually occurs in tangled chains. The colonies are small and smooth, however, some of the colonies may be spreading.

GRAM-POSITIVE BACILLI

Corynebacteria

Corynebacteria are gram-positive rod-like forms. They are aerobic, non-motile, non-sporing, non-acid-fast, often staining irregularly and having a beaded appearance. Frequently, irregular swelling at one end gives the organism a club-shaped appearance. *Corynebacterium diphtheriae* is the most important in this species and there are three types, gravis, intermedius and mitis. Gravis is the most virulent and mitis the least.

Morphology

Slender, straight or slightly curved rods, non-motile and non-sporing. They may show a beaded appearance due to uneven staining and arranged in a "Chinese letter" formation pattern. Albert's stain is the stain of choice, the bacilli appearing green with blue-black beading due to volutin granules.

Culture

Various media on which they can be grown are:

1. Ordinary nutrient media, e.g. nutrient agar and blood agar.
2. Loeffler's serum agar—on this the colonies are small, circular, white and opaque with thick centers and crenated borders.
3. Potassium tellurite—on this three types of colonies are seen:
   a. Gravis—relatively large, grayish black, flat lusterless colonies appearing like "daisy heads".
   b. Intermedius—relatively small, black, lusterless colonies with domed centers resembling "poached eggs".
   c. Mitis—Convex, smooth, translucent colonies.

Elek's gel precipitin test is positive and demonstrates the powerful exotoxin produced. Some strains produce hemolysis.

Diseases Caused

1. Diphtheritic inflammations, e.g. pseudomembranous inflammation in the fauces.
2. Acute myocarditis.

Laboratory Diagnosis

1. Elek's test.
2. Intradermal injection (of material to be tested) into guinea pig.
3. Schick test: Intradermal injection of toxin. Positive reaction is an area of redness 1–5 cm in diameter by the fourth day.

Diphtheroid bacilli

These are nontoxigenic corynebacteria with little or no pathogenicity.

Corynebacterium hofmannii

This is a commensal of the throat. Morphology and staining—compared to the diphtheria bacillus it is shorter and may present an oval shape. It is strongly gram-positive and no volutin granules are detected by Albert's staining. It grows on ordinary media aerobically.

Corynebacterium xerosis

This is a commensal in the conjunctival sac. Closely resembles diphtheria bacillus and many show volutin granules.

Corynebacterium acne

This is an organism associated with acne. It is gram-positive, rod-shaped and shows marked pleomorphism some show beaded appearance.

MYCOBACTERIA

Mycobacteria are non-motile, rod-shaped bacteria, usually slender, straight or slightly curved, but occasionally with slender filaments or even branching. Most species do not stain easily, but when stained with strong dyes resist decolorization with acid, and hence the name—Acid fast bacilli (AFB). They are aerobic, non-spore bearing, and most strains grow slowly, but a few stains grow rapidly, not only at 37°C but even at 22°C.

Strains of Mycobacteria

a. Pathogenic species
   - M. tuberculosis:
     - *hominis*
     - *bovis*
     - *avium*
   - *M. fortuitum*
   - *M. paratuberculosis*
   - *M. ulcerans*
   - *M. balnei*
   - *M. leprae*
   - *M. lepraemurium.*

b. Non-pathogenic species
   - *M. smegmatis*
   - *M. phlei*
   - *M. butyricum.*

c. Anonymous strains
   - Photochromogens
   - Scotochromogens
   - Battey types
   - Rapid growing saprophytes.

Morphology

It is a slender or slightly curved bacillus, in direct smears measuring about 2.5 to 3.5 by 0.3 µm although both shorter and longer forms may be seen. In cultures, short forms are found especially on solid media, but longer forms may be found in liquid media. The bacillus may occur singly, or in pairs, or in larger or smaller masses.

The tubercle bacillus (TB or Koch's Bacillus) does not stain easily by the ordinary dyes, but it stains well with a strong dye with a mordant, such as carbol fuchsin when the stain is hot but it takes a longer time when the stain is cold. When once it has been stained it resists decolorization with 20% H_2SO_4 or HNO_3 (Nitric acid); it also resists decolorization with alcohol, and so it is both acid and alcohol fast. The method generally used for staining is Ziehl-Neelsen's method or one of its modifications. The bacilli may stain evenly, or they may show beading or barred staining or sometimes may have terminal granules. The bacilli are gram-positive but by this method they are stained only with great difficulty and Gram's method is of no use for their identification. A more recent method is the use of auramine-phenol fluorescent stain.

Cultures

The tubercle bacillus will not grow on ordinary media. Primary cultures are usually made on some form of egg

medium of which Lowenstein-Jensen (LJ) medium is probably the most widely used. Dorset's egg medium or Petragnani's media may also be used. After the primary culture has been established, it is possible to make subcultures on media without egg, such as Dubos, Proskauer and Beck, Kirschner and Youman's media.

All the varieties, viz. hominis, bovis and avium may be found in man but in India, the bovine seems to be rare in man, and the avian is not a common finding.

Cultural Characteristics

M. tuberculosis is aerobic, and the optimum temperature for the human and bovine varieties is 37ºC, and for the avian 40–44ºC. It grows slowly. It does not grow on ordinary media but for the primary isolation requires an enriched medium, a medium including eggs is used most commonly. Glycerol stimulates the growth of the human and avian varieties but not the bovine. On LJ medium, the general appearance of the growth is dry, irregular, tough and tenacious, a buff to light orange in color, but if the surface of the medium is moist the appearance of the colony is smoother. The growth is said to be eugonic that is growing well. The bovine variety is dysgonic that is growing with difficulty and the colonies are smaller, discrete, rather smooth, slightly moist, and grayish yellow in color. The avian grows rapidly, the colonies are moister, more luxuriant and individual colonies have a smooth shiny surface, yellowish to faint pink in color.

Laboratory Diagnosis

In many specimens the finding of acid-fast bacilli typical in shape and staining, is accepted as sufficient for calling them tubercle bacilli, but it must always be remembered that there are many other acid-fast bacilli, which may be found particularly in stomach-wash, urine, feces, and even in sputum. Also when bacilli are few in number, they may not be found in direct smear. Concentration methods such as sodium hydroxide and trisodium phosphate may help but on the whole considerable labor is involved without a great increase in positive findings. Therefore, cultures are being increasingly used even if 3 to 4 weeks elapse before a positive result is given, and most workers report a negative only after 6 to 8 weeks.

The specimens most commonly examined for tubercle bacilli are sputum, stomach wash, laryngeal swabs, urine, CSF, pleural or peritoneal fluid, pus and tissue. The specimens are collected in clean, sterile vessels, and most are treated with 4% NaOH or 6% H_2SO_4 (v/v) before the culture is made. The treatment homogenices the specimen and also destroys organisms other than mycobacteria.

Concentration Methods

Except in very severe cases, the organism is not always present in large numbers and for this reason, techniques have been devised for concentrating the organisms present to facilitate their detection and isolation. Concentration serves two other purposes.

1. The mucoid material is broken down and the sample homogenized.
2. Other unwanted bacteria are killed, thus allowing the tubercle bacillus to grow in pure culture.

Petroff's Method

This is considered to be one of the most reliable methods.

1. Half fill a universal container with the sputum (or other material) and add an equal quantity of 4% NaOH (For lesser samples a proportionately equal quantity should be added).
2. Invert the closed container twice or three times and place in the incubator for 30 minutes, inverting it every 10 minutes.
3. Centrifuge at 3000 rpm for 30 minutes (The centrifuge must be completely at a standstill before opening it again after centrifuging tubercular samples.
4. Discard the supernatant.
5. Add a few drops of neutral red indicator to the deposit.
6. Neutralize with 8% HCl.
7. Inoculate neutralized deposit on the LJ or some other egg medium.

Oxalic Acid Method

This is primarily used for laryngeal swabs, but is rather unreliable in that it does not completely ensure the destruction of untoward organisms.

The swab is simply left in oxalic acid for 30 minutes, and then smeared on the egg medium.

Trisodium Phosphate Method

This, too, is less preferable than Petroff's method, because it needs much longer incubation periods.

An equal quantity of sample and 10% trisodium phosphate are incubated for 24 hours, after which the container is centrifuged for 30 minutes and the supernatant discarded.

The deposit is neutralized with 8% HCl using bromothymol as indicator.

Other Pathogenic Mycobacteria

M. fortuitum

This bacillus has been isolated from soil and from suppurative infections in men and animals and also in

glandular infections. It grows rapidly and is not pathogenic for guinea-pigs.

M. paratuberculosis

This is also known as bacillus of Johne's disease and is the cause of a chronic enteritis in cattle and sheep. The primary isolation is difficult and medium has to contain mycobactin—an extract from other mycobacteria.

M. ulcerans

This produces a chronic or subacute ulceration in both the skin and the adjacent subcutaneous tissue, particularly of legs and arms. Incubation temperature should be between 25 and 35°C and the best growth is at 33°C. It grows on glycerin agar.

M. balnei

This bacillus has been isolated from swimming pools and produces ulcerative lesions on the extremities. It grows more rapidly than *M. ulcerans,* but will not grow above 35°C.

M. leprae

This is also known as Hansen's bacillus and causes leprosy. Smears are made from a scraping from the skin of suspected lesions and from nasal smears. It has been found in sputum. The usual method is skin clips from the affected areas.

Films are stained by Z-N stain, but it is customary to use 5% sulfuric acid for decolorizing as *M. leprae* is not so strongly acid fast as *M. tuberculosis*, but stained *M. leprae* bacilli may resist decolorization with 20% sulfuric acid. The bacilli are usually present in large numbers (in lepromatous leprosy) and are generally found in packets like cigar bundles within phagocytic cells called lepra cells. They may stain uniformly but there is often marked beading. The bacilli may also be stained fairly easily by Gram's method.

Until recently, no claims of culture were substantiated, but it is now believe that the organism may be isolated on the footpads of mice.

M. lepraemurium

This is an organism found in rats in a disease somewhat resembling leprosy. It can be passed on experimentally to other animals of the same species.

Non-pathogenic Species

M. smegmatis

The smegma bacillus is an acid-fast bacillus found in the smegma secretion around the genital and anal parts of human beings and in some animals such as dogs. It can also be found in other parts of the body, e.g. in the ear. The bacillus may be found in the urine, but if the urine is carefully collected after cleansing the external parts it can usually be avoided.

It is generally shorter and thicker than the tubercle bacillus, and many strains of the smegma bacillus are decolorized by alcohol, which distinguishes them from the tubercle bacillus. They grow rapidly in culture.

M. butyricum

The butter bacillus. These bacilli may be found in grass, water, butter, milk, manure, etc. They grow rapidly in culture.

Acid-fast bacilli can frequently be found in the mouths of water taps, but some at least seem to be affected by 6% H_2SO_4 when an attempt is made to grow them, but survive treatment with weaker acid.

Anonymous Strains

Many of the so-called anonymous strains are classified by their reaction to light. Photochromogens are those which produce orange colonies when they are exposed to light (day-light/artificial light). Scotochromogens are those which produce colored colonies even in the dark. It is therefore, important in dealing with this classification not to grow them in an incubator that is opened frequently, not to let them stand on the laboratory bench unless the observation is actually being made. The yellow-orange pigment develops after exposure to light in 6–24 hours. The scotochromogens produce a yellow growth but this turns more orange on exposure to light. The Battey type either does not change, or if it does, the change is very slow.

Animal Inoculation

Sometimes for final identification it is necessary to inoculate an animal to see if the organism will produce disease. The human strain of *M. tuberculosis* will produce disease in both guinea pigs and rabbits, but the rabbit will show little sign of tuberculosis. With the bovine strain, however, the rabbit is also highly susceptible. In some specimens, the material may be directly inoculated, in other, only after a primary culture has been grown.

OVERVIEW OF *M. TUBERCULOSIS*: DIAGNOSTIC APPROACH, AFB STAINING, CULTURE AND SENSITIVITY

Introduction

Humans are very susceptible to the tuberculosis infection but are remarkably resistant to the tuberculosis disease; which is dependent largely on the state of the hosts immune system. Of all the mycobacterial species,

Mycobacterium tuberculosis remains the most common cause of pulmonary tuberculosis and remains the most virulent of all the mycobacterial species.

The disease, as now well known, is highly contagious. Although the disease involves all susceptible individuals, the incidence is higher among disadvantaged minorities. Industrialization, increased crowded housing and nutritional deprivation have influenced the spread. With the emergence of HIV and resultant immunocompromise, TB has emerged as a major killer not only in the third world countries but is also resurging in the Western world. According to World Health Organization (WHO) reports, each year an estimated eight million new cases of tuberculosis occur, leading to three million deaths; and almost a third of the world's population is infected by the causative organism, *Mycobacterium tuberculosis.*

According to a study, in India, the number of tuberculosis patients is increasing at the rate of 1.5 million per year, and a quarter of these are sputum positive. Thus, about 4% of all Indians are infected with *Mycobacterium tuberculosis.*

With the emergence of the multiple drug-resistant strains due to poorly administered therapeutic measures and patient non-compliance, *Mycobacterium tuberculosis* is challenging its containment, on the basis of empirical treatment alone.

Brief Microbiology

The genus *Mycobacterium* is composed of slow growing organisms, which are "acid fast". Currently about 55 species of *Mycobacteria* are recognized. They are non-motile, slightly curved or straight rods (0.2–0.6 × 1–10 μm) and may occasionally demonstrate branching. The organisms are aerobic and have a gram-positive cell wall, although they do not Gram stain well.

The mycobacteria contain a lipid rich cell surface which includes true waxes and glycolipids 60–90 carbon, long chain mycolic acids, unique to the mycobacterial cell wall are responsible for their:

- Acid fastness
- Failure to react with Gram stains
- Resistance to the action of antibodies and complement.

The four species in the *Mycobacterium tuberculosis* complex are *M. tuberculosis, M. microtic, M. africanum* and *M. bovis.* Laboratories can use biochemical tests for differentiation between isolated strains.

Diagnosis of *Mycobacterium Tuberculosis* Infection

The diagnosis of tuberculosis is often made on the basis of clinical symptoms, chest X-ray and sputum AFB, since available tests based on immunological principles for *Mycobacterium tuberculosis* diagnosis have yet to overcome the problem of poor sensitivity and specificity associated with them. For the time being, speedy and appropriate laboratory diagnosis of tuberculosis infection through AFB staining, culture and sensitivity have more and more important role to play in sensitive detection and appropriate treatment of patients with tuberculosis. However, sample collection, preparation, processing techniques and detection methods employed have a profound effect on the sensitivity and specificity of the results for the detection of *Mycobacterium tuberculosis* infection by AFB and culture methods.

Specimen Selection

A critical factor in the ability of laboratories to isolate *Mycobacterium tuberculosis* is obtaining appropriate specimen for AFB smear and culture. Approximately 85% of the TB cases are pulmonary. However, many patients cannot produce sputum spontaneously and alternative respiratory tract specimens such as induced sputum, gastric lavage or fiberoptic bronchoscopy may be needed. As the proportion of patients with extrapulmonary form of tuberculosis is increasing, adequate specimen from extrapulmonary sites need to be provided.

Sample Concentration and Decontamination

Specimens obtained from sterile sites such as CSF, peritoneal or pleural fluids do not require decontamination. However, most specimens for AFB smear and culture are from the respiratory tract and do contain mixed microbial flora. Successful recovery of mycobacteria depends upon properly collected specimen and suppression of contaminating bacteria.

Since mucous traps AFB and protects other organisms from effective decontamination a combination of 2% NaOH (decontaminant) and 0.5% N-acetyl-L-cysteine (mucolytic agent) is preferably employed. Neutralization of strong decontaminating solutions before using the sample for AFB stain and culture is usually accompanied with sequential buffered wash of the concentrated sample because if the pH of the concentrate remains alkaline or acidic it can destroy the culture medium and prevent the growth of mycobacteria and staining efficiency of the AFB smears. The buffered wash also helps in reducing the specific gravity of specimen and sediments the *Mycobacterium* more effectively.

Another important aspect post-decontamination is the specimen concentration and relative centrifugal force applied to the specimen. Improvement in correlation between specimen showing a positive smear for AFB and a positive culture has been demonstrated by increasing the centrifugal force applied to pellet the specimen.

Recommendations for sample collection for mycobacterial isolation and acid fast staining

Specimen type	*Specimen requirements*	*Special instructions*	*Unacceptable specimen*
Abscess contents aspirated fluid	As much as possible in syringe with Luer tip cap	Cleanse skin with alcohol before aspirating sample. Laboratory may provide 7H9 broth/Kirchner medium for transport of small volumes of aspirates	Dry swab
Blood	10 mL SPS (yellow top) blood collection tube or 10 mL isolator tube	Disinfect site as for routine blood culture. Mix tube contents immediately after collection. SPS is preferred anticoagulant Heparinized blood is also acceptable	Blood collected in EDTA, which greatly inhibits mycobacterial growth even in trace amounts Coagulated blood
Body fluids (pleural, pericardial, peritoneal)	As much as possible (10–15 mL/min) in sterile container or syringe with Luer tip cap. Collect bloody specimens into SPS blood collection tubes	Disinfect site with alcohol and collect by needle and syringe	
Bone	Bone in sterile container without fixative or preservative	—	Specimen submitted in formalin
Bone marrow	As much as possible in SPS blood collection tube or 1.5 mL in pediatric Isolator tube	Collect aseptically. Mix SPS tube contents immediately following collection	
Bronchoalveolar lavage or bronchial washings	≥ 5 mL in sterile containers	Avoid contaminating bronchoscope with tap water, Saprophytic mycobacteria may produce false positive culture or smear results	
Bronchial brushings CSF	Sterile container or Middlebrook 7H9 broth or Kirchner medium ≥ 2 mL in sterile container	— Use maximum volume attainable	—
Gastric lavage fluid	≥ 5–10 mL in sterile container. Collect in the morning soon after the patient awakens in order to obtain sputum swallowed during sleep	Collect fasting early morning specimen on three consecutive days. Use sterile saline. Adjust to neutral pH with 10 mg of sodium carbonate immediately following collection Laboratory should provide collection tube containing sodium carbonate	Specimen that has not been neutralized
Lymph node	Node or portion on sterile container without fixative or preservative	Collect aseptically, and avoid indigenous microbiota. Select caseous portion if available. Do not immerse in saline or other fluid or wrap in gauze	Specimen submitted in formalin
Skin lesion	Submit biopsy specimen in sterile container without fixative or preservative. Submit aspirate in syringe with Luer tip cap	Swabs in transport medium (Amies or Stuarts) are acceptable only if biopsy sample or aspirate is not obtainable. For cutaneous ulcer, collect biopsy sample from periphery of lesion, or aspirate material from under margin or lesion	Dry swab

Contd...

Contd...

Smear on slides	Smear specimen over 1.5 by 1.5 cm area of clear slide	Heat fix smears.Transport in slide container taped closed and labeled BIOHAZARD	
Sputum	5–10 mL in sterile wax-free disposable container. Collect an early morning specimen from deep, productive cough on at least 3 consecutive days. Do not pool specimens. For follow-up of patients on therapy, collect at weekly intervals beginning 3 weeks after initiation of therapy	For expectorated sputum, instruct patient on how to produce sputum specimen as distinct from saliva or nasopharyngeal discharge. Have patient rinse mouth with water before collecting sputum to avoid contaminating specimen with food particles, mouthwash or oral drugs, which may inhibit the growth of mycobacteria. For induced sputum, use sterile hypertonic saline. Indicate on request if specimen is induced sputum	24 hours pooled specimens; saliva
Stool	≥ 1 g in sterile, wax-free, disposable container	Collect specimen directly into container or transfer from bedpan or plastic wrap stretched over toilet bowl. Wax from container may produce false positive smear	Frozen specimen.Utility of culturing stool for acid-fast bacilli remains controversial

MUCOLYTIC, DISINFECTANT, SPECIMEN PRETREATMENT AND BUFFERING SYSTEM FOR AFB STAINING AND CULTURE

Lyfectol®

(*Courtesy:* Tulip Group of Companies)

Summary

Infection with *Mycobacterium tuberculosis* remains a major public health problem. The epidemic of tuber-culosis and multidrug resistant tuberculosis reflects the failure of public health and social programs towards prompt treatment of infected cases and screening of high-risk population. Culture, isolation and sensitivity of *Mycobacterium tuberculosis* from patient groups using standard methods remain the gold standard for *Mycobacterium tuberculosis* detection and effective and swift treatment worldwide.

Reagent

LYFECTOL is a reagent for laboratory use only. LYFECTOL is provided as a three component reagent.

a. Reagent A (2% NaOH solution)
b. Reagent B (N- acetyl L-cysteine)
c. Reagent C (Phosphate buffer pH 6.8).

Accessories: Spatula for approximate weighing (12 mg) and transfer of reagent B.

LYFECTOL is used for decontamination and concentration of specimen containing normal microbial flora such as sputum as per international recommendation.

Principle

Proper decontamination and concentration of specimen containing normal microbial flora such as sputum are crucial in detecting *Mycobacterium tuberculosis.*

LYFECTOL provides a liquefaction-decontamination and specimen buffering procedure that maintains the viability and pathogenicity of *Mycobacterium tuberculosis,* simultaneously eliminating all unwanted microorganisms. Since mucous is sticky, acid fast bacilli trapped in mucoid portion of sputum are released by mucolytic action of N-acetyl L-cysteine. NaOH decontaminates other microorganisms, and final wash with phosphate buffer ensures that specimen is at optimum pH for staining and culturing. Specimen pretreatment and disinfection with LYFECTOL increases relative acid fast bacilli concentration and ensures its more sensitive detection during acid fast bacilli staining and culture.

Storage and Stability

1. Store the LYFECTOL kit at 2–8°C, away from light.
2. Stability of the LYFECTOL kit is as per the expiry date mentioned on the label.

Additional Material Required

Sterile plating loops (10 µL), biosafety hood with Bunsen burner, centrifuge at 3000–4000 g, activated 2% glutaraldehyde solution. 5 mL measuring cylinder, vortex mixer, 1 mL micropipette, 15–25 mL universal container.

Specimen Collection

Collect specimen prior to use of antimicrobial agent. Wherever possible, indicate clearly that patient is on antitubercular drugs. Sputum: Collect 5 to 10 mL in a sterile container from an early morning specimen of deep productive cough. For induced specimen use sterile saline. Have patients rinse mouth with water to minimize specimen contamination with food particles, mouthwash, or oral drugs.

Procedure

The procedure mentioned below is for 2.5 mL of the sputum sample. In case of variation in quantity of specimen used, process using proportionate amounts of reagent, mucolytic and disinfection reagent.

Preparation of Mucolytic Reagent

The mucolytic reagent must be prepared just prior to use.

1. Bring the reagents to room temperature.
2. Add one scoop full (~12 mg) of reagent B to 2.5 mL of Reagent A with the provided spatula.
3. Mix to dissolve.
4. The mucolytic reagent can be used within 24 hours of preparation, if stored at 2–8°C.

Processing of Specimen

1. Take approximately 2.5 mL of the specimen in a clean sterile 15–25 mL universal container.
2. Add 2.5 mL of the mucolytic Reagent and close the container tightly with a screw cap fitted with an intact liner.
3. Mix well by gently vortexing at every 5 minutes interval for 20 minutes.
4. After 20 minutes, unscrew the cap of the container carefully and add 5 mL of reagent C.
5. Close again the container tightly as in step 2.
6. Mix well and centrifuge for 25 minutes at 3000–4000 g.
7. After centrifugation unscrew the cap of the container with the content carefully and discard the supernatant gently in an activated 2% glutaraldehyde solution, taking care as not to disturb the pellet at the bottom.
8. To the pellet at the bottom, add 1 mL distilled water and resuspend the contents.
9. Use this suspended material for microscopy (acid fast bacilli), acid fast bacilli culture or polymerase chain reaction.

Remarks

1. Treat the unused specimen and contaminated containers by immersing in 2% activated glutaraldehyde for at least 2 hours before incineration and disposal.
2. Good laboratory practices and hazard precautions must be observed at all times.
3. Discolored or contaminated reagent should not be used.
4. The reagent containing the phosphate buffer may appear turbid on prolonged storage at 2–8°C. Gently warm at 25–30°C before usage to remove such a appearance.

Effect of centrifugal force on positive smears/cultures for mycobacteria

Specimen	*Relative*	*Centrifugal*	*Force (g)*
	1260	3000	3800
Positive smear	1.8%	4.5%	9.6%
Positive cultures	7.1%	11.2%	11.6%
Correlation of positive smear/cultures	25%	4 0. %	82%

Thus, proper decontamination and preparation of specimen is crucial to AFB detection by culture and AFB staining.

Troubleshooting

Problem: False negative results in LJ media

Possible causes	*Solutions*
1. Mucoid sputum exposed to 2% NaOH for longer duration	Contact time of 2% with sputum should be for only 20 minutes since prolonged period of contact may kill or injure the mycobacteria
2. Saliva used as specimen	Thick yellowish green mucoid sputum collected from an early morning deep productive cough should be used as a specimen

Contd...

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Problem: False positive results in LJ media

Possible causes	*Solutions*
1. Sputum collected in a contaminated container	Collect the sputum in a clean sterile container
2. Contaminated container used for treatment of the sputum	Clean sterile container is to be used during treatment of sputum to avoid contamination

Problem: Turbidity in phosphate buffer

Possible cause	*Solutions*
1. Prolonged storage of phosphate buffer at 2–8°C	Gently warm phosphate buffer in a water bath at 25–30°C before usage

Problem: No liquefaction of mucoid sputum

Possible causes	*Solutions*
1. Mucolytic reagent used is prepared and stored at 2–8°C for more than 24 hours	Freshly prepared mucolytic reagent should be used. If stored at 2–8°C, should be used within 24 hours

The AFB Smear

The sensitivity of AFB smear for specimen from extrapulmonary sites is lower than from sputa. The lipid-containing cell walls of mycobacteria have a unique characteristic in binding carbol fuchsin stain so tightly that it resists destaining with strong decolorizing agents such as strong alcohols and strong acids. This "acid-fast" staining reaction of mycobacteria, along with their unique beaded and slightly curved shape, is a valuable aid in the early detection of infection and monitoring of therapy.

It has been estimated that there must be 10,000 acid-fast bacilli per milliliter of sputum to be detected by microscopy. Patients with extensive disease will shed large numbers of mycobacteria and show a good correlation between a positive smear and a positive culture. In patients with minimal or less advanced disease, the correlation of positive smears to positive cultures may range from 30 to 80%.

Acid-fact stains performed on a weekly basis are also useful in following the response of patients to drug therapy. After drugs are started, cultures will become negative before smears, indicating that the bacilli are injured sufficiently to prevent replication but not to the point of preventing binding of the stain. With continued drug treatment, more organisms are killed and fewer shed, hence monitoring the number of stainable organisms in the sputum during treatment can provide an early and objective measure of response.

It should be noted that in patients receiving antimycobacterial therapy not all stainable organisms are viable. Should the number of organism fail to decrease after therapy is started, the possibility of drug resistance must be considered. Additional cultures should be taken and drug susceptibility studies obtained.

Two types of acid-fast stains are frequently used:

1. Carbol fuchsin based stains;
2. Fluorochrome based stains.

The carbol fuchsin stains, so called because of the Reagent formed by mixing of the stain basic fuchsin with the disinfectant phenol (carbolic acid). Carbolfuchsin stained mycobacteria appear bright red/pinkish against a bluish background.

Two procedures using carbol fuchsin based stains are in common use:

a. Three component Ziehl-Neelsen, or "hot stain", and
b. Three component Kinyoun or "cold stain".

The Kinyoun stain is a modification of the classical Ziehl-Neelsen "hot stain". The classical Ziehl-Neelsen "hot stain" requires application of heat to the fixed smears flushed with the stains during staining process, whereas the Kinyoun stain does not require the application of heat and is less tedious to perform and standardize.

Recent advances in staining techniques have been reported where the cold Kinyoun stain has been further modified to accommodate the decolorizer within the counter stain. The novel two component two step stain is time, labor and cost saving, more user friendly and easy to standardize. It also has good correlation with the classical Ziehl-Neelsen "hot stain" and AFB cultures.

RAPID TWO STEP COLD AFB STAIN

(*Courtesy:* Tulip Group of Companies)

Novachrom®

Summary

Infection with *Mycobacterium tuberculosis* remains a major public health problem. The epidemic of tuberculosis and multi drug resistant tuberculosis reflects the failure of public health and social programs towards prompt treatment of infected cases and screening of high-risk population. While culture, isolation and sensitivity of *Mycobacterium tuberculosis* from patient groups using standard methods remain the gold standard for *Mycobacterium tuberculosis* detection and effective and swift treatment worldwide, acid fast bacilli staining is the first line microscopic procedure performed towards this goal.

Reagent

NOVACHROM is a reagent for laboratory use only. NOVACHROM Rapid Two Step Cold AFB Stain comprises of:

a. AFB stain (A) — carbol fuchsin
b. AFB stain (B) — counterstain with decolorizer

Rapid Two Step Cold AFB Stain is provided as a ready to use stain set. It is used for screening of *Mycobacterium tuberculosis* from biological specimen such as sputum, CSF and urine. It is also used in the identification of *Mycobacterium tuberculosis* from isolated culture. It is a modification of Kinyoun's cold stain.

Principle

Carbol fuchsin forms acid insoluble complex with mycolic and present on the Acid Fast Bacilli and renders red/pinkish red color to *Mycobacterium tuberculosis.* Other elements present in the smear take up counterstain (Methylene blue) and are stained bluish. Rapid Two Step Cold AFB Stain avoids the extra decolorization step associated with traditional staining techniques as the decolorizing component is incorporated within the counterstain. The staining system is simple to perform and very much reproducible. It has a sensitivity comparable to the traditional Ziehl-Neelsen hot staining method and also as compared to Acid Fast Bacilli culture results. It is a clinically proven, easy to use, time, labor and cost saving. This simplicity of staining makes this an ideal screening tool for *Mycobacterium tuberculosis.*

Storage and Stability

1. Store the kit at room temperature (25–30°C), away from light.
2. Stability of the kit is as per the expiry date mentioned on the label.

Additional Material Required

Sterile plating loops (10 µL), biosafety hood with Bunsen burner, activated 2% glutaraldehyde solution, distilled water, microscope with oil immersion lens, cedar wood oil.

Specimen Collection and Preparation

Collect specimen prior to use of antimicrobial agent. Wherever possible, indicate clearly that patient is on antitubercular drugs.

CSF

Collect as much as possible in a syringe, clean skin with alcohol before aspirating specimen.

Body Fluids

Disinfect the site and collect specimen with aseptic precautions.

Sputum

Collect 5 to 10 mL in a sterile container from an early morning specimen of deep productive cough. For induced specimen use sterile saline. Have patients rinse mouth with water to minimize specimen contamination with food particles, mouthwash, or oral drugs.

Urine

As organisms accumulate in the bladder overnight, first morning void provides best yield. Collect midstream clean catch urine, first morning catheterization/suprapubic taps in sterile containers.

Specimen Preparation

Proper decontamination and concentration of specimen containing normal microbial flora are crucial to detection of *Mycobacterium tuberculosis.* Specimen obtained from sterile sites such as CSF, peritoneal or pleural fluids do not need decontamination. However, since most specimens for Acid Fast Bacilli smear and culture are from respiratory tract and the mucous traps Acid Fast Bacilli and protects other organisms from decontamination and concentration, decontamination and liquefaction is a must. Most satisfactory for this purpose is a combination of N-acetyl-L cysteine (mucolytic agent) and 2% NaOH (decontaminant). Petroffs method of decontamination can also be used.

Test Procedure

For direct sputum screening use 10 µL of purulent sputum or use 10 µL of decontaminated and concentrated specimen.

1. Place the specimen under test on a clean, scratchless glass slide using a sterile plating loop.
2. Spread by tracing concentric circles well separated over an area of 200 mm square (20 mm × 10 mm), take care as to not to reach the edge of the slide. Alternatively for dense mucoid specimen press the specimen between two glass slides and pull apart gently to form a thin film of mucous.
3. When the smear is completed, plunge the inoculating loop into liquid disinfectant (2% Glutaraldehyde) and shake to remove any sputum, then flame sterilize loop.
4. Air-dry the smear.
5. Fix the smear by passing the slide approximately three times on the flame.
   (**Note:** While passing the smear slide on the flame see that the side opposite the smear is facing the flame).
6. Mix well and add AFB stain (A) over the smear to cover it completely (4–5 drops ≈ 0.2 mL, may be required).
7. Keep for 6 minutes and then rinse with plenty of distilled water slowly to remove excess of AFB stain (A).
8. Tilt slide to drain, mix well and add AFB stain (B) over the smear to cover it completely (4–5 drops ≈ 0.2 mL, may be required).
9. Keep for 6 minutes and then rinse the smear once more with distilled water to remove excess of AFB stain (B).
10. Air-dry and observe under oil immersion (magnification 100 X objective).

Interpretation of Results

1. Presence of pink to red colored slender Bacilli—Smear Acid Fast Bacilli positive.
2. Absence of pink to red colored slender Bacilli—Smear Acid Fast Bacilli negative.
3. Pus cells and other bacteria stain purple to blue color.

Grading of Results

After 5 minutes of examination covering about 100 fields.

Number of Acid Fast Bacilli observed	*Report*
No Acid Fast Bacilli	Negative
1–10 Acid Fast Bacilli	Actual Number
> 10 Acid Fast Bacilli	+
Masses of Acid Fast Bacilli in several fields	++

Remarks

1. Improper decontamination and concentration procedure will yield erroneous results.
2. Treat the specimens and used slants by immersing in 2% activated glutaraldehyde for at least 2 hours before incineration and disposal.
3. Good laboratory practices and hazard precautions must be observed at all times.
4. Observe stain timings, which are essential to obtain correct staining results.
5. Understain or over decolorization may give false results.
6. The stains stored between 25 and 30°C should not show precipitation.
7. Artefacts could be mistaken for Acid Fast Bacilli.

The fluorochrome based stains for AFB comprise of Auramine O, sometimes used in combination with a second fluorochrome stain, Rhodamine.

Smears stained with Auramine O can be scanned using a 25 × objective. Fluorochrome-stained *Mycobacteria* appear bright yellow against a dark background obtained by counterstaining with potassium permanganate, thereby permitting the slide to be scanned under the lower magnification without losing sensitivity. The sharp visual contrast between the bright colored mycobacteria and the dark background offers a distinct advantage in scanning a much larger area of the slide during the same time necessary for looking at the carbol fuchsin stain.

When using the Auramine stain, a significantly larger area of the smear can be scanned in the same period of time used to scan a carbol fuschin-stained smear.

Enthusiasm for the carbol fuchsin and fluorochrome staining methods varies between laboratories, with different professionals strongly partial to one method or the other. Specificity for mycobacteria seems to be the same for both.

The crucial factors in maximizing smear sensitivity and specificity are:

- Centrifugation of digested fluid specimen at a minimum of 3000 g
- The smear should be prepared on a new clean undamaged glass slide
- Scanning of at least 30 fields per slide
- The reporting of the AFB smear should be preferably done according to the CDC, USA method, or as per the National Reference Institution norms.

Quantitation scale for acid-fast bacillus smears according to stain used

Carbol fuchsin (X 1000)	*Fluorochrome (X 250)*	*Quantity reported*
No AFB/3 fields	No AFB/30 fields	No AFB seen
1–2 AFB/300 fields	1–2 AFB/30 fields	Doubtful; repeat test
1–9 AFB/100 fields	1–9 AFB/10 fields	Rare (1+)
1–9 AFB/10 fields	1–9 AFB/field	Few (2+)

Contd...

Contd...

1–9 AFB/fields	10–90 AFB/field	Moderate (3+)
>9 AFB/fields	>9 AFB/field	Numerous (4+)

However, Indian Reference Institutions recommend reporting after 5 minutes of examination covering about 100 fields. Grading is done as follows:

Number of Acid Fast Bacilli observed	*Report*
No Acid Fast Bacilli	Negative
1–10 Acid Fast Bacilli	Actual Number
>10 Acid Fast Bacilli	+
Masses of Acid Fast	+ +
Bacilli in several fields	

Smears with fewer than 3 AFB per slide account for about 85% of false positive smear reporting and are considered doubtful. A repeat specimen should be registered. However, *Mycobacterium tuberculosis* infection must be considered for any patient with repeat smear AFB positive regardless of the number of AFB observed.

Factors Influencing Sensitivity and Specificity of AFB Smears

False Positive Results

Acid Fast Particles Other Than Tubercle Bacilli

Occasionally, a sputum specimen or smear may contain particles that are acid-fast, i.e. when treated with the Ziehl-Neelsen method, they retain the red stain (carbol fuchsin) and resist decolorization with acid-alcohol. These red particles may sometimes resemble tubercle bacilli. They include certain food particles (e.g. waxes, oils), precipitates, other microorganisms, inorganic materials and artifacts.

Food particles

To eliminate these, the patient should rinse their mouth with pure water and clean their teeth (without using tooth-paste or disinfectant) before producing the sputum specimen. It is even better if the patient produced the specimen before breakfast or on an empty stomach.

Precipitated stains

Though these are quite easy to differentiate from acid-fast bacilli, they may hamper reading or occasionally mislead an inexperienced microscopist. Precipitates can be removed by filtration of staining solutions. However, it is safer to use freshly prepared solutions, filled into carefully cleaned bottles, rather than stale staining solutions.

Saprophytic acid-fast bacilli

These occur in soil and water, and may occasionally get into the specimen or smear during processing. This can be avoided by using distilled or boiled water from scrupulously clean containers.

Mycobacterium kansasii or Nocardia species

These occasionally occur in specimens. When they cause pulmonary disease, they are usually present in large numbers.

Spores of Bacillus subtilis

These are very rare, mostly of ovoid shape, and larger than tubercle bacilli.

Fibers and pollens

Fibers, including those of wood, cotton, filter paper and bamboo, usually occur singly, most often in only one microscopic field. The pollen of certain pine trees is seen as short, coccoid rods occurring very rarely in specimens.

Scratches on the slide

Scratches may sometimes retain the red stain and confuse beginners. They are usually seen in parallel rows, are generally longer than acid-fast bacilli, and are undulated. They can be identified easily, because they are found in a deeper layer on the slide, below the smear disappearing when the cells (e.g. leukocytes) in the smear get focused on.

Contamination through the Transfer of Bacilli from One Smear to Another

It may happen that acid-fast bacilli are transferred accidentally from a positive slide to a negative one, when several slides are treated simultaneously in staining or decolorization tanks. This can be avoided by processing each slide separately, e.g. on a rack. Such racks are usually made of wire and can be decontaminated easily by flaming.

Acid-fast bacilli may also be transferred accidentally when the glass rod or dropper used for placing immersion oil on the slide touches the surface of a positive slide and rubs off some material. The same can happen when blotting paper is used for drying several stained smear consecutively. Therefore, the blotting paper should not be used at all, or for no more than one slide. The oil dropper should not touch the smear, and the oil should be allowed to drip freely on to the slide. For the same reason, the surface of the slide should not be rubbed with the oil immersion objective. Before a new slide is examined, the oil should be wiped off the lens with a piece of cotton tissue or, even better, with special lens-cleaning paper.

When microscopy is used for the detection of acid-fast bacilli, slides should never be used more than once.

False Negative Results

False negative results are commonly due to deficiencies in the preparation of the smear, in staining, and in

scanning. Adequate collection of the specimen and subsequent "selection of sputum particles are essential to the preparation of a smear and should receive special attention.

Deficiencies leading to false negative results include the following:

Inadequate Sputum Collection

The patient is sometimes not told clearly enough what constitutes a proper sputum specimen and how he should produce one. It must be made clear to him that saliva and nasopharyngeal discharge are unsuitable for examination. Patients should be encouraged and given time to produce bronchial sputum from the "depths of the chest". If repeated attempts have failed, tickling of the inner surface of the epiglottis or trachea with a swab, or intratracheal instillation of 5–10 mL of cool saline or sterile water may provoke a vigorous cough with sputum. Other techniques to stimulate the production of sputum, such as aerosol induction, gastric aspiration, and bronchoscopy, require more complex equipment or special skills.

If a patient discharges acid-fast bacilli in his sputum, these are more likely to be found in a specimen produced in the early morning than in one produced later in the day. If early morning sputum in required, the patient should be given a container and instructed to place in it the very first sputum he produces in the morning, before breakfast and before taking any medicaments.

Improper Storage of Sputum Specimens and Stained Smears

Acid-fast bacilli may lose their acid-fastness as a result of exposure of the specimen to direct sunlight, radiation (e.g. ultraviolet light), excessive heat, or storage for more than a week in hot and dry conditions.

If Ziehl-Neelsen stained smears have to be stored for reexamination, the immersion oil must be washed from the smears with xylol because the immersion oil removes the stain from the acid-fast bacilli.

Fluorochrome stained smears will lose their fluorescence with storage.

Failure to Select Suitable Sputum Particles for Smear Preparation

Tubercle bacilli are most likely to be found in little blobs ("lentils") of greenish-gray or yellowish matter of a thick, creamy consistency. (Such blobs usually consist of dead caseous tissue eliminated from a cavity in the lung). If the sputum is not treated by a special concentration procedure involving centrifugation, these blobs have to be carefully separated from the rest of the sputum and transferred to a slide. They can be seen more easily in the sputum against a dark background.

Inadequate Preparation of Smear or Staining of Slides

False negative results may be obtained also when:

a. Too little material has been spread on the slide, so that the smear is too thin;
b. The smear is too thick, so that sufficient light cannot pass through it;
c. The slide has been over heated when fixing the smear;
d. The smear has not been sufficiently fixed and parts of the material have been washed off;
e. The staining with carbol fuchsin was too short or was overdone by boiling;
f. The counterstaining was too intensive, so that the acid-fast bacilli have been obscured;
g. Staining and counterstaining times have not been followed precisely.

Inadequate Examination of the Smear

If the scanning is done erratically or too briefly, too few fields may be examined (Occasionally the examiner is unable to distinguish the red-stained acid-fast bacilli because of color blindness or other visual disturbances).

Other Reasons for False Results

Administrative errors

Such errors may include:

a. Misidentification of patients, misspelling of names, or confusion of names or of codes numbers of specimens and slides;
b. Mistakes in labeling containers;
c. False recording of reporting.

Reading errors

Reader or observer error, which is mainly due to visual or psychological reasons, occurs in practically all diagnostic, clinical and laboratory work. The nature of this phenomenon, sometimes called the "human factor", is to a large extent unknown. Nevertheless, under certain conditions it is measurable. The degree and frequency of error-overreading as well as under-reading varies from one person to another and also within the same individual at different times.

Interindividual reader variations in smear microscopy has been repeatedly studied and its frequency has been found relatively low compared, for instance, with interindividual error in say, chest radiography.

It seems likely that many reader errors would be avoided if each microscopist were properly trained and strongly advised to report what he actually saw, and never what he thought he was expected to see. However, discrepancies in the results of smear microscopy are far more often due to deficient sputum collection and smear preparation than due to reader error.

AFB Culture and Isolation

The modern bacteriology has many mycobacteriological media available to it. An ideal medium should be able to

produce rapid and abundant growth, enhance phenotype characteristics, inhibit the growth of contaminants and should be usable for antimicrobial techniques. However, despite advances, the isolation of *Mycobacterium tuberculosis* is still a slow process ranging from 10 days to 8 weeks.

Solid media: LJ medium produces a slightly higher rate of TB isolation however, it is prone to slant contamination. A good LJ medium is non-selective, light green in color, smooth slant without bubble formation so as to view mycobacterial growth easily. The concentration of Malachite green is critical for achieving a good color contrast for visualization of mycobacterial colonies. Suboptimal concentration of Malachite green in the medium produces higher contamination rates whereas excessive Malachite green can suppress and delay the *Mycobacterium* growth itself.

Agar based medium such as Middlebrook are transparent, allow quicker examination of colony morphology. Middlebrook is more resistant to contamination and produces growth of *Mycobacterium tuberculosis* faster than LJ medium. Some commercially available 7H11 medium have been modified to increase the amount of Malachite green. Laboratory workers should be careful to determine this, for while the increase content of aniline dye retards growth of contaminating bacteria, it can also inhibit the growth of *Mycobacterium*.

When laboratories rely primarily on solid medium it will take a minimum of 3 weeks to produce colonies of *Mycobacterium tuberculosis*.

READY TO USE LJ SOLID *MEDIUM FOR MYCOBACTERIUM TUBERCULOSIS ISOLATION*

Mycocult®

(*Courtesy:* Tulip Group of Companies)

Summary

Infection with *Mycobacterium tuberculosis* remains a major public health problem. The epidemic of tuberculosis and multidrug resistant tuberculosis reflects the failure of public health and social programs towards prompt treatment of infected cases and screening of high-risk population. Culture, isolation and sensitivity of *Mycobacterium tuberculosis* from patient groups using standard culture methods remain the gold standard for *Mycobacterium tuberculosis* detection and effective and swift treatment worldwide.

Reagent

MYCOCULT is a reagent for laboratory use only.

The Lowenstein-Jensen medium is provided as a ready to use slant. It is a standard non-selective inspissated egg based solid medium for the isolation of *Mycobacterium tuberculosis* from biological specimen such as sputum, CSF, urine.

Principle

Lowenstein-Jensen medium supports the growth of *Mycobacterium tuberculosis*.

The glycerol present in the medium enhances the growth of *Mycobacterium tuberculosis*. Accurate amount of malachite green not only has an inhibitory effect on growth of organisms other than *Mycobacterium tuberculosis*, but also provides the desired color contrast for easy identification of *Mycobacterium tuberculosis* colonies.

Storage and Stability

a. Store the LJ kit at 2–8°C away from light.
b. Stability of the unopened medium is as per the expiry date mentioned on the label.
c. Avoid jerks and vibration while storage, shipping and incubation.
d. Upon opening, the medium must be put into use instantly.

Additional Material Required

Sterile plating loops (10 µL), incubator at 37 ± 0.5°C, biosafety hood with Bunsen burner, activated 2% glutaraldehyde solution, 0.2 mL micropipettes.

Specimen Collection and Preparation

Collect specimen prior to use of antimicrobial agent. Wherever possible, indicate clearly that patient is on antitubercular drugs.

CSF

Collect as much as possible in a syringe, clean skin with alcohol before aspirating specimen.

Body Fluids

Disinfect the site and collect specimen with aseptic precautions.

Sputum

Collect 5 to 10 mL in a sterile container from an early morning specimen of deep productive cough. For induced specimen use sterile saline. Have patients rinse mouth with water to minimize specimen contamination with food particles, mouthwash, or oral drugs.

Urine

As organisms accumulate in the bladder overnight, first morning void provides best yield. Collect midstream clean catch urine, first morning catheterization/suprapubic taps in sterile containers.

Specimen Preparation

Proper decontamination and concentration of specimen containing normal microbial flora are crucial to detection of *Mycobacterium tuberculosis*. Specimen obtained from sterile sites such as CSF, peritoneal or pleural fluids do not need decontamination. However, since most specimens for AFB smear and culture are from respiratory tract and mucous traps AFB and protects other organisms from decontamination and concentration, decontamination and liquefaction is a must. Most satisfactory for this purpose is a combination of N-acetyl-cysteine (mucolytic agent) and 2% NaOH (decontaminant). Petroffs method of decontamination can also be used.

Test Procedure

1. Bring the Lowenstein-Jensen medium slant to room temperature.
2. Label the Lowenstein-Jensen medium slant appropriately.
3. Draw 10 µL of the decontaminated and concentrated specimen from the reconstituted pellet with a sterile calibrated loop and plate it on the Lowenstein-Jensen medium slant aseptically.
4. For quantitative evaluation prepare bacterial suspension to match McFariland 0.5 standard, dilute this 1:10000 and Seed 100 µL on the Lowenstein-Jensen medium slant aseptically (seed stock consists of approx.-15000 organisms/mL).
5. Close the Lowenstein-Jensen slant cap tightly and incubate at 37 ± 0.5°C.
6. Observe for growth weekly till 8 weeks.

Interpretation of Results

1. *Mycobacterium tuberculosis* colonies may be detected from third week onwards up to 8 weeks. The colonies are characterized by rough granular buff colored growth, which has an initial size of 1–3 mm and full-grown size of 5–8 mm.

Remarks

1. Discolored, dislodged, or contaminated medium should not be used.
2. Improper decontamination and concentration procedure will yield erroneous results.
3. Treat the specimens and used slants by immersing in 2% activated glutaraldehyde for at least 2 hours before incineration and disposal.
4. Good laboratory practices and hazard precautions must be observed at all times.
5. In specimens from patients already on antitubercular drugs, the initial growth may be further delayed.
6. Growth on the Lowenstein-Jensen slant within the first week post inoculation usually indicates atypical *Mycobacterium* or contamination due to insufficient decontamination of specimen.
7. All culture growth should be characterized based on morphology, AFB stain and biochemical tests.

Liquid media: Such as Middlebrook 7H9, Dubos Tween albumin broth and Kirchner medium have been developed for the enrichment of growth of small number of mycobacteria. They are valuable in isolating bacteria from uncontaminated specimen such as CSF, pleura and peritoneal fluids. There is an increased growth rate of *Mycobacterium tuberculosis* in liquid medium. Inclusion of antibiotic cocktails such as PACT (Polymyxin B, Ampho-tericin B, Carbenicillin, Trimethoprim) or PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) is required to make the liquid media sufficiently inhibitory to the growth of other bacteria and fungi especially when sputum specimens are used.

It is recommended internationally that specimen for mycobacterial culture should be inoculated in both types of media. According to most acceptable guidelines at least three different media should be inoculated, and at least one of them being a liquid medium.

The different composition of the media and combination of different media have an impact on the yield and positive cultures, thereby increasing sensitivity of culture and mycobacterial isolation.

Recent Indian studies have also indicated that *'Lowenstein-Jensen'* medium and *'Kirchner's* liquid medium are the best combination for the isolation of mycobacteria from specimens other than sputum.

Ideally the cultures are incubated at 36 ± 10°C; with an atmosphere of 5–10% of CO_2 being stimulating to the growth of mycobacteria.

Troubleshooting

Problem: Growth on the Slant within First Week of Incubation

Possible causes	*Solutions*
1. No proper decontamination of sputum specimen due to which contaminants over grow	Proper decontamination of sputum specimen is to be carried out using Lyfectol (decontamination reagent) to ensure that all unwanted organisms are killed
2. Fast growing organism of *Mycobacterium* species	Confirm the results with biochemical tests

Problem: Collapse of Slants

Possible causes	*Solutions*
1. Improper transportation, i.e. kits subjected to violent jerks and vibrations or not handled with care	Discard the slant and use fresh slant
2. Contaminated slants	If the slants are found contaminated because of various reasons, discard the slants and use fresh slants to perform the test
3. Contaminated untreated sputum sample used	Pretreat sputum sample prior to inoculation

COMBIPACK OF SOLID AND LIQUID MEDIUM FOR *MYCOBACTERIUM TUBERCULOSIS* ISOLATION

(*Courtesy:* Tulip Group of Companies)

Combicult®

Summary

Infection with *Mycobacterium tuberculosis* remains a major public health problem. The epidemic of tuberculosis and multidrug resistant tuberculosis reflects the failure of public health and social program's towards prompt treatment of infected cases and screening of high-risk population. Culture, isolation and sensitivity of *Mycobacterium tuberculosis* from patient groups using standard culture methods remain the gold standard for *Mycobacterium tuberculosis* detection and effective and swift treatment worldwide.

Reagent

COMBICULT is a reagent for laboratory use only. Lowenstein-Jensen medium is provided as a ready to use slant Kirchner medium is provided as a three-component medium.

a. Kirchner medium base
b. Kirchner selective enrichment containing antibiotic cocktail Polymyxin B, Amphotericin B, Carbenicillin, Trimethoprim (PACT)
c. Sterile distilled water for reconstitution of Kirchner selective enrichment.

Lowenstein-Jensen medium is a standard non-selective inspissated egg based solid medium for the isolation of *Mycobacterium tuberculosis* from biological specimen such as sputum, CSF, urine. Lowenstein-Jensen medium supports the growth of *Mycobacterium tuberculosis.* The glycerol present in the Lowenstein-Jensen medium enhances the growth of *Mycobacterium tuberculosis.* Accurate amount of malachite green not only has an inhibitory effect on growth of organisms other than *Mycobacterium* but also provide the desired color contrast for easy identification of *Mycobacterium* colonies.

Kirchner medium is a liquid medium enriched with serum. Kirchner medium has polymyxin B, amphotericin B, carbenicillin, trimethoprim as inhibitory antibiotic cocktail for most of the bacteria and fungus other than *Mycobacterium.* Being a buffered medium, it allows direct inoculation of larger inoculum up to 500 µL, and also keeps up the acid base balance during the growth phase.

Principle

The gold standard for primary isolation of *Mycobacterium tuberculosis* is the use of liquid media in conjunction with solid media. Most *Mycobacterium* species grow more quickly in liquid media than solid media. Liquid media also support higher detection rates especially with specimen material containing smaller number of bacilli. Simultaneous inoculation of solid media and liquid media yields significantly higher recovery rates for *Mycobacterium tuberculosis* growth as compared to when each media is used independently.

Storage and Stability

1. Store the kit at 2–8°C, away from light.
2. Stability of the unopened media is as per the expiry date mentioned on the label.
3. Avoid jerks and vibrations while storage, shipping and incubation.
4. Upon opening, the media must be put into use instantly.

Additional Material Required

Sterile plating loops (10 µL), incubator at 37 ± 0.5°C, biosafety hood with Bunsen burner, activated 2% glutaraldehyde solution, 0.2 mL micropipettes.

Specimen Collection and Preparation

Collect specimen prior to use of antimicrobial agents. Wherever possible, indicate clearly that patient is on antitubercular drugs.

CSF

Collect as much as possible in a syringe, clean skin with alcohol before aspirating specimen.

Body Fluids

Disinfect the site and collect specimen with aseptic precautions.

Sputum

Collect 5 to 10 mL in a sterile container from an early morning specimen of deep productive cough. For induced specimen use sterile saline, have patients rinse mouth with water to minimize specimen contamination with food particles, mouthwash, or oral drugs.

Urine

As organisms accumulate in the bladder overnight, first morning void provides best yield. Collect midstream clean catch urine, first morning catheterization/suprapubic taps in sterile containers.

Specimen Preparation

Proper decontamination and concentration of specimen containing normal microbial flora are crucial to detection of *Mycobacterium tuberculosis*. Specimen obtained from sterile sites such as CSF, peritoneal or pleural fluids do not need decontamination. However, since most specimens for AFB smear and culture are from respiratory tract and the mucous traps AFB and protects other organisms from decontamination and concentration, decontamination and liquefaction is a must. Most satisfactory for this purpose is a combination of N-acetyl - L cysteine (mucolytic agent) and 2% NaOH (decontaminant). Petroffs method of decontamination can also be used.

Preparation of Kirchner Medium

1. Reconstitute the Kirchner selective enrichment with 1 mL of sterile distilled water provided with the kit.
2. Transfer reconstituted selective enrichment aseptically to the Kirchner medium base, which is now ready to use as 'Kirchner Medium'.

Test Procedure

Kirchner Medium

1. Bring the Kirchner medium to room temperature.
2. Label the Kirchner medium appropriately.
3. Draw 10 µL of the decontaminated and concentrated specimen from the reconstituted pellet with a sterile calibrated loop.
4. Inoculate in Kirchner medium aseptically.
5. Close the Kirchner medium cap tightly and incubate at 37 ± 0.5° C.
6. Observe for growth every third day till 8 weeks.

Lowenstein-Jensen Slant

1. Bring the Lowenstein-Jensen medium slant to room temperature.
2. Label the Lowenstein-Jensen medium slant appropriately.
3. Draw 10 µL of the decontaminated and concentrated specimen from the reconstituted pellet with a sterile calibrated loop and plate it on the Lowenstein-Jensen medium slant aseptically.
4. For quantitative evaluation prepare bacterial suspension to match McFari land 0.5 standard, dilute this 1:10000 and Seed 100 µL on the Lowenstein-Jensen medium slant aseptically (seed stock consists of approximately-15000 organisms/mL).
5. Close the Lowenstein-Jensen slant cap tightly and incubate at 37± 0.5°C.
6. Observe for growth weekly till 8 weeks.

Interpretation of Results

a. *Mycobacterium tuberculosis* colonies on Lowenstein-Jensen slant may be detected from third week onwards up to 8 weeks. The colonies are characterized by rough granular buff colored growth, which has initial size of 1–3 mm and full-grown size of 5–8 mm.
b. *Mycobacterium tuberculosis* growth in Kirchner medium is characterized by fluffy growth to small granules. The granules sediment to the bottom.
c. Since both the media differ in their composition, growth of *Mycobacterium tuberculosis* in either medium should be considered as a positive culture result. The growth needs to be identified.

Remarks

1. Discolored, dislodged, contaminated or turbid medium should not be used.
2. Improper decontamination and concentration procedure will yield erroneous results.
3. Good laboratory practices and hazard precautions must be observed at all times.
4. While observing growth in liquid medium, care needs to be taken to differentiate between *Mycobacterium* growth and specimen material's own turbidity.
5. Treat the specimens and used slants by immersing in 2% activated Glutaraldehyde for at least two hours before incineration and disposal.
6. Preparation of Kirchner medium has to be carried out just prior to inoculation of specimen or culture.
7. In specimens from patient, already on antitubercular drugs, the initial growth may be further delayed.

8. Growth on the Lowenstein-Jensen slant/Kirchner medium within the first week postinoculation usually indicates atypical *Mycobacterium* or contamination due to insufficient decontamination of specimen.
9. All culture growth should be characterized based on morphology, AFB stain and biochemical tests.

Radiometric media: Developed in 1970, represent a significant improvement in the rapid isolation of *Mycobacterium tuberculosis.* Detection time is directly proportional to the number of metabolically active bacteria present and the metabolic rate is influenced by the type of specimen, number of organisms, therapy status of patient, decontamination procedures and the incubation temperature.

The average time for reporting the isolation of *Mycobacterium tuberculosis* using radiometric technique is reportedly 22 ± 9 days as compared to 31 ± 9 days for solid media.

However, the radiometric system is more labor intensive, requires disposal of radioactive material and still cannot detect some *Mycobacterium tuberculosis* isolates that can only be detected on agar slants. Some laboratories prefer to use LJ slants as a backup to Radiometric media. Considering the cost aspects and the fact that *Mycobacterium tuberculosis* is largely a problem of the third world, use of radiometric media is still restricted and use of solid and liquid media is widely practiced.

Troubleshooting

Problem: Growth on the slant within first week of incubation

Possible causes	*Solutions*
1. Improper decontamination of sputum specimen due to which contaminants over grow	Proper decontamination and concentration of specimen containing normal microbial flora are crucial to detection of *Mycobacterium tuberculosis*. Therefore, proper decontamination of sputum specimen should be carried out using Lyfectol
2. Fast growing organism of *Mycobacterium* species	Confirm the type of organism grown with biochemical tests

Problem: Collapse of slants

Possible causes	*Solutions*
1. Improper transportation, i.e. kits subjected to violent jerks and vibrations or not handled with care during storage, shipping and incubation	Discard the collapsed slant and use fresh slant for testing
2. Contaminated slants	If the slants are found contaminated because of various reasons, discard the slants and use fresh slants to perform the test
3. Contaminated untreated sputum sample used	Pretreat sputum sample prior to inoculation

Susceptibility Testing of *Mycobacterium tuberculosis*

Resistance to antitubercular agents was recognized soon after their introduction in early 1960s, and standardized methods for antimicrobial susceptibility have been developed. Routine laboratory susceptibility testing of primary TB isolates has not been generally suggested unless drug resistance in a particular community exceeds 5%. However, with the resurgence of TB drug resistance, CDC USA has recently recommended that susceptibility tests should be performed on all primary isolates.

In a recent Indian study, a total of 3181 samples were processed for isolation of tubercle bacilli; and 707 samples were culture positive. The pattern of drug resistance is shown in the following table:

Pattern of drug resistance for mycobacterium tuberculosis

Drug	*Percent resistance*
Isoniazid	30.41
Rifampin	58.55
Streptomycin	46.95
Ethambutol	3.67
D-Cycloserine	24.32
Kanamycin	14.42
Ethionamide	60.67
Amikacin	15.84
Ciprofloxacin	7.49

In India, it has been observed that private practitioners use different drug regimens to treat tuberculosis and very few regimens match with the standard (recommended by WHO).

The problem of acquired drug resistance (ADR) is truly man made. Poor administered tuberculosis control program, inadequate dosages, monotherapy, insufficient durations of treatment, irregularity in drug intake, frequent defaults are some of the common reasons for emergence of ADR. In addition, HIV is quickening the pace at which Tuberculosis is spreading. Therefore, Tuberculosis is becoming the leading killer disease of HIV-positive people.

Clinicians should ensure that *Mycobacterium tuberculosis* susceptibility tests are carried out for patients:

- Who fail to respond after 3 months of treatment
- Who do not convert to having negative smears after 3 months of treatment; with regimens that include INH and Rifampin, and 5 months for treatment without INH and Rifampin
- Whose smears demonstrate increasing number of AFB after an initial decrease
- Patients whose cultures do not become negative after 4–6 months
- Patients who relapse

TB susceptibility testing has three main goals:

- It provides data as to what drug should be used for treatment
- Screens for drug resistance
- Measures incidence and prevalence of drug resistance within the community.

Susceptibility Testing Methodology

Susceptibility tests can be performed directly, from a smear positive specimen, or indirectly, from the growth of colonies from the specimens. The former has the advantage of measuring the sensitivity prior to cultivation on laboratory media. The direct method also produces results more rapidly but; because of uncertainty of the species of *Mycobacterium,* and due to less control of the viable inoculum size, the results require confirmation with an indirect test, the direct test is not generally utilized.

Three methods make use of critical concentrations to define drug resistance and can be performed directly or indirectly:

- Absolute concentration method
- Resistance ratio method
- Proportion method.

The absolute concentration method determines if 1% or more of an inoculum will grow after being cultured on media containing critical concentrations of a drug on the plate. It requires growth of the patient strain on drug free medium to demonstrate the viability, but does not compare the colony numbers on drug free and drug containing media so that the inoculum must be carefully standardized.

The resistance ratio is similar to the absolute concentration method except that the patient strain is compared with the growth of a standard laboratory strain. Results are reported as the ratio of the MIC of the patient strain to that of the laboratory strain. A patient strain with a ratio of 8:1 is considered resistant, while 4:1 is suggestive of resistance. This method is more tolerant to variation in concentration of drugs within different batches of media.

The proportion method compares the growth of a patient strain in the presence and absence of a drug. If 1% or more of the inoculum produces colonies on media that contains an agent at the critical concentration compared with controls, the isolate is considered to be resistant. This method is the most popular and is relatively simple to perform and interpret.

Susceptibility Testing of Mycobacteria

Eleven drugs are used in the treatment of tuberculosis. Five are considered "primary" and include streptomycin, isoniazid, rifampin, pyrazinamide and ethambutol, while the remaining six, Ethionamide, ciprofloxacin, kanamycin, D-cycloserine, para-aminosalicylic acid and amikacin are considered "secondary" and used only when resistance develops to the primary drugs.

Although drugs have been incorporated in inspissated egg-based media for conducting susceptibility tests, many laboratories internationally now prefer using Middlebrook 7H11 or 7H10 as a base medium, adding the drugs after cooling the agar to 45°C. Adding the drugs to the agar medium after autoclaving decreases the loss of activity that can occur in egg-based medium such LJ during inspisstion. An additional loss of drug activity may occur in egg-based media with binding of some agents to egg albumin and other proteins.

Drug Concentrations for Proportion Method Susceptibility Testing using Various Culture Media*

Drug concentration (µg/mL)

Drug	*7H10*	*7H11*	*Lowenstein-Jensen*
Isoniazid	0.2,1.0	0.2,1.0	0.2,1.0
p-Aminosalicylic acid	2.0	8.0	0.5
Streptomycin	2.0	2.0	4.0
Rifampin	1.0	1.0	40.0
Ethambutol	2.0	7.5	2.0
Ethionamide	5.0	10.0	0.0
Kanamycin	5.0	6.0	20.0
Capreomycin	10.0	10.0	20.0
D-Cycloserine	20.0	30.0	30.0
Pyrazinamide	50.0	—	100.0

A simplified method for preparing drug susceptibility plates has also been developed. This method uses filter paper disks containing the primary antitubercular drugs, and the test for susceptibility is run in a similar fashion as the Kirby Bauer method for routine drug susceptibility tests.

As discussed, the direct mycobacterial susceptibility test is inoculated from digested and concentrated sputum found to be positive for acid-fast bacilli. The indirect susceptibility test is inoculated from colonies isolated from a primary culture. The direct test will usually give good results only if large numbers of mycobacteria are present in the specimen. The advantage of the direct susceptibility test is an earlier report (3 to 4 weeks) in contrast to the indirect test, which may take up to 6 to 8 weeks. The disadvantage of the direct susceptibility test is that it usually requires a large number of mycobacteria for successful growth and is often overgrown by large numbers of contaminating bacteria.

Other novel methods of susceptibility testing have been developed based on the mycobacteriophage technique, using the luminescent luciferase activity. Other researchers have localized specific *Mycobacterium tuberculosis* mutations responsible for drug resistance. These sites have been used as amplification targets and promise to provide a rapid method for testing the susceptibility of patient isolates to these drugs.

PRIMARY/SECONDARY DRUG CONTAINING LOWENSTEIN-JENSEN MEDIA PANEL MTB SENSITIVITY TESTS

(*Courtesy:* Tulip Group of Companies)

Sensicult®

Summary

Inadequate chemotherapy, irregularity of treatment and use of improper antitubercular regimen lead to high failure rates of antitubercular treatment. As a result, the prevalence of chronic patients discharging drug-resistant organisms increases. Alarming figures of drug resistance in newly detected patients are being reported, mainly from developing countries. This calls for testing of antibiotic sensitivity in vitro prior to starting therapy.

Reagent

SENSICULT: LJ Primary/secondary drug panels are Reagents for laboratory use only. Primary/secondary drug containing Lowenstein-Jensen media panel for MTB sensitivity tests is a set of ready to use Lowenstein-Jensen solid medium slants incorporated with individual antitubercular drugs of recommended specified strength.

Contents

I. Primary drug Lowenstein-Jensen medium panel contains Lowenstein-Jensen medium with the following antibiotics/antitubercular drugs.

Drug	*Symbol*	*pH*	*Concentration*
1. Isoniazid	IN	7.0+ 0.1	1.0 ng/mL
2. Ethambutol	EB	7.0± 0.1	2.0 µg/mL
3. Rifampin	RP	7.0± 0.1	4.0 µg/mL
4. Streptomycin	ST	7.0 ± 0.1	4.0 µg/mL
5. Pyrazinamide	PY	5.5 ± 0.1	100. µg/mL
6. Control for Pyrazinamide	PC	5.5 ± 0.1	
7. LJ Control	LJ	7.0 ± 0.1	-
8. Sterile distilled water with glass beads for inoculum preparation			

II. Secondary drugs Lowenstein-Jensen media panel contains Lowenstein-Jensen medium with the following antibiotics/antitubercular drugs.

Drug	*Symbol*	*pH*	*Concentration*
1. p-Aminosalicylic acid	PA	7.0 ± 0.1	0.5 µg/mL
2. Ciprofloxacin	CP	7.0± 0.1	2 0. µg/mL
3. Amikacin	AM	7.0 ± 0.1	20.0 µg/mL
4. D-cycloserine	DC	7.0 ± 0.1	30.0 µg/mL
5. Kanamycin	KA	7.0 ± 0.1	20.0 µg/mL
6. Ethionamide	ET	7.0 ± 0.1	20.0 µg/mL
7. LJ Control	LJ	7.0 ± 0.1	—
8. Sterile distilled water with glass beads for inoculum preparation			

Principle

Due to increase in drug resistant strains of *Mycobacterium tuberculosis* and increasing failure rates of antitubercular drug regimens, it is desirable to start antitubercular therapy only after sensitivity assay of the most suitable drug against particular isolate infecting the patient.

Storage and Stability

1. Avoid jerks and vibration while storage, shipping and incubation.
2. Store the LJ kits at 2–8°C, away from light.
3. Stability of the unopened media is as per the expiry date mentioned on the label.
4. Upon opening, the medium must be put into use instantly.

Additional Material Required

Sterile plating loops (10 µL), incubator at 37± 0.5°C, biosafety hood with Bunsen burner, activated 2%

glutaraldehyde solution, vortex mixer, 0.1–0.5 mL micropipettes, sterile micropipette tips.

Inoculum Preparation for Sensitivity Testing

a. Take a loopful aseptically from the *Mycobacterium tuberculosis* colony grown on Lowenstein-Jensen slant.
b. Transfer it aseptically to the screw capped bottle containing 0.1 mL of sterile distilled water and glass beads, for inoculum preparation.
c. Close cap tightly and subject the contents of the bottle to mechanical shaking (vortex) for 10 minutes.
d. Keep standing for 10 minutes before opening the bottle.
e. Dilute this in saline to match McFarland 0.5 Standard. This contains approximately 1.5×10^8 org/mL.
f. Further dilute to 1: 10000 with saline. This is seed culture. (10000–12000 org/mL).
g. Mix well and use this as inoculum.
h. Discard the container with glass beads in 2% activated glutaraldehyde solution.

Test Procedure

1. Bring the primary/secondary drug containing Lowenstein-Jensen media panel for MTB sensitivity tests slants to room temperature.
2. Apply 100 µL from the seed stock to each slant of primary/secondary drug containing Lowenstein-Jensen media panel for MTB sensitivity tests also control LJ.
3. A fresh disposable loop should be used for each slant.
4. Close the cap tightly and incubate at 37± 0.5° C.
5. Observe for the growth after 2 weeks till 8 weeks, every week.

Interpretation of Results

As and when there is sufficient growth on control (>100 colonies) compare the growth with the antibiotic containing media.

1. If ratio of the growth in antibiotic containing media as compared to control is less than 0.01 the isolate will be termed as sensitive.
2. If ratio of the growth in antibiotic containing media as compared to control is more than 0.01 the isolate will be termed as resistant.

Example:

$$\text{Ratio} = \frac{\text{No. of colonies on antibiotic containing media}}{\text{No. of colonies on control media}}$$

Sensitive if ratio is less than 0. 01
Resistant if ratio is more than 0.01
Border line if ratio is equal to 0.01

Remarks

1. Discolored, dislodged or contaminated medium should not be used.
2. Good laboratory practices and hazard precautions must be observed at all times.
3. Treat the specimen and used slants by immersing in 2% activated glutaraldehyde for at least 2 hours before incineration and disposal.

Other Markers

Adenosine deaminase, a surrogate marker, for the diagnosis of tuberculosis has also shown promise. It is based on the measurement of activity of Adenosine deaminase, an enzyme produced by lymphocytes. The test has excellent sensitivity for TB meningitis and for examining pleural infections.The sensitivity and specificity is reported well above 90%, the test is easy to perform and relatively inexpensive.

To conclude, the objective of adapting different types of technology and instruments is to shorten the times for isolation, identification and susceptibility testing of bacteria and other microorganisms has been particularly relevant for mycobacteria. Hopefully, alternative methods to the standard procedures now used, could be developed soon enough for routine use, to provide cultures and susceptibility information in a shorter time interval. Till such time the AFB staining, culture and sensitivity remain the gold standard for accurate and early diagnosis of tuberculosis, improvements and standardization of techniques for these classical methods is important for better laboratory diagnosis and clinical information support. Significant cost savings might be effected by a reduction in hospitalization and return of the patient to a productive career.

Troubleshooting LB

Problem: Growth not obtained on LJ control slant after diluting the culture suspense to 1:10000 of the Standard 0.5 McFarland turbidity

Possible causes	*Solutions*
1. During scraping of culture growth, the media (egg yolk base) is being scrapped which gives turbidity matching standard 0.5 McFarland	Select only the cultural colony without scraping the media (egg yolk base)

Contd...

Contd...

Problem: Collapse of slants

Possible causes	*Solutions*
1. Improper transportation, i.e. kits subjected to violent jerks and vibrations or not handled with care during storage, shipping and incubation	Discard the slant and use fresh slant for testing
2. Contaminated slants	If the slants are found contaminated because of various reasons, discard the slants and use fresh slants to perform the test
3. Contaminated untreated sputum sample used	Pretreat sputum sample prior to inoculation

IN DETERMINATION OF ADENOSINE DEAMINASE ACTIVITY IN SERUM, PLASMA AND BIOLOGICAL FLUIDS

Courtesy: Tulip Group of Companies

ADA-MTB®

Summary

Tuberculosis occurs worldwide and is rampant in many countries. Though curable, its infection is on the rise. The most specific test is the positive bacterial culture of a patient's sputum sample. This is cumbersome and time consuming. X-rays, smears for AFB and Tuberculin tests though comparatively rapid are not conclusive. Adenosine Deaminase (ADA) is an enzyme widely distributed in mammalian tissues, particularly in T lymphocytes. Increased levels of ADA are found in various forms of tuberculosis making it a marker for the same. Though ADA is also increased in various infectious diseases like infectious mononucleosis, typhoid, viral hepatitis, initial stages of HIV, and in cases of malignant tumors, the same can be ruled out clinically.

Reagent

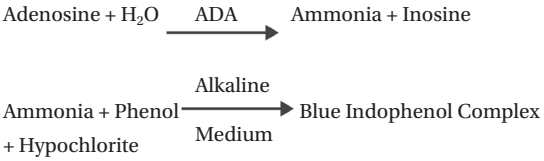
ADA-MTB is a reagent for laboratory use only.

ADA-MTB comprises of:

a. ADA-MTB reagent (L1)—Buffer reagent, ready to use.
b. ADA-MTB reagent (L2)—Adenosine reagent, ready to use.
c. ADA-MTB reagent (L3)—Phenol reagent.
d. ADA-MTB reagent (L4)—Hypochlorite reagent.
e. ADA-MTB standard (S)—ADA standard, ready to use.

Principle

Adenosine deaminase hydrolyzes adenosine to ammonia and inosine. The ammonia formed further reacts with a phenol and hypochlorite in an alkaline medium to form a blue indophenol complex with sodium nitroprusside acting as a catalyst. Intensity of the blue colored indophenol complex formed is directly proportional to the amount of ADA present in the sample.



Reference Values

Serum, Plasma, Pleural,	Normal	< 30 U/L
Pericardial and	Suspect	30 U/L to 40 U/L
Ascitic Fluids		
	Strong Suspect	40 U/L to 60 U/L
	Positive	> 60 U/L
	Normal	< 10 U/L
CSF	Positive	> 10 U/L

It is recommended that each laboratory establish its own normal range representing its patient population.

Storage and Stability

1. Store the kit at 2–8°C, away from light.
2. Stability of the kit is as per the expiry date mentioned on the label.

Note

1. It is important that kit components from the same lot are used for achieving accurate and reproducible results. Do not intermix reagents from different lots.

2. The sequence of addition of Reagents should be followed meticulously for achieving accurate results.

Additional Material Required

Test tubes, test tube stand, water bath/incubator (37°C), distilled or deionized water, variable volume pipettes, spectrophotometer with filter at 570–630 nm (Hg 578 or 623 nm) at 37°C or colorimeter with yellow or red filter, stopwatch.

Reagent Preparation

Reagents L1, L2 and standard are ready to use. Adenosine Reagent (L2) may form crystals at 2–8°C. Dissolve the same by gently warming (37 to 50°C) the Reagent for some time before use. Both the Phenol Reagent (L3) and Hypochlorite Reagent (L4) need to be diluted 1:5 with distilled water before use (1 part of Reagent + 4 parts of distilled water). The Working Phenol Reagent and Working Hypochlorite Reagent are stable for at least 6 months when stored at 2–8°C in tightly closed bottles.

Specimen Collection and Preparation

Collect specimen prior to use of antimicrobial agent. Wherever possible, indicate clearly that patient is on antitubercular drugs.

CSF: Collect as much as possible in a syringe, clean skin with alcohol before aspirating specimen.

Body fluids: Disinfect the site and collect specimen with aseptic precautions.

Serum, Plasma: No special preparation of the patient is required prior to sample collection by approved techniques. It is recommended to use fresh sample specimen for testing. Do not use hemolyzed, contaminated or turbid sample specimens. Fresh EDTA, citrate, heparinized or oxalate anticoagulated (dry anticoagulant) plasma specimens are suitable for performing the test.

ADA is reported to be stable in serum for 3 days at 2–8°C and in biological fluids for 2 days at 2–8°C, as after this, ammonia may be released in the samples even without any microbial contamination.

Test Procedure

1. Bring all Reagents and samples to room temperature before use.
2. Prepare the working phenol reagent and working hypochlorite reagent.
3. Set the spectrophotometer filter at 570–630 nm (Hg 578 or 623 nm) at 37°C.
4. Pipette into clean dry test tubes labeled Blank (B), standard (S), sample blank (SB) and test (T) as follows:

Calculations

$$\text{Total ADA activity in U/L} = \frac{\text{Abs.T–Abs.SB}}{\text{Abs. S–Abs.B}} \times 50$$

Linearity

The procedure is linear up to 150 U/L. If values exceed this limit dilute the sample with deionized water and repeat the assay. Calculate the value using the appropriate dilution factor.

Remarks

1. One unit of ADA activity releases three nanomoles of ammonia in the reaction in 1 hour at 37°C.
2. Patients with hyperammonemia, kidney disorders and hepatitis can present high level of ADA values. Patients with chronic malnutrition or HIV can present low levels of ADA values.
3. Higher levels of ADA are also found in leprosy, brucellosis, HIV infections, viral hepatitis, infectious mononucleosis and liver cirrhosis. Before arriving to a diagnostic decision, these clinical conditions must be ruled out.
4. Using a cut off level of 60 units/L of ADA, values has been reported to show the specificity and the sensitivity of the test as above 90% for the MTB infection.
5. Below 60 U/L of ADA, the serum of ADA specificity and sensitivity is lower and should be interpreted in the light of other tests for confirmation of *Mycobacterium tuberculosis* infection.

Troubleshootings

Problem: False positive results

Possible cause	*Solutions*
1. Addition of too much sample in relation to reagent	Add exact quantity of sample as mentioned in the package insert.
2. Markedly lipemic and contaminated sample could give improper reading	Do not use lipemic and contaminated sample for testing
3. Less amount of standard added in the respective tube	Add exact quantity of standard as mentioned in the package insert
4. Incubation period or incubation time exceeded	Ensure 1st incubation exactly at 37°C and 2nd incubation at 37°C or at RT for exact period as mentioned in the package insert
5. Incorrect interpretation of results	Read the result within 30 minutes after performing the test as per instructions given in the package insert

Problem: False negative results

Possible causes	*Solutions*
1. Addition of less amount of sample with respect to the reagent	Add exact quantity of sample as mentioned in the pack insert
2. Addition of higher amount of standard in respective tube	Add exact quantity of standard as mentioned in the package insert
3. Improper incubation	Ensure 1st incubation exactly at 37°C and 2nd incubation at 37°C or at RT for exact period as mentioned in the pack insert
4. Improper dissolution of the adenosine reagent	Dissolve the adenosine reagent properly by gently warming at 37–50°C in water bath
5. Improper mixing of the sample with reagent	Mix the sample properly with reagent after every addition

Problem: Reagent not working

Possible causes	*Solutions*
1. Improperly mixed reagent	Bring the reagent at RT. Mix the reagent properly by tilting the vial upside down before performing the test
2. Improper dissolution of adenosine reagent	Dissolve the adenosine reagent completely by warming at 37–50°C in water bath

GRAM-NEGATIVE BACILLI

The organisms mentioned below can be divided into five families according to the criteria given.

A. The organisms which grow well on any ordinary media containing peptone.
   1. The organisms which ferment few or no carbohydrates.
      a. Achromobacteriaceae.
   2. The organisms which ferment many carbohydrates.
      a. Enterobacteriaceae.

B. The organisms which are obligate parasites requiring body fluids for growth. These are small gram-negative rods.
   a. Brucellaceae.

C. The organisms which tend to elongate and are motile and most species produce water soluble pigments.
   a. Pseudomonadaceae.

D. Miscellaneous rod-shaped organisms (both gram-positive and gram-negative).

Achromobacteriacea

This family contains three genera of which only one is encountered in human microbiology.

Alcaligenes faecalis

This is a gram-negative rod inhabiting the intestinal tract of man and animals. It is motile and like all members of this family fails to ferment most carbohydrates. It is non-pathogenic in the stool of man because it fails to ferment lactose, it may at first have the appearance of a pathogenic stool organisms. It is readily distinguished, however, because it not only fails to ferment lactose but it also fails to ferment dextrose and other sugar, which are commonly used for species identification, and it fails to grow on the selective media designed for the isolation of intestinal pathogens.

Enterobacteriaceae

(Gram-negative intestinal bacilli) These organisms comprise the following main groups:

1. The coliform bacilli, which include the common commensal aerobic bacteria of the colon of man and animals.
2. The *Salmonella* group, which includes the typhoid-paratyphoid bacilli (of enteric fever) and the organisms of bacterial enteritis or food poisoning.
3. The *Shigella* or dysentery group. General characteristics: gram-negative, non-sporing bacillary organisms, about 2–4 microns by 0.5 micron (average), aerobes and facultative anaerobes growing best at about 37°C fermenting various carbohydrates but not usually liquefying gelatin or serum.

Proteus vulgaris

Straight rods about the same size as *Pseudomonas aeruginosa,* pleomorphic, motile with numerous lateral flagella, non-sporing, gram-negative. Members of this genus grow abundantly at 37°C on the usual cultural media. They are actively motile and have a tendency to swarm, causing spreading of their colonies over the media, often obscuring other organisms. It is important to remember that these organisms do not ferment lactose despite the fact that they are not intestinal pathogens. They can be readily distinguished from the pathogenic non-lactose fermenters because they do not grow readily on the selective media to be described, but they split urea. That is colonies of organisms produce urease, which have the property to break down urea to ammonia. Pathogenic non-lactose fermenters lack this property.

The next three genera, closely related, are *Escherichia, Aerobacter* and *Klebsiella*. These organisms have the following features in common—they are all non-pathogenic in stool, they all ferment lactose actively, and they grow abundantly on ordinary media at 37°C.

Escherichia coli

These gram-negative rods are found in almost every stool specimen and are of the utmost importance to medical microbiology. They are frequently pathogenic when found in either the urinary tract or in purulent material from other sources. Their growth on blood agar is rather non-specific and they may occasionally show true hemolysis. They are usually motile, they do not produce hydrogen sulfide, and they produce promptly both acid and gas from lactose. On EMB media they are highly pigmented and have a metallic sheen. They are important as an indication of fecal contamination of water, food and milk and is therefore, important that this organism be distinguished from some others, which are closely related. The IMVC is a group of tests, which are useful as a means of distinguishing *E. coli* from other members of this group.

Indole

This can be tested for by growing the organism in peptone water and after 2 days, withdrawing with a sterile pipette 2 to 3 mL into a test tube. An equal volume of Ehrlich's reagent is then added. A rose color develops in the presence of indole, and can be separated out with amyl alcohol. The addition of a saturated solution of potassium persulfate hastens the reaction. If the indole reaction is negative after 2 days growth, the test should also be repeated after 7 days, as some strains are slow in their production of indole.

Methyl Red

This test is dependent upon the degree of acidity produced by the organism in dextrose broth. The indicator methyl red is red on the acid side and yellow on the alkaline side but the shift is at about pH 3. Therefore, an organism, which ferments dextrose with small amount of acidity approximately 5, is negative to methyl red, whereas one which ferments, dextrose with the production of a high degree of acidity, approximately pH 2, is positive methyl red.

Voges-Proskauer Reaction

This is a qualitative test for the presence of acetyl-methyl-carbinol. In the fermentation of glucose, some organisms form this chemical, which gives a purple color when added to peptone in a strongly alkaline medium. The test is performed by growing the organisms in a glucose-peptone broth for about 48 hours. At the end of this time, if the medium is alkalinized by adding 5 cc of 10% KOH, a deep pink color develops after some standing. The culture must be at least 48 hours old.

Citrate Test

This is a test to determine the ability of an organism to utilize citrate as the sole source of carbon in a synthetic medium. The medium is prepared containing inorganic substances and sodium citrate.

The characteristic IMVC formula for *E. coli* is +, +, –, –.

Aerobacter aerogenes

The organism is ubiquitous in nature found in the soil and upon many grains. It resembles *E. coli* although it usually forms a more gelatinous colony on EMB. The IMVC test is –, –, +, +. Some organisms are intermediate in their IMVC reactions. Bergey separates these two genera according to Methyl Red and Voges-Proskaur, therefore of the four tests, these two are the most reliable.

Klebsiella species (Klebsiella pneumoniae)

This organism is rather variable in some of its physiologic characteristics. It usually closely re-sembles *Aerobacter aerogenes* but IMVC often intermediate or variable. *Klebsiella pneumoniae* should be suspected in the following instances:

a. In culture material obtained from lungs.
b. In culture material from a patient with crusting lesions of the nose.

Morphology
A small non-motile, non-sporing gram-negative bacillus with rounded ends and varying greatly in size 1–4 μm by 0.5 μm. The shorter forms simulate cocci. The bacilli occur usually in pairs but also singly, and in short chains. They are typically capsulated, especially when seen in tissues.

Klebsiella rhinoscleromatis

This organism closely resembles the pneumobacillus in morphology and cultural characteristics, but produces no gas from glucose, and does not ferment lactose. It does not grow in media containing bile, and does not give the Indole and Voges-Proskauer reactions. The organism is associated with a chronic granuloma of the mucous membrane of the nose, mouth or throat.

Klebsiella ozaenae

Closely resembles the pneumobacillus, but is non-gas producing in glucose. It is found associated with ozaenae, but is not to be regarded as the causative agent of this condition.

Paracolon Bacilli

These organisms occupy a borderline position. In all essential features they resemble either *E. coli* or *Aerobacter aerogenes*, with one important exception, they ferment lactose very slowly and weakly. Since, one species of the enteric pathogens may also ferment lactose very slowly there is a possibility of confusion. They following features serve to distinguish the paracolon organisms from this one pathogenic species (*Shigella shigae*).

1. Growth on selective media: *Shigella* grows well on SS media, while paracolon organisms do not.
2. Motility: *Shigella* is always non-motile. The paracolon may or may not demonstrate motility.

The paracolon group of organisms represents a variant of the *Escherichia* tribe. They are named according to which the common genera, the unknown most closely resembles for example Paracolobacterium aerogenodies or P. coliforms.

Enteric Fever Bacilli—Typhoid and Paratyphoid

These include *Salmonella typhi, Salmonella paratyphi* A and *Salmonella paratyphi* B.

Salmonella typhi

This is the pathogen responsible for typhoid fever.

Morphology
A gram-negative non-sporing bacillus about 2–4 μm, actively motile, with numerous long peritrichous flagella as observed in special stained preparations.

Cultures
Aerobe and facultative, grows well on ordinary media at optimum temperature of 37°C. Colonies on agar-like those of coliform bacilli, but smaller, thinner and most transparent. No liquefaction of gelatin. Colonies on MacConkeys are smaller than those of coliform bacilli and pale or colorless, the typhoid bacillus being a non-lactose fermenter. Colonies are pale or colorless on deoxycholate citrate medium. *S. typhi* ferments glucose and mannitol with acid, but no gas formation; does not ferment lactose or sucrose and does not produce indole.

Salmonella paratyphi A and Salmonella ♥paratyphi B

The morphology and general characters are identical with those of the typhoid bacillus. Important reactions are no change on lactose, glucose and mannitol—acid and gas production, sucrose—no change. The typhoid bacilli do not produce gas while the paratyphoid bacilli produce gas.

Pathogenicity

The diseases of typhoid fever and paratyphoid fever are characterized by widespread dissemination of the organisms by the bloodstream, with production of a systemic febrile disease. The common symptoms are headache, anorexia, muscular weakness, diarrhea and development of typical 'rose spots' on the abdominal skin. After the general invasion of the body, the bacteria tend to localize in the lymphatic system. The symptoms of the disease are apparently the result of toxemia from the endotoxin of the organisms. The organisms may be found in the blood cultures during the first 10 days. They are found in the urine, especially during the 2nd week, and they are found in the stool from about the 2nd week on, with increasing frequency as the organisms tend to localize in the lymphoid tissue of the intestinal wall. After recovery from the disease, many people harbor a few bacteria in the intestinal tract and especially in the gallbladder. These people are not sick, for they have developed an adequate immunologic protection to the organism. Artificial immunization is established by the use of typhoid vaccine.

Diagnosis of Enteric Infections

The bacteriological diagnosis depend upon

1. The isolation from the body and the identification of the causative organisms.

2. The demonstration of its presence in the body by the Widal agglutination reaction, which is based on the occurrence of specific agglutinins to the organism in the serum of the infected person.

Blood Culture

In the early stages of illness, blood culture is the best diagnostic method, and should be used in all cases met with during the first 10 days of fever.

Feces Culture

Typhoid and paratyphoid bacilli can be isolated from the feces and are most frequent at the end of the second week or during the third week, but may be detected at all stages of the disease. Examination of feces however, may yield negative results unless repeated, and the isolation of typhoid-paratyphoid bacilli from this source is often rendered difficult owing to their being relatively scanty as compared with coliform bacilli. MacConkeys bile salt, neutral red, lactose agar is a medium used for the differentiation of typhoid-paratyphoid bacilli, and ordinary coliform bacilli. Deoxycholate-citrate agar is also used. Wilson and Blairs Bismuth sulfite method may also be used. Special media includes selenite F medium and tetrathionate broth, which gives very good results when used as an enrichment. In the examination of feces from enteric cases, the best results are obtained by employing two or three different methods simultaneously.

Urine Culture

Typhoid-paratyphoid bacilli may also be isolated from urine. The specimen is centrifuged, several loopfuls of the deposit are inoculated on a plate of deoxycholate-citrate medium and successive strokes made in the usual way so that isolated colonies are obtained.

Widal Reaction

Discussed in detail in Serology chapter.

Organisms of Bacterial Enteritidis or Food Poisoning

These organisms are found in the intestinal contents during the disease and in some cases, in the food.

Salmonella enteritidis

The organism is generally similar to *S. paratyphi* B in various cultural and biochemical characters but does not ferment inositol.

Salmonella typhimurium

Resembles *S. paratyphi* B in cultural and biochemical reactions. The identification of *S. typhimurium* and other *Salmonella* types is carried out by the method of antigenic analysis. *S. typhimurium* produces enteritidis in a wide variety of animals.

Diagnosis of Salmonella Food Poisoning

The stool is cultured on deoxycholate citrate medium as in the diagnosis of enteric fever, pale colonies are subinoculated and the resulting cultures are tested and identified. Blood culture may in some cases yield positive results and should be carried out as a routine measure.

Group of Dysentery Bacilli

The causative organisms of an acute form of dysentry most prevalent in tropical countries. In some instances, particularly those caused by *Shigella shigae*, the disease may be virulent, characterized by high fever and toxemia. The stool is characteristically full of pus cells, without much blood or mucus. In severe cases the stool may resemble purulent material. The degree of toxemia may be extreme so as to cause death. These generalized effects are probably the result of absorption of endotoxins.

Shigella Shigae or Dysenteriae and Shigella Flexneri or Paradysenteriae

Morphology

Non-motile, non-sporing, gram-negative bacilli about 2–4 μm × 0.5 μm but often showing a tendency to shorter coccobacillary forms.

Culture

Resembles the *Salmonella* group. Gelatin is not liquefied. Biochemical reactions—the dysentery bacilli ferment glucose without gas production, and in the case of sugar fermentations generally are non-gas producing. H_2S is not produced.

Brucellaceae

These organisms are small gram-negative coccoid to rod-shaped cells, which may be found either singly or in pairs or short chains. The organisms may either be aerobic or facultative anaerobes. An increased CO_2 tension is necessary for the growth of some of the species. Most of the species require additional nutritional factors for growth.

Pasteurella pestis

This organism is the causative agent of plague in man and rats.

Morphology

The plague bacilli are short plump, non-motile, gram-negative rods, which may be very pleomorphic. They may

appear singly or in pairs or short chains. A capsule may be demonstrated especially from animal tissues.

Culture
This organism grows best at 30°C. The colonies produced on blood agar are small and round and are transparent and glistening with a wavy margin.

Pasteurella tularensis

This organism causes tularemia, which is a disease of rodents that is easily transmitted to man, through handling of infected animals or blood sucking insects.

Morphology
This organism is a minute, pleomorphic, non-motile, gram-negative rod with capsules occurring in vivo.

Culture
The organism requires a special enrichment medium for growth. The colonies produced are minute transparent drop-like colonies, which appear 2–5 days after incubation. The optimal growth temperature is 32°C.

Bordetella pertussis

This is the causative agent of whooping cough.

Morphology
These organisms are minute gram-negative, motile or non-motile coccobacilli.

Culture
The organism requires special enrichment media (potato-blood glycerol agar) called Bordet-Gengou agar for growth.

Brucella species

This genus consists of three species of small gram-negative coccoid bacilli. All 3 are strict parasites and are pathogenic for man and animals. This genus is divided into species by their action on sugar and by their additional requirements of CO_2. The organisms are aerobic and grow best at 37°C.

Brucella abortus, *Br. melitensis* and *Br. suis* cause contagious abortion in cattle and undulent fever in man.

Laboratory Diagnosis
Blood culture should be carried out repeatedly in all cases during the febrile phase and it is essential that at least 10 mL of blood should be withdrawn for this purpose, as the organisms may be relatively scanty. In suspected *Br. abortus* infection, the blood culture must be incubated in an atmosphere of 10% CO_2, in *Br. melitensis* the organism may be isolated from urine.

An agglutination reaction may be done with *Brucella* antigen after 7–10 days from the onset of illness. It has to be noted that normal serum may agglutinate *Brucella* suspensions in low dilutions. In cases of undulant fever, however, the serum often agglutinates both *Br. abortus* and *Br. melitensis* in high dilutions 1:1000. In suspected cases, if the reaction occurs only with low dilutions, e.g. less than 1:80, the result cannot be regarded as conclusive. When the test is repeated, a rising titer should be observed.

Haemophilus

This genus includes the small non-motile, gram-negative bacilli, which require the presence of hemoglobin for growth. Some of the species require additional factors for growth. This factor is Factor X which is a heat stable substance associated with hemoglobin and Factor V which is a heat labile substance found in yeasts and vegetable extracts. All of the species of this genus are parasites and will grow only in the presence of growth accessory substances. They may or may not be pathogenic for man.

Haemophilus influenzae

This organism is commonly isolated from the respiratory tract. It plays an important role in acute respiratory infections, conjunctivitis and purulent meningitis. This organism grows well on chocolate agar enriched with a yeast supplement. The colonies grow in 24 hours and resemble droplets of moisture on agar plates. If the colonies are growing near colonies of staphylococci or pneumococci, they will be large. These colonies produce an extra supply of Factor V, which stimulates the growth of *Haemophilus influenzae.* This phenomenon is called as satellitism.

Haemophilus aegyptius (Koch-week bacillus)

This organism is the causative agent of conjunctivitis (pink eye). This organism closely resembles *H. influenzae* and needs both factors X and V for growth.

Haemophilus ducreyi

This organism is the cause of chancroid in man, a sexually transmitted disease. The small gram-negative rods may be found in smears from a genital ulcer. *H. ducreyi* is grown on chocolate enriched agar only with considerable difficulty.

Donovania granulomatis

This too causes a venereal disease—Granuloma Venereum. Laboratory diagnosis of this disease is made by demonstrating 'Donovan bodies' which are bacillary bodies surrounded by a dense capsule in the mononuclear cells from the lesion, which have been stained with Leishman's stain. The organism will not grow on ordinary media.

Moraxella lacunae (Morax-Axenfeld bacillus)

This is the causative agent of conjunctivitis. In stained smears of pus, the organisms appear as short, thick gram-

negative diplobacilli. It grows on Loeffler's serum medium and the colonies appear as small indentations indicating liquefaction of the medium.

Pseudomonadaceae

This group includes a large number of bacilli, which tend to elongate and some of these may produce a water soluble pigment. The majority of these species are not pathogenic to man.

Pseudomonas aeruginosa (Bacillus pyocyaneus)

The organism is commonly found in air and water but is frequently encountered in urinary, eye and ear infections.

Morphology

This is a gram-negative motile rod with 1–3 polar flagella occurring singly, in pairs and chains. The colonies give a ground glass blue green appearance. This organism can often be distinguished by the characteristic grape-like odor. It should be remembered, however, that a few of the species are non-chromogenic and therefore, the identification on the basis of pigment production may be difficult.

Culture

Pseudomonas grows well on ordinary media. It does not ferment lactose and since on MacConkey agar the pigment may be masked or absent, it may be confused with enteric pathogens. *Pseudomonas* strains isolated from clinical material are hemolytic on blood agar.

Bacteriaceae

The members of this group include the small gram-negative, non-sporing bacteria with rounded or pointed ends. They vary in size from filtrable forms to long filamentous branching organisms. Majority of them are strict anaerobes and are found in mouth, vagina and intestinal tract of man. These organisms can cause abscesses and occasionally septicemia. Complex culture media are needed for isolating these bacteria.

Fusobacterium fusiforme

Gram-negative bacilli with pointed ends. It is found in the normal mouth and is especially associated with the fusospirochetal disease, Vincent's angina. This organism may be seen in smears from Vincent's lesions.

Vibrio cholerae

Vibrio cholerae, or the comma bacillus is the causative agent of cholera. This disease is characterized by an acute gastroenteritis of sudden onset and often running a fatal course.

Morphology

It is a bent or slightly curved bacillus, resembling very closely a comma, from which it derived its name, comma bacillus. It is about 2 μm in length. It is actively motile, and the movement is of a darting or scintillating type. It is a gram-negative bacteria.

Culture

It is aerobic and slight growth also occurs under anaerobic conditions. It grows on ordinary media at a temperature range of 16–40°C. Abundant growth occurs on highly alkaline media of pH 8.2. Colonies on nutrient agar are white circular discs and transparent. True *V. cholerae* is non-hemolytic. It ferments glucose, sucrose, mannitol, and maltose with only acid production. *V. cholerae* can produce indole and nitrites in peptone water. This is studied by cholera red reaction. Add a few drops of H_2SO_4 to 4 day's peptone water culture. A reddish-pink color develops due to the formation of nitroso indole.

Laboratory Diagnosis

Collection of materials, from infected patient; 'rice water' stool, vomit and other fomites; from suspected water: About a liter should be collected from the surface in a sterile vessel, and packed in ice until used for examination.

Methods of Examination

Microscopic examination of fresh stool. This gives an idea of the character of the cellular exudate and is very helpful to differentiate the condition from acute bacillary dysentery. There are very few leukocytes or macrophages but only mucus and degenerated epithelial cells. In some cases, red cells may be present. Many bacilli with characteristic morphology and well marked motility may be seen.

A white flake of mucus is taken and smeared over a clear slide which is allowed to dry in the air. The film is then fixed over a flame and stained with dilute carbol fuchsin for a minute or two. After a thorough wash, when the slide is dry, it is examined under an oil immersion lens. The typical comma shaped, gram-negative vibrios are found in a case of cholera. A hanging drop preparation is useful to observe motility.

Cultural Methods

An alkaline peptone water tube is inoculated with a flake of mucus and incubated for 8–10 hours. A drop from the surface should be examined for the vibrios. Subcultures are made from this to Dieudonne's medium. The resultant growth is identified by smear and biochemical reactions. From the peptone water culture, a cholera red reaction may be done to identify the organism.

SPIROCHETES

These are spiral, elongated, motile, flexible, organisms. They are not easily stained and are best demonstrated by dark field microscopy or silver impregnation methods. The three important groups are (1) *Treponema*. (2) *Leptospira*, and (3) *Borrelia*.

Treponema

These are slender spirals about 0.2 μm in width and 5–15 μm in length, causing syphilis and related diseases. The spirals are so thin that dark field microscopy is used. They are actively motile.

Treponema pallidum

This is the causative organism of syphilis, a human infection, transmitted sexually. It occurs in three stages, namely primary, secondary and tertiary stages.

Primary Stage
About 2–10 weeks after infection, an ulcer is formed which heals spontaneously. Spirochetes can usually be demonstrated in the serous exudate from the lesion.

Secondary Stage
About 2–10 weeks after healing, skin eruption may occur giving rise to a red rash with papules. Spirochetes can be demonstrated in the serous exudate from these skin lesions.

Tertiary Stage
About 50% of the untreated cases proceed to this stage which is characterized by degenerative changes in the central nervous system, bones and liver. Spirochetes are very rarely demonstrated, serological reactions such as Kahn, VDRL are usually positive after about 2 weeks from the onset of the primary stage.

Congenital syphilis: It is caused by the transference of *T. pallidum* by a syphilitic woman to the fetus through the placenta.

Morphology
An exceedingly delicate, spiral filament. 6–14 μm by 0.13 μm with 6–12 coils which are comparatively sharp and regular. In the unstained material, it requires dark field illumination for its demonstration. *T. pallidum* cannot be demonstrated by the ordinary staining methods. The organism may be demonstrated by Fontana's method or the India Ink method, using the exudate from the chancre.

Laboratory Diagnosis of Syphilis
In primary stage, where there is an ulcerated sore, *T. pallidum* can usually be demonstrated in the serous exudate from the lesion. The dark ground illumination method is the most suitable technique for the purpose, and provides a convenient means of diagnosis. The procedure of dark field illumination has been discussed under special uses of a microscope.

Material Collection
The material consists of scrapings from primary or secondary lesions or from aspirations from glands. In the case of a primary chancre the part above the ulcer is constricted with fingers (make sure to wear gloves) and the scrapings taken from the depth of the ulcer. In the case of the secondary lesions on skin or mucous membrane the tissue may be scraped taking precautions to avoid secondary contamination. In all cases several preparations should be made and the specimens examined immediately. The examiner should protect himself against infection by wearing gloves and by washing his hands in an antiseptic immediately after the examination.

In the dark field microscopy *T. pallidum* appears as bright and corkscrew like, slowly revolving and occasionally flexible. The ends are pointed and tapering. The undulating movement is characteristic.

Treponema pertenue

This is the cause of yaws, a tropical disease, an ulcerating papule occurring on the arms or legs. In morphology, it is identical to *T. pallidum.*

Treponema carateum

Causative organism of pinta, a disease giving rise to non-ulcerating papule. It is seen predominantly in Negros and has highest incidence in Mexico.

Treponema microdentium

This organism may flourish in caries teeth and may be found in secretions between teeth.

Treponema calligyrum

These organisms may occur in secretions of the genitals.

Leptospira

These are tightly coiled, thin spirochetes with one end often turned at a sharp angle resembling, when at rest, a button hook. They vary from 5–15 μm in length and are about 0.1 μm in width. They can be stained by Giemsa, but are best demonstrated by silver impregnation.

Leptospira icterohaemorrhagiae

This is the causative organism of infectious jaundice (Weil's disease) and is transmitted to man by ingestion of

water or food contaminated from animal sources. Urine and feces excreted by infected rats, mice or dogs contain the leptospira, which may remain viable for many weeks.

Laboratory Diagnosis

Examination of blood by dark field microscopy. During the first week of the disease, *Leptospira* can be detected in blood by the dark field illumination. Only a very small percentage can be diagnosed this way. After this, the urine is examined. Micro-agglutination test and immunochromatography as well as ELISA format are also available for diagnosis.

Cultivation

The *Leptospira* medium is inoculated with 2–3 mL of blood. The organisms are searched for dark ground microscopy weekly, at least for 4 weeks. The *Leptospira* are best demonstrated by examining the centrifuged deposit of urine by the dark field method. Other methods include MAT coagglutinations, ELISA and immunochromatography techniques.

Borrelia

These spirochetes are large, 10 to 30 μm in length with irregular wide open coils staining easily with aniline and in Romanowsky stains. They can be cultured in blood or serum and also in tissue culture.

Borrelia vincentii

Occurs in Vincent's angina, a pseudomembranous condition of the throat. Appearances of the spirochetes together with large fusiform bacilli are indicative of infection. Sputum and throat swabs are examined for the diagnosis of Vincent's angina.

Borrelia recurrentis

This is the causative organism of European relapsing fever transmitted by body lice either by bite or by scratching with infected fingers after crushing the lice. Incubation period is 3–10 days after which chills and fever arise.

Borrelia duttonii

This is the causative organism of West African relapsing fever. The organisms are transmitted by ticks.

Laboratory Diagnosis of Relapsing Fevers

During the pyrexial phases, the spirochetes may be frequently demonstrated in the blood. Thin and thick films are made, as for malaria, and stained by Leishman's or Giemsa's stain. Better methods, however, are dark ground or phase contrast microscopy.

Spirillum minus

This is the causative organism of rat bite fever. It is a short, spiral organism about 2–5 μm in length and relatively broad, with regular short coils. This organism is actively motile and is included in the group *Spirillum.* It can be demonstrated by dark field illumination.

In rat bite fever, the *Spirillum* may be demonstrated in the local lesion, the draining lymph nodes, and even the blood, either by direct microscopic examination or by animal inoculation. The organism is transferred to human beings by the bite of a rat, causing a local lesion, which leads to swelling of glands, skin rashes and a relapsing type of fever.

GRAM STAINER

Gram stainer: Aerospray microbiology can be used for quick and high quality Gram staining offers advantages over hand staining or dip type stainer, like speed, economy, consistent performance and no cross contamination. It can stain 12 slides in 5 minutes and the slides are ready for microscopy.

The instrument is available from WESCOR USA.

QUALITY ASSURANCE IN BACTERIOLOGY

Quality Control of Media and Stains

Culture media are used in the laboratory for a variety of purposes. These are used to support the growth of microorganisms showing typical colonial and morphological appearance.

Media are also used to demonstrate many other properties of organisms, e.g. production of acid and gas in carbohydrate fermentation media or hemolysis on blood agar. Variations in the composition of the medium may alter these characters.

Quality Control of Media

Sources of Media

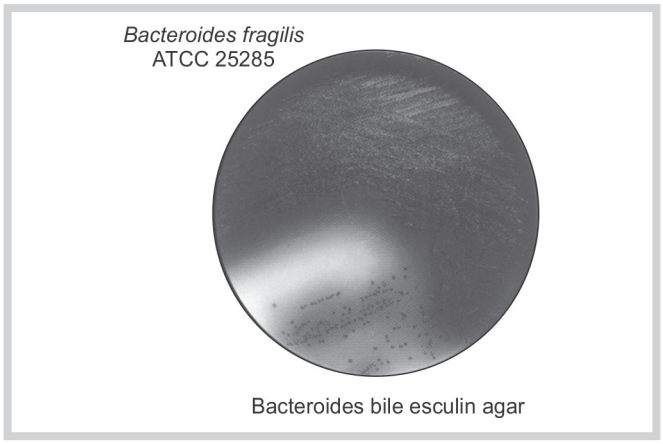
A few years back media used to be prepared from basic chemical ingredients, but laboratories are no longer required to do this now.

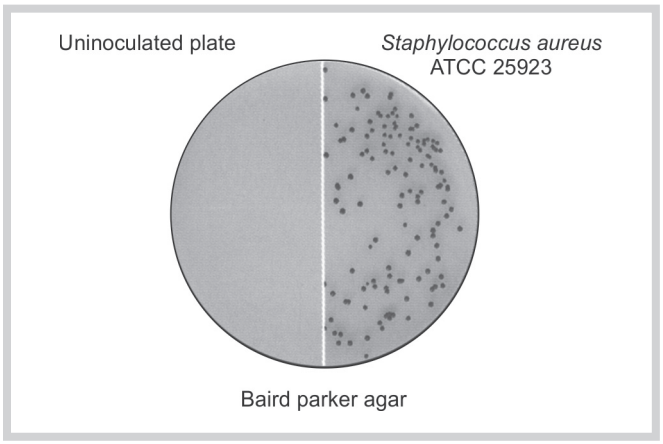
Dehydrated Media

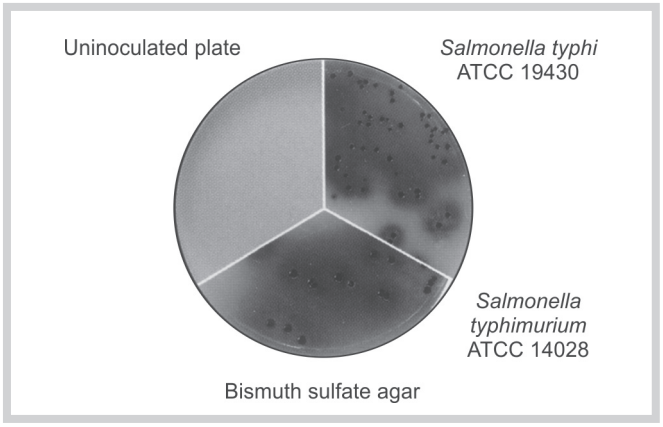
These are commercially available and require only the addition of water to be reconstituted for use. The responsibility for quality control lies with the manufacturer.

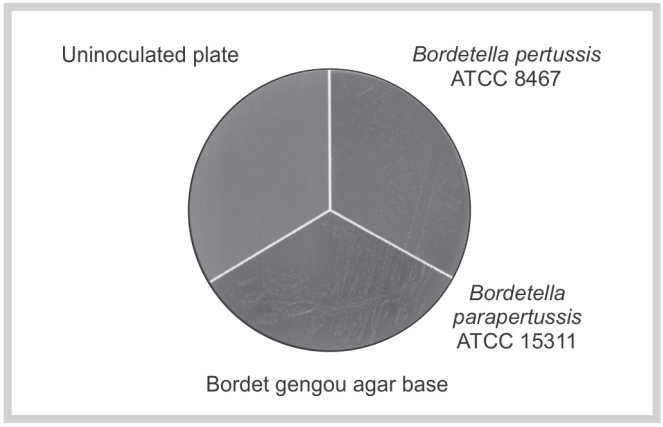
However, it has to be tested for its quality, after preparation, because of changes that can be brought about by the process of reconstitution and sterilization.

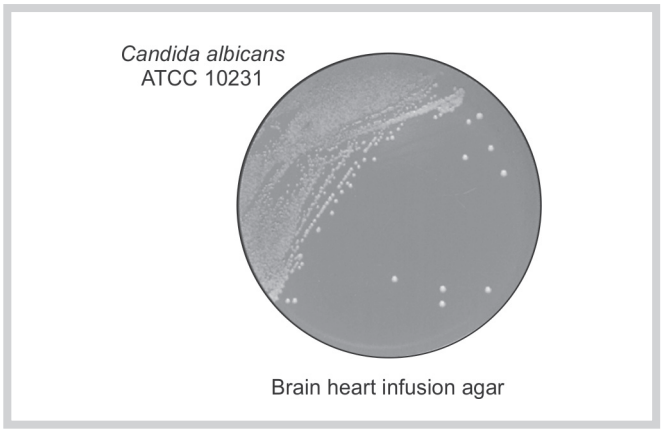
COLOR ATLAS—MEDIA AND COLONIES

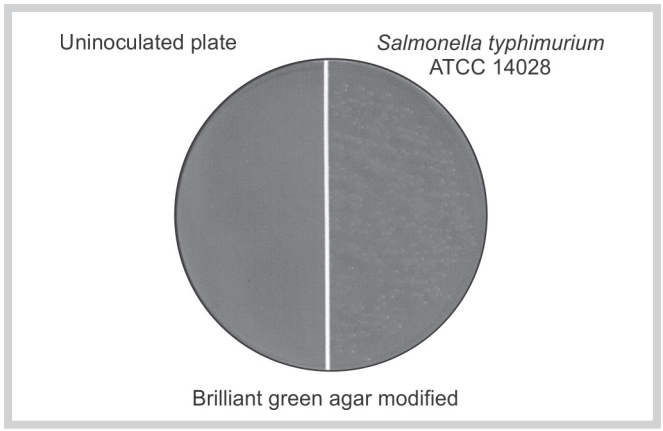


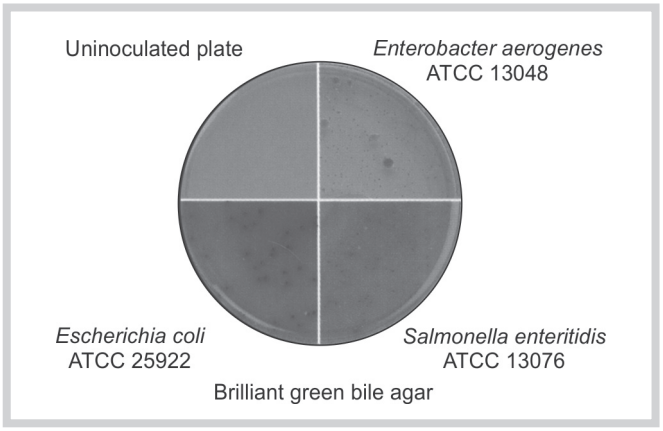








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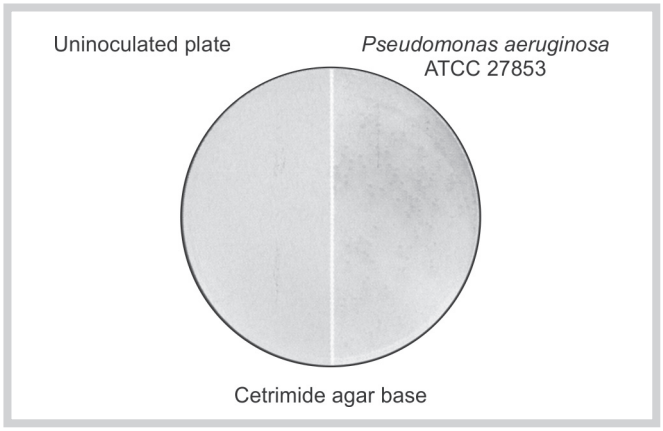
Escherichia coli ATCC 25922
Salmonella enteritidis ATCC 13076
Brilliant green bile agar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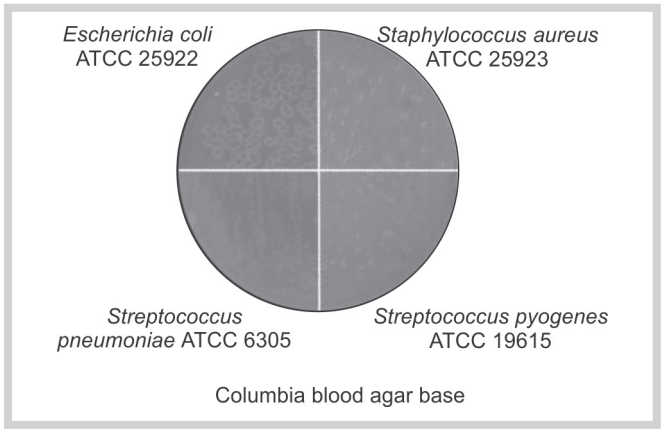
Cetrimide agar base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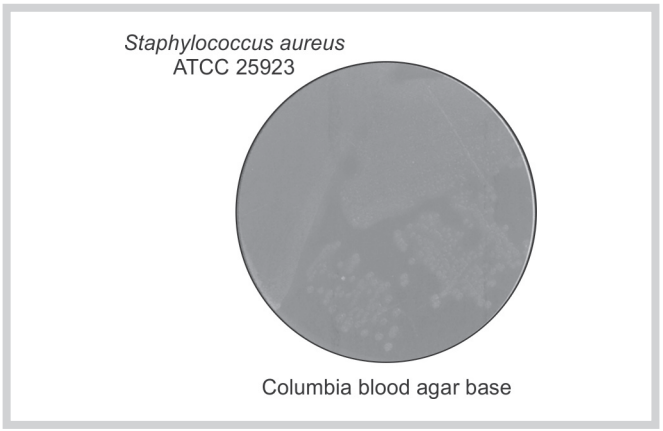
Streptococcus pneumoniae ATCC 6305
Streptococcus pyogenes ATCC 19615
Columbia blood agar base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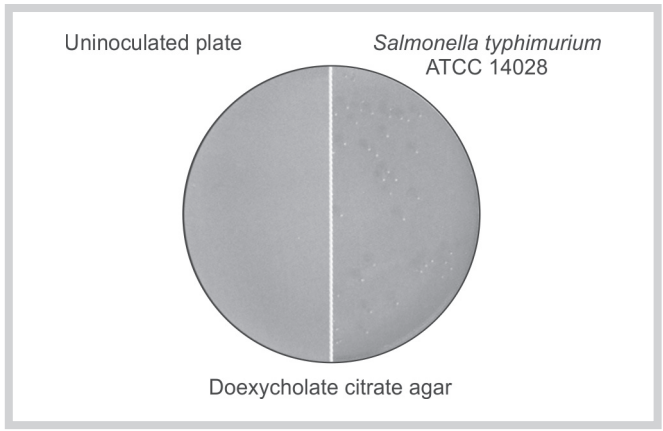
Columbia blood agar base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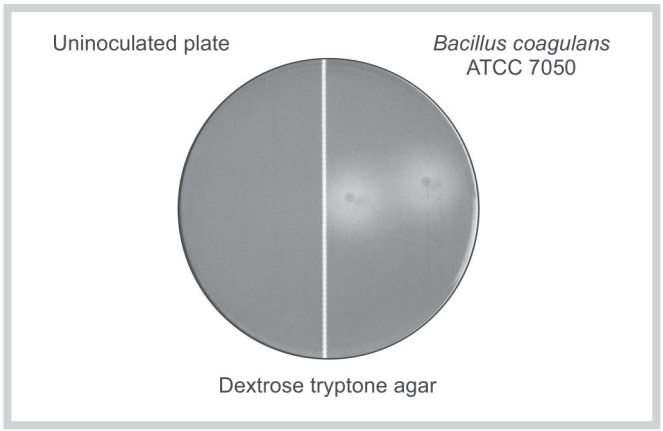
Doexycholate citrate agar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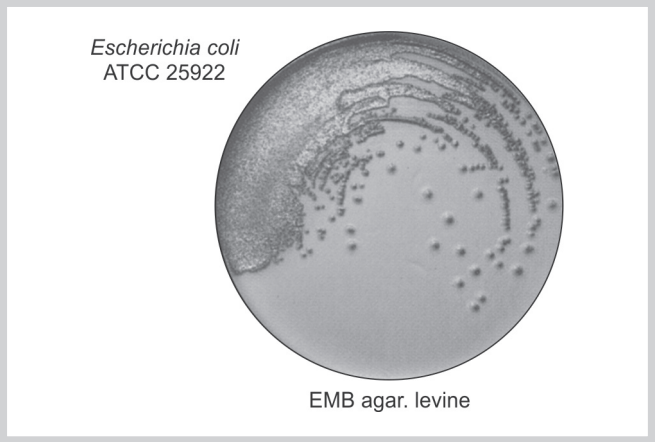
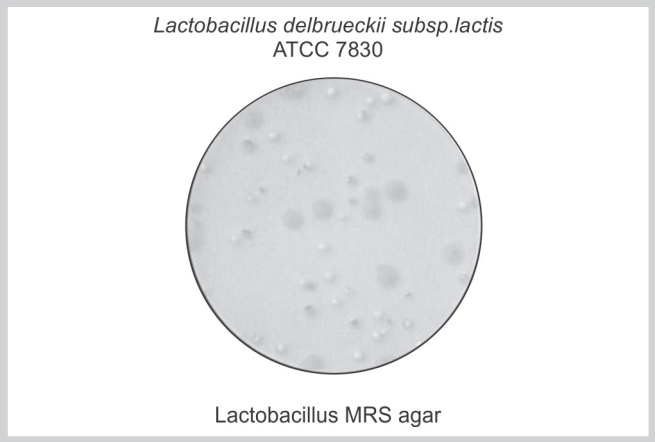
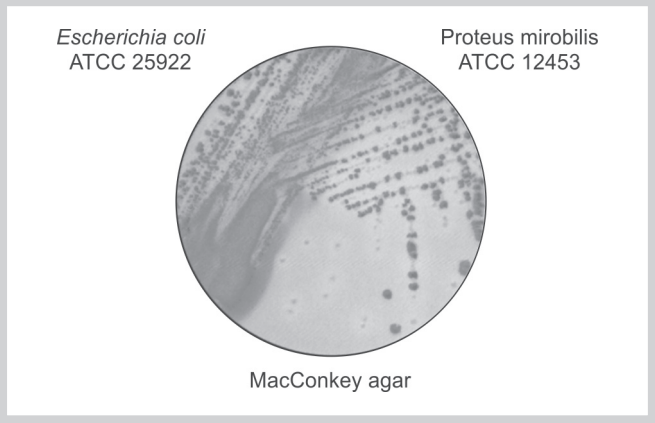
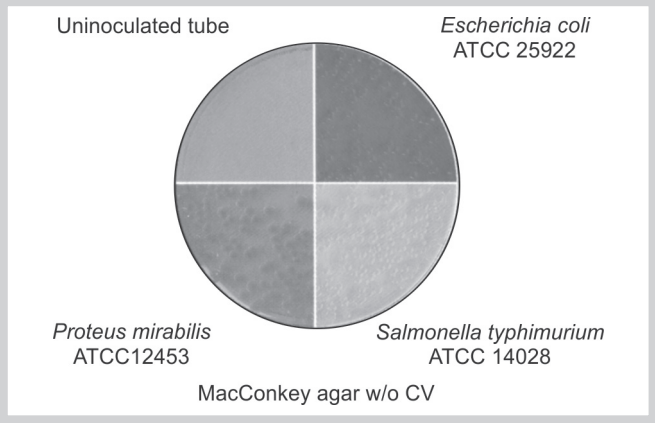
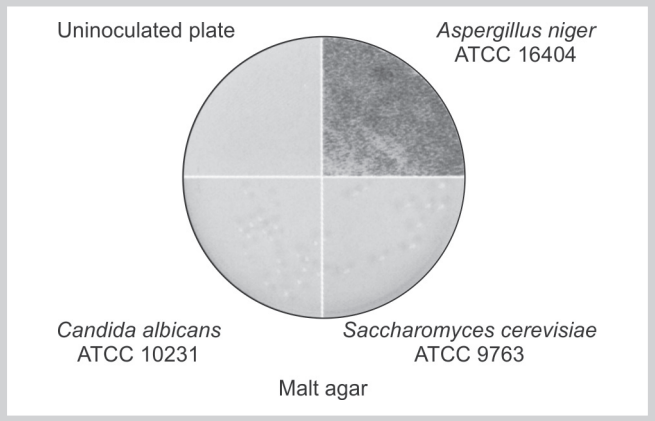
Dextrose tryptone agar

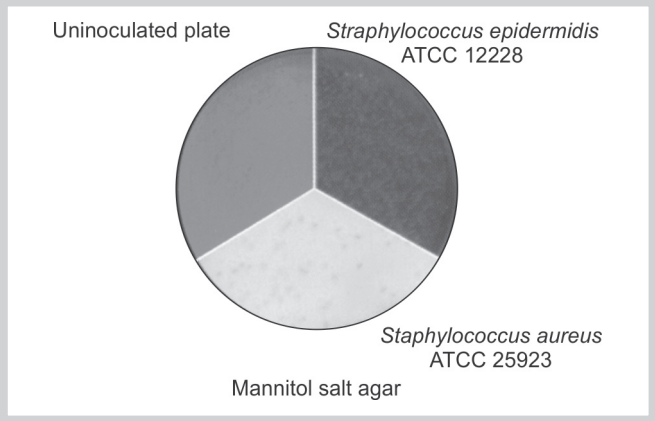


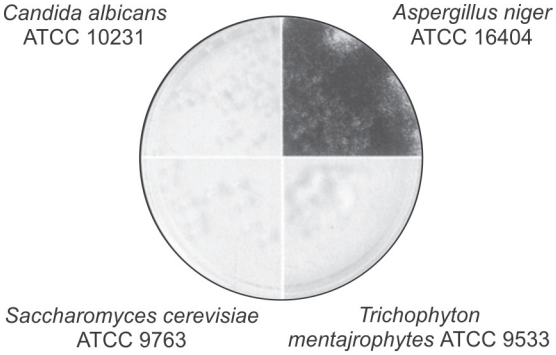




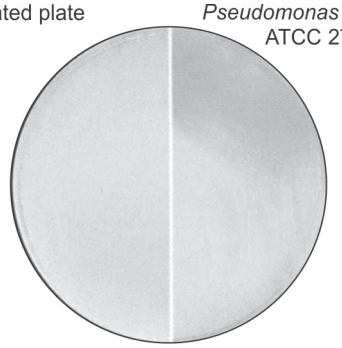




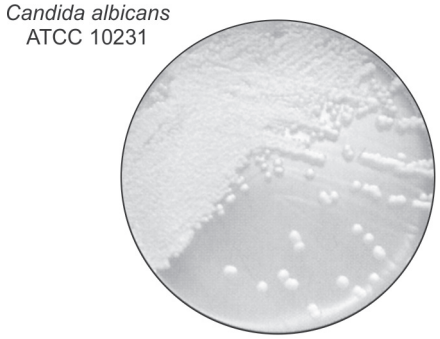




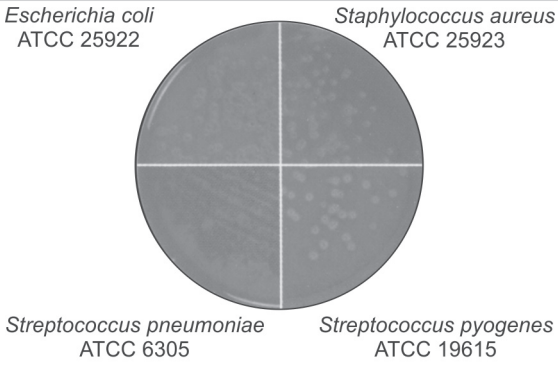
Potato dextrose agar



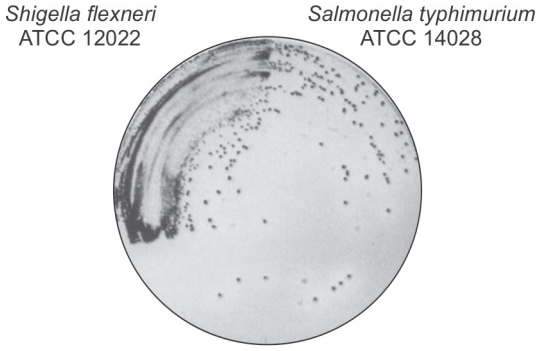
Pseudomonas isolation agar



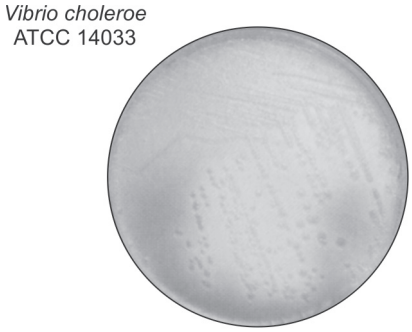
Sabouraud dextrose agar



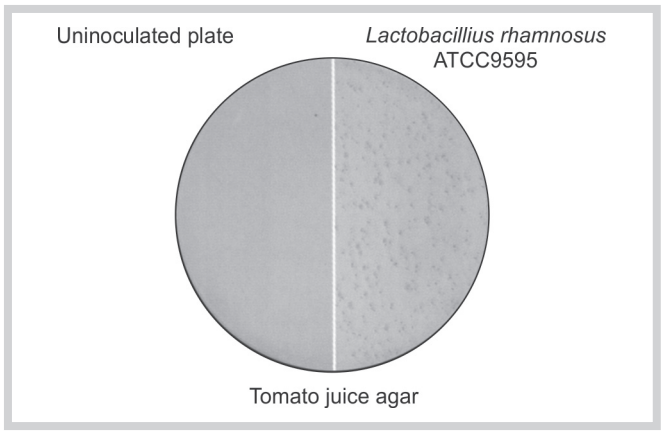
Soybean casein digest agar

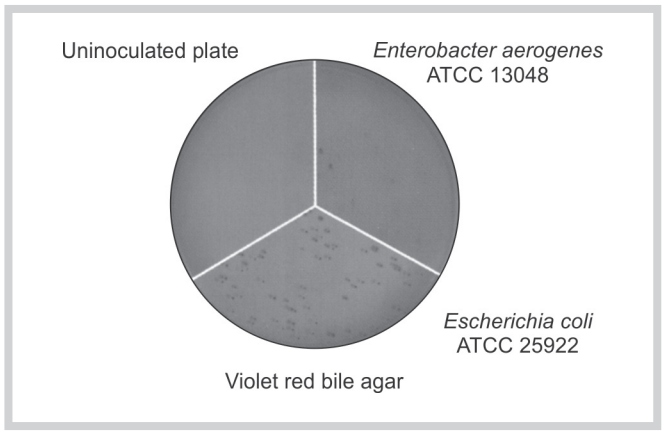


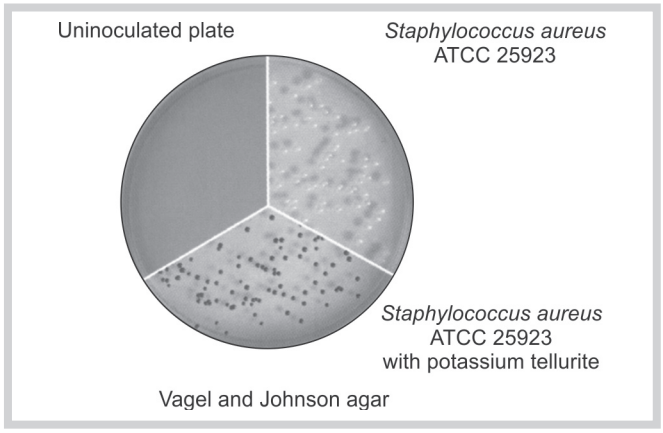
SS agar

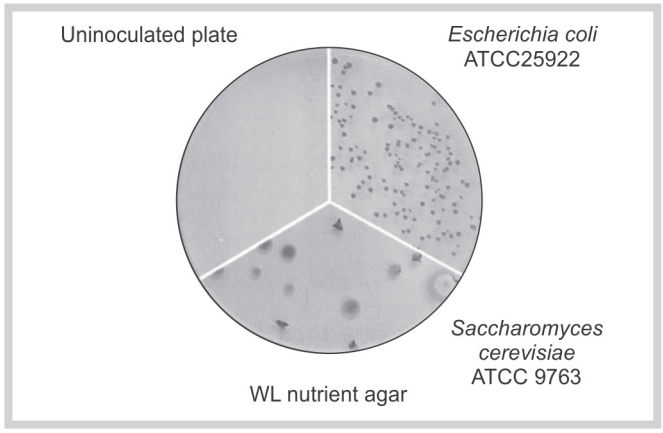


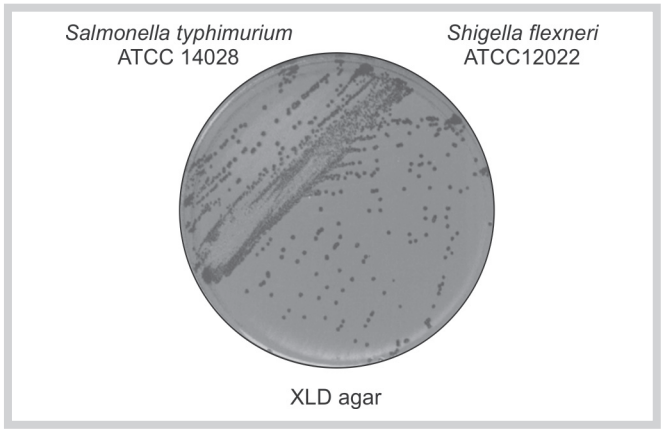
TCBS agar

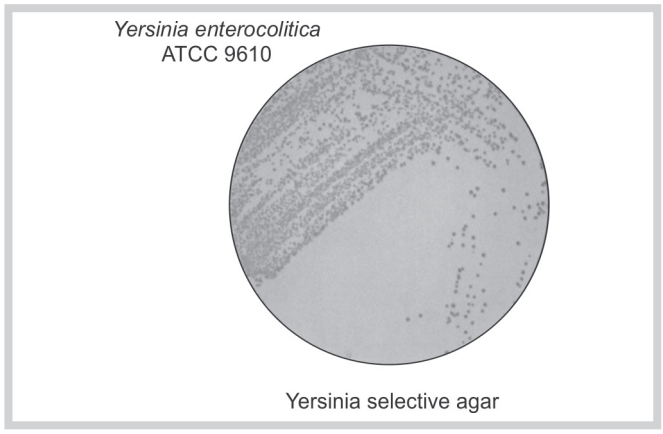






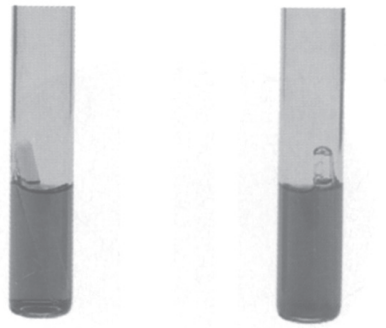






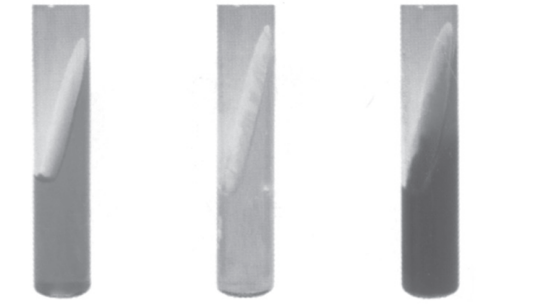
Uninoculated tube | *Neisseria meningitidis* ATCC 13090 | *Streptococcus pyogenes* ATCC 19615

Brain heart infusion broth



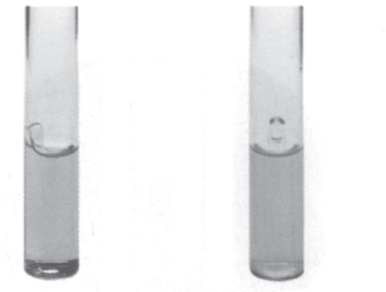
Uninoculated tube | *Escherichia coli* ATCC 25922

Brilliant green bile broth 2%



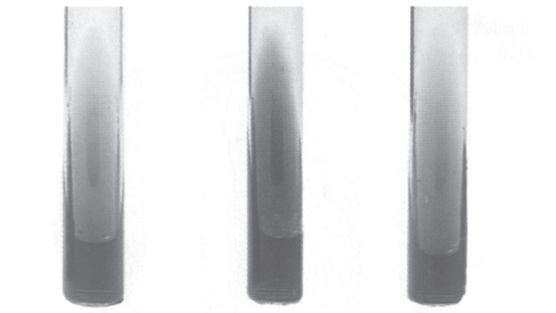
Uninoculated tube | *Escherichia coli* ATCC 25922 | *Salmonella typhimurium* ATCC 12022

Kliger iron agar



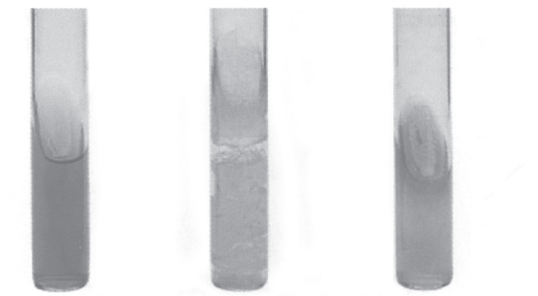
Uninoculated tube | *Escherichia coli* ATCC 25922

Lactose broth



Uninoculated tube | *Enterobacter aerogenes* ATCC 13048 | *Escherichia coli* ATCC 25922

Simmons citrate agar



Uninoculated tube | *Escherichia coli* ATCC 25922 | *Shigella flexneri* ATCC 12022

Triple sugar iron agar

Dehydration with Additive

For isolation of fastidious organisms, certain additives need to be used when media are prepared in the laboratory. The additives usually are unstable materials such as blood, serum or other growth factors. Hence, quality control needs to be maintained.

Commercially Prepared Media

Ready to use media are commercially available. In these medias, also the responsibility for quality control maintenance lies with the manufacturer but laboratories need to keep a watch on their behavior.

Sources of Error

Inappropriate Medium

Since dehydrated media are usually arranged alphabetically on a shelf, one may select the wrong bottle inadvertently, or an improper additive might be selected, making the medium unsuitable for use.

It is always important to read the label, particularly when a new lot of medium has been received in the laboratory.

Water

Measure carefully the amount of water that is added when reconstituting media. Since impurities render tap water unsuitable for the preparation of most biological media, laboratories should use either distilled water, deionized water, or water that has been treated in both ways.

Weighing

Accurate balances should be used for weighing dry materials. Weighing errors significantly alter the composition of the final product.

Dispensing

Media should be dispensed accurately and aseptically in plates and tubes. Failure to measure the amount accurately may result, for example, in too shallow or too deep agar medium, either of which may make the medium unsuitable for use.

Proper Sterilization

A common error in media preparation is sterilizing media at too high a temperature or for too long a period, or both. This may result in deterioration or decomposition of some constituents of the media, which will render the media useless for the intended purpose.

Glassware

Care should be taken to use clean glassware, since residues on glass may be inhibitory to some fastidious microorganisms, particularly viruses grown in cell culture, or to the cells themselves.

Quality Control

Any quality program for culture media must in the final analysis assure that a medium will support the growth of the organisms likely to be in the specimen. It must, if specified inhibit the growth of commensal organisms, exhibit a typical biochemical response, be stable and have a reasonable shelf-life. Because laboratories usually have no control over the preparation, shipping or storage of these products, it is very important that they document the information that is available for each.

Physical Appearance

If the medium is stored for an excessively long time under adverse conditions or has been improperly prepared, the following signs may develop and these should be documented.

- Presence of turbidity or a precipitate indicates that some constituent has come out of the solution.
- Colors darker than normal may indicate overcooking of sugar containing media, incorrect pH or incorrect mixture of ingredients.
- Colors lighter than normal may also indicate incorrect mixture of ingredients or a wrong pH.
- Prolonged storage of medium after pouring in plates causes its dehydration and makes it unfit for use. Dehydration of the medium can be reduced by preparing only required number of plates of media and storing them by sealing plates in plastic bags.

Sterility

A few media are used without terminal sterilization, but these are exceptions; most media must be sterile when they are inoculated. Each batch of medium, whether prepared in the laboratory or received from a commercial source, should be sampled for sterility.

This is best done by removing 1–5% of the batch and placing it in a bacteriologic incubator at 35°C for 48 hours. If contaminants appear in the medium as a result of inadequate sterilization, a new lot should be obtained.

Those containers that are used for sterility testing should be discarded at the completion of the test, since they are unsuitable for inoculation because of the dehydration that occurs after up to 48 hours in the incubator.

Growth

Determine the ability of the medium to support the growth of suspected organisms by inoculating the medium with

a typical stock culture isolate. A frequent quality control error is the use of a heavy inoculum for this purpose. For most media, inoculating with a stock culture that is too heavy may result in misleading growth.

In a specimen, the organism may be much more fastidious or present in very small numbers; therefore, the medium may not support its growth. When testing for the ability to support growth, it is good to prepare a dilute suspension to use as the inoculum. This suspension will give greater assurance that the medium is adequate for the growth of a small number of organisms in a patient's specimen.

In selecting an organism for testing, one should select from among the more fastidious species of organisms that one may be looking for in specimens received from patients.

Biochemical Response

When inoculating media used to identify a specific reaction, such as fermentation or H_2S production, it is necessary to use only a species or strain of organism that will produce the desired reaction.

Selective Media

Since selective media are designed not only to support the growth of organisms but to inhibit the growth of others, it is necessary to inoculate the medium with representatives of both groups of organisms.

To demonstrate the inhibitory effect, one can challenge the medium with a heavy inoculum, since, if the medium will prevent the growth of a large inoculum, it will inhibit the small number of organisms that may be present in the primary specimen. The medium must also support the growth of the selected organisms.

As a matter of general principle, each batch of culture medium should be checked before use with control strains to ensure that it supports the growth of bacteria and, in the case of selective media, inhibits the growth of undesirable organisms. However, if economics does not permit this approach, those media which are known from experience to be trouble free and reliable need not be subjected to such a regular quality control regimen. The laboratory has to identify such reliable media and accordingly establish quality control schedules. This concept must be periodically reviewed. However, whenever a new batch of medium, new supplier or a new product is to be used, it is prudent to subject it to rigorous quality control measures until confidence in the quality of the product is established. A "batch" of the medium refers to all the tubes, plates or containers of medium prepared at the same time in the laboratory, or all the plates, tubes or containers having the same lot number that are received in a single shipment from an outside supplier.

Spectrum of Quality Control

The frequency of performing quality control procedures needs to be determined from the experience of the laboratory. To meet certification requirements, laboratories need to perform quality control procedures according to a prescribed pattern. Careful records of quality control procedures should be made and maintained, which should be reviewed periodically to determine the stability of media so that corrective measures can be taken in time.

Quality control of culture media should not be a blind procedure, but should be approached in a rational and disciplined manner.

Performance of Plated Media

Samples of plates from each batch are selected for performance testing and are inoculated with the appropriate stock cultures. For each type of medium, at least two or three microorganisms having growth characteristics with 'positive' and 'negative' results for the medium should be used. The size of inoculum and method of inoculating the test plates must be standardized as closely as possible.

In general, control organisms should be selected from an actively growing broth culture and a standard loopful of culture seeded directly onto the test medium, which is then streaked, so as to obtain isolated colonies. After appropriate incubation, the results of the performance test are recorded.

The medium is released for use in the clinical laboratory only if the results indicate satisfactory performance. In initiating a quality control program, one must establish some priorities, such as beginning by testing those media that are most likely to demonstrate deficiencies.

Top priority should be given to blood agar, chocolate agar and Thayer Martin agar media. Secondary priority should be accorded to selective enteric media such as MacConkey agar, EMB, XLD and bile salt agars.

A quantitative approach may be more useful for testing of performance of selective or inhibitory media such as Thayer Martin agar. *N. gonorrhoeae* and *N. meningitidis* usually grow on Thayer Martin agar when the inoculum is heavy, but when a fairly light inoculum is used, the pathogens might be inhibited.

Consequently, a somewhat quantitative performance test could detect deficiencies that would be overlooked if one simply inoculated test plates with undiluted stock cultures.

Quality Control of Stains

Test all stains at appropriate intervals for their ability to distinguish positive and negative organisms and document the results. The performance standards for some of the commonly used stains in the bacteriology laboratory along with their desired frequencies of testing so as to have continuous reliable results have been shown in Table given above.

Quality control of stains need to be performed on weekly basis and also as and when a new lot of reagents for staining are procured.

Quality Control of Bacteriological Techniques

Various biochemical tests are performed in the laboratory on the isolates obtained from the clinical specimen. These tests help in identification of the organism. Quality control procedures are essential for these tests to avoid generation of wrong results, which may lead to erroneous diagnosis. Organisms known to give positive or negative reactions with various biochemical tests have been identified. These must be used frequently in the laboratory to assess the authenticity of results of biochemical reactions.

It is also essential to undertake quality control procedures at regular intervals. These should be performed:

- With each new batch of reagents
- With each new vial of reagent
- Daily for catalase, oxidase, and coagulase
- Weekly for bacitracin, optochin and ONPG.

A test procedure not giving anticipated results with the control organisms should not be used till such time that remedial steps have been taken to correct the problem.

Quality control of stains

Stain	*Control organism/material*	*ATCC No*	*Expected result*
Ziehl-Neelsen	*Mycobacterium sp.* *Esch. coli*	25177 25922	Pink red bacilli Blue bacilli
Acridine orange	*Esch. coli* *Staph. aureus*	25922 25923	Fluorescent bacilli/cocci
Giemsa	Thin film blood smear		Distinct staining of WBCs and RBCs
Gram	*Esch. coli* *Staph. aureus*	25922 25923	Gram negative bacilli Gram positive cocci
Iodine solution	Formalin treated stool specimen with cysts		Visible cyst nuclei
Spores	*Bacillus* specius		Spores stain one color and bacillus stains with counterstain

Quality control of commonly used media suggested control organisms and expected reactions

Medium	*Control organism*	*Expected reaction*
Blood agar	Group A streptococci *S. pneumoniae*	Good growth, β - hemolytic Good growth, α - hemolytic
Bile-esculin agar	*Enterococcus* species β - hemolytic *Streptococcus*, not Group D	Good growth, black No growth
Chocolate agar	*H. influenzae* *N. gonorrhoeae*	Good growth Good growth
Christensen urea agar	*Proteus mirabilis* *Klebsiella pneumoniae* *Escherchia coli*	Pink throughout (positive) Pink slant (partial positive) Yellow (negative)
Simmon's citrate agar	*K. pneumoniae* *E. coli*	Growth or blue color (positive) No growth, remains green (negative)
Deoxyribonuclease	*Serratia marcescens* *E. cloacae*	Zone of clearing (add 1 N HCl) No zone of clearing
Motility (semisolid agar)	*P. mirabilis* *K. pneumoniae*	Media cloudy (positive) No feather edge on streak line (negative)

Contd...

Contd...

Medium	*Control organism*	*Expected reaction*
MacConkey agar	*E. coli* *P. mirabilis*	Pink colonies (lactose positive) Colorless colonies, no spreading
Sucrose	*E. coli* *N. gonorrhoeae*	Yellow (positive) No color change (negative)
Maltose	*Salmonella* species *N. gonorrhoeae*	Yellow (positive) No color change (negative)
Lactose	*N. lactamicus* *N. gonorrhoeae*	Yellow (positive) No color change (negative)
Lysine	*K. pneumoniae* *Enterobacter sakazakii*	Bluish (positive) Yellow (negative)
Arginine	*E. cloacae* *P. mirabilis*	Bluish (positive) Yellow (negative)
Ornithine	*P. mirabilis* *K. pneumoniae*	Bluish (positive) Yellow (negative)
Medium	*Control organism*	*Expected reaction*
o-Nitrophenol-p-Dgalactopyranoside (ONPG)	*Serratia marcescens* *S. typhimurium*	Yellow (positive) Colorless (negative)
Phenylalanine deaminase	*P. mirabilis* *E. coli*	Green (add 10% $FeCl_3$) No color change (negative)
Salmonella-Shigella (SS) agar	*S. typhimurium* *E. coli*	Colorless colonies, black center No growth
Voges Prauskauer	*K. pneumoniae* *E. coli*	Red (add reagents) No development (negative)
Xylose-Lysine-Dextrose	*Salmonella* species *E. coli* *Shigella* species	Red colonies (positive lysine) Yellow colonies (positive sugars) Transparent colonies (negative)

Quality control procedures for commonly used tests

Procedure/Test	*Control organism*	*Expected result*	*Expected reaction*
Catalase	*Staph. aureus* *Streptococcus* species	+ –	Bubbling reaction No bubbling
Coagulase	*Staph. aureus* *Staph. epidermidis*	+ –	Clot formation in 4 hours No clot
Indole	*Esch. coli* *Enterobacter aerogenes*	+ –	Red ring at surface Yellow ring at surface
Methyl red	*Esch. coli* *Ent. aerogenes*	+ –	Instant red color No color change
Oxidase	*P. aeruginosa* *Esch. coli*	+ –	Purple color in 20 seconds No color in 20 seconds
Voges Proskauer	*Enterobacter aerogenes* *Esch. coli*	+ –	Red color No color change
Bacitracin disc	*Streptococcus* group A *Enterobacter faecalis*	+ –	Zone of inhibition No zone of inhibition
Optochin disc	*Strept. pneumoniae* *Strept. viridans*	+ –	Zone of inhibition No zone of inhibition
ONPG disc	*Esch. coli* *Proteus vulgaris*	+ –	Yellow color No change in color
Oxidase disc	*P. aeruginosa* *Esch. coli*	+ –	Purple color in 30 seconds No change in color

CHAPTER 28

Mycology

In evolutionary set up fungi are placed higher in plant kingdom than bacteria. They exist in two distinct morphological forms: 1. the yeast form, and 2. the mycelium—which consists of a mass of long, branching tube-like filamentous structures called hyphae. Hyphae can be divided into segments—septate, or may not have any divisions—aseptate. Some fungi exist only as yeast forms when growing at 37°C but grow as mycelial forms at room temperatures. Other fungi grow only as hyphae at all temperatures. The yeast form of fungi generally multiply by budding; the bud is known as a blastospore. The hyphae have specialized structures known as conidia. They may also develop resting spores, especially when they are exposed to a hostile environment, which have thick walls, called chlamydospores. A colony of yeast-like fungi resembles a bacterial colony in culture. A colony of hyphae with its conidia and spores presents a filamentous growth, or mould.

All fungi are gram-positive and give a positive PAS reaction. Most fungi are aerobic with the exception of *Actinomyces*. Some species of *Nocardia* are acid fast, but most fungi are acid fast.

Barring a few of fungi, most are nonpathogenic to man. They are classified according to their medical importance as follows:

1. **Superficial mycoses and dermatophytes.**
2. **Deep or systemic mycoses.**
3. **Contaminant (opportunistic) fungi which under special circumstances cause disease.**

SUPERFICIAL MYCOSES AND DERMATOPHYTES

The fungi infect the skin, hair and nails. They do not involve deeper structures. They are subdivided into three genera:

Microsporum

This invades hair and skin, but not nails. This causes 'ringworm' infection. Colonies on Sabouraud's agar are a reddish-brown in color, and often a reddish-brown pigment is produced in the medium. They produce powdery aerial mycelium.

Trichophyton

This fungus invades skin, hair and nails. They cause various types of ringworm, athlete's foot, etc. Colonies are powdery or waxy with pigmentation from white, pink, red to brown and yellow.

Epidermophyton

This fungus invades skin and nails, but not hair. Colonies are slow-growing with a green, slightly granular appearance.

Laboratory Diagnosis

- Scrapings of skin and nails.
- Hair plucked from infected area.
- Culture on Sabouraud's, agar for 2–3 weeks at room temperature.

Hair and scrapings are placed on a slide in a drop of 10% NaOH or KOH, coverslip, and examined after 10–30 minutes. In skin and nails branching hyphae are seen. In hairs, spores are seen. If they are all on the outside of the hair, it is called ectothrix infection. If they have developed and grown inside the hair, it is called endothrix infection. Some species cause the infected hair to fluorescence under ultraviolet light.

INTERMEDIATE SUPERFICIAL DEEP MYCOSES

Candida albicans (Monilia)

This is an oval budding, yeast-like fungus. It may be a commensal or part of the normal flora in small numbers in the GIT, mouth and vagina; but it can also cause disease in the skin, nails, mucous membranes (thrush of the mouth, vagina or anus), rarely it may take the form of systemic disease and involve heart, lungs or other structures in the body. On Sabouraud's agar soft, cream-colored colonies develop at both 37°C and room temperature.

Examine swabs and scrapings from surface lesions, sputum or pus from deeper tissues. Sputum or exudates may be examined by Gram's stain for presence of budding, gram-positive, yeast-like fungus. Skin or nail scrapings are first placed in a drop of 10% NaOH or KOH and then examined for the yeast-like fungi.

Specimens are cultured on Sabouraud's agar at room temperature and at 37°C. At both temperatures, the colonies consist of yeast-like cells, though at room temperature small 'pseudomycelia' (nonseptate projections from yeast-like cells) are produced.

DEEP OR SYSTEMIC MYCOSES

Actinomyces

These organisms are related to true bacteria, but because of filamentous branching mycelial growth resemble fungi. The filaments sometimes break up into bacteria-like pieces. It causes actinomycosis, which is characterized by chronic sinuses, draining pus, leading from deep abscess cavities, usually in the neck, chest or abdomen. *Actinomyces* are sometimes normal inhabitants—commensals or saprophytes—in the mouth, throat and tonsils but under impaired host resistance, they become opportunistic pathogens and invade tissues. Direct examination of sputum or pus from a draining abscess will reveal small yellow granules, called 'sulfur granules.' When they are found and examined microscopically after crushing between two glass slides, the granules are seen to be made up of thin interlacing strands or branching filaments (about 1 μ in diameter) with club-shaped expansions at the end of the filaments at the periphery giving a ray-like appearance to the edge of the granule. The filaments are gram-positive and nonacid fast. Culture must be made anaerobically since this organism is an obligate anaerobe, usually in thioglycollate broth or on blood agar, at 37°C.

Laboratory Diagnosis

Examine pus or sputum for 'sulfur granules' and Gram's stain for gram-positive branching filaments.

Nocardia

These are morphologically similar to *Actinomyces*, though they; (i) are found in soil and not as commensal in the body, (ii) are aerobic rather than anaerobic, (iii) may have granules in the pus though less frequently and those present not usually as yellow in color, and (iv) some of the organisms may be weakly acid fast.

Nocardia is one of the organisms causing Madura foot.

Cryptococcus neoformans

It causes cryptococcosis or torulosis. It can cause a pulmonary infection and often spreads to the meninges and brain. It is a yeast-like budding fungus which is characterized by a large capsule both as it grows in tissue and in culture. Even in culture, it does not produce mycelia, but the colonies on Sabouraud's agar are smooth and glistening and cream-colored. The capsule can be best identified by mixing the specimens of sputum, pus, or CSF with India ink.

Laboratory Diagnosis

Sputum, CSF and tissue are examined for these budding yeast-like organisms (5–20 μ). They are not refractile like red blood cells (RBCs), and neither RBCs nor lymphocytes show budding like these organisms. Toluidine blue 0.1% colors these organisms pink, stains lymphocytes blue and does not give any color to red cells. India ink will reveal the wide capsules. Culture is done on Sabouraud's agar at 37°C and growth is rapid (1–2 days).

Histoplasma capsulatum

This organism causes histoplasmosis, which can be both a pulmonary infection (similar to tuberculosis) or show widespread dissemination throughout the body. The organism generally lives intracellularly in cells of the reticuloendothelial system; in macrophages, reticulum cells in bone marrow, spleen, etc. They appear as tiny (1 μ) intracellular oval bodies with a recognizable clear halo or capsule surrounding the small central stained organism. They cannot be seen in the unstained material, but can be seen in Giemsa, Leishman, hematoxylin and PAS stained preparations. Culture in broth or blood agar, plates at 37°C

growth is yeast-like with smooth, white colored colonies. On Sabouraud's agar at room temperature, there is mycelial growth of septate hyphae with spores.

Laboratory Diagnosis

Sputum, pus, blood and bone marrow smears, and tissue specimens are examined as stained preparations for the presence of the small, ovoid, capsulated intracellular organism.

Coccidioides

This organism causes coccidioidomycosis, which like *Histoplasma* can cause pulmonary, disseminated disease or occasionally deep skin ulcers. In tissue the organism is typified by the presence of thick-walled spherules (10–80 μ) which are filled with endospores (2–5 μ).

Laboratory Diagnosis

Examination of sputum, gastric washings or pus for the presence of these spherules. Culture at room temperature on Sabouraud's agar gives white cottony growth which soon becomes brown and microscopic examination reveals septate hyphae and spores.

FUNGI USUALLY PRESENT AS CONTAMINANTS BUT WHICH RARELY CAUSE DISEASE—USUALLY IN PATIENTS CHRONICALLY ILL FROM OTHER DISEASES

1. *Geotrichum*

Occasionally causes pulmonary, bronchial or oral disease. Direct examination of sputum reveals rectangular cells (4–8 m) with rounded edges.

2. *Penicillium*

3. *Aspergillus*

4. *Mucor*

The common mould on bread.

MYCOLOGICAL METHODS

Collection and Despatch of Specimens

Skin, Nail and Hair

Scrape the skin, into piece of clean and sterile tissue paper. Fold the paper and send to the laboratory. Debris beneath the nail after clipping off the affected nail and hair from infected parts are also collected in the same way.

Sputum, Pus, Spinal Fluid Exudates and Biopsy Materials

These are collected in sterile bottles.

Microscopic Examination

Skin, Nail and Hair

The specimens are placed into a clean slide and one drop of 10–20% NaOH or KOH is added and is covered with a coverslip. The slide is heated over a flame to soften and clear the material. Examine the slides microscopically.

The slides are stained by adding one drop of lactophenol cotton blue to one edge of the coverslip. Mycelial structure takes the blue stain.

Sputum, Pus and Exudates

Wet mount preparations are made on clean slide and examined microscopically. Gram's stain is also used to stain the fungus materials.

Cultural Examination

The most common medium is Sabouraud's agar. The scrapings from skin and nails and hairs are planted onto medium and are incubated for 2–3 weeks. Sputum, pus and exudates are cultured and incubated at room temperature and at 37°C.

CHAPTER 29

Diagnostic Skin Test

Skin tests are diagnostic procedures performed by the intradermal administration of diagnostic materials or their application to the surface of the skin. In order to be useful, skin tests have to be properly performed, accurately read, and correctly interpreted.

TECHNIQUE OF SKIN TESTS

Instruments and materials must be sterile. The skin must be carefully cleaned, but irritation is to be avoided. Gentle sponging with 70% alcohol is sufficient. Most skin tests are performed on the forearm but other skin areas are equally satisfactory.

Intracutaneous Injection

A short bevel, fine gauge (No. 26 or 27) needle is introduced below the upper layers of the epithelium but not into the subcutis. A properly placed needle permits the injection of 0.1 mL of fluid, raising a round bleb. This is the most reliable technique, and is commonly used for tuberculin purified protein derivative (PPD) and histoplasmin tests, coccidioidin test in adults and many others.

Transcutaneous Administration

The site is cleaned and dried, with a lance or needle, short scratches are made in the epidermis, not deeply enough to draw blood. The scratches should be 1/8 inch long and about 2 inches apart on the flexor aspect of the forearm. One drop of the allergen test fluid is applied to each scratch and left undisturbed for 10 minutes. This technique is used commonly in testing for pollen or food allergens.

Patch Tests

These depend on the ability of the test substance to diffuse into the skin. The test substance is adsorbed onto a small patch of gauze which is affixed to the skin with adhesive tape. Allowing the patch to remain in contact with the skin for 24 hours. Sterile, unimpregnated gauze must surround the patch and separate it from the area where the adhesive tape touches the skin (to distinguish reactions to the test substance from reactions to adhesive). This technique is used sometimes for tuberculin tests in children, but it is less reliable than the intradermal test.

IMMUNOLOGIC BASIC FOR SKIN TESTS

Most skin tests may be placed in one of the following three groups

Toxin—Antitoxin Neutralization

The action of toxin or antitoxin is responsible for the observed reaction. In the Schick and Dick tests, toxin is injected into the skin. Unless neutralized by circulating antitoxin, the toxin provokes erythema and induration within 12–72 hours. A positive reaction thus indicates the absence of adequate antitoxin levels. In the Schültz-Charlton reaction, antitoxin is injected into an area of suspected scarlet fever rash. If blanching occurs, the erythrogenic toxin has been neutralized by specific antitoxin. This identifies the rash and confirms the diagnosis.

Anaphylatic Type of Hypersensitivity (Immediate Reactions)

This type of reaction is characterized by the following features:

a. The reaction is “immediate”. Erythema and wheal formation appear within 5–20 minutes and disappear within 1 hour after injection or application of the allergen.

b. The reaction is associated with specific circulating antibodies and can be passively transferred by means of serum (see Prausnitz-Kustner reaction).
c. The reaction is mainly a vascular one, without much cellular infiltration and induration.

Examples of "immediate reactions" of this type are allergy to pollen and horse serum sensitivity.

Tuberculin Type of Hypersensitivity (Delayed Reactions)

This type of reaction is characterized by the following features:

a. The reaction is "delayed". Erythema and induration appear in 24–48 hours and may last for several days.
b. The reaction is not intimately associated with circulating antibodies and cannot be transferred passively by means of serum. Passive transfer is possible by means of leucocytes or leucocyte extracts from a sensitized person.
c. The reaction is largely infiltrative and inflammatory with little acute change in vascular permeability (which is paramount in the anaphylactic type).

Examples of "delayed reactions" are tuberculin, coccidioidin, histoplasmin and Frei's tests.

COMMON SKIN TESTS

Toxin-Antitoxin Neutralization Tests

Schick Test

For determining susceptibility to diphtheria.

Material

Diluted diphtheria toxin containing 1/50 MLD (minimal lethal dose for a guinea pig) in 0.1 mL, available commercially.

Technique

After cleansing the skin of the forearm, 0.1 mL is injected intradermally. A positive test consists of an area of erythema 1–2 cm in diameter reaching its maximum intensity about the fourth day. Pigmentation of the area may persist for weeks.

Interpretation

A positive test means that the individual does not have sufficient circulating antitoxin to neutralize the injected toxin and therefore, that he is susceptible to diphtheria. The converse is true for the negative reaction.

Present-day purified toxins rarely give false-positive reactions attributable to sensitivity tend to fade much quickly than a positive Schick test. If it is desired to include a check on possible sensitivity, 0.1 mL of heated (and therefore inactivated) toxin is injected into the other arm. If the control site develops the same reactions as the site injected with active toxin, sensitivity is present to the injected protein.

Test for Toxoid Sensitivity

Before immunization of adults, the Moloney test for sensitivity to toxoid should be performed. This is usually done by injecting 0.1 mL diphtheria toxoid diluted 1:20 instead of the heated toxin control. A positive test indicates sensitivity to the toxoid, and immunization has to start with exceedingly small doses.

Dick Test

For the determination of susceptibility to the erythrogenic toxin of hemolytic streptococci. It consists of observing the reaction to intradermally injected erythrogenic *Streptococcus* toxin. The development of erythema indicates as positive test. A negative Dick test merely indicates probable immunity to the erythrogenic toxin but not to streptococcal infection. A Dick negative person is unlikely to develop scarlet fever but is as likely as a Dick-positive person to develop other streptococcal diseases or sequelae. Therefore, the test is of little importance and is rarely done.

Schültz-Charlton Reaction

For the diagnosis of scarlet fever rash. The test consists of the injection of antitoxin to streptococcal erythrogenic toxin (or scarlet fever convalescent serum) into an area of rash. Blanching in 12 hours suggests specific neutralization and confirms the diagnosis of scarlet fever. This test is of little importance and is rarely done.

IMMEDIATE REACTION TYPE OF SKIN TESTS

Test for Sensitivity to Horse Serum

Before injecting horse serum (e.g. tetanus or diphtheria antitoxin) into any patient, ascertain possible sensitivity to horse serum. Similar precautions and tests apply to certain drugs, especially penicillin. Inquire about previous injections of serum or reactions to drugs, history of allergy, hay fever, etc. and perform sensitivity test as follows:

Technique

Always have a vial of 1:1000 epinephrine and a small syringe and needle ready when doing any test for serum sensitivity or administering serum. Of the two tests skin is

more reliable. If no evidence of hypersensitivity is obtained by the tests, serum is administered undiluted by the route indicated.

Skin Test
Cleanse skin, then inject intradermally 0.1 mL of horse serum diluted 1:30 with saline. Appearance of wheal 1–3 cm in diameter within 15 minutes suggests sensitivity to horse serum.

Conjunctival Test
Examine conjunctivas for presence of inflammation. Instill 1 drop of horse serum diluted 1:10 into the conjunctival sac, 1 drop of saline into the other as control. The control conjunctiva should appear normal after 3–5 minutes. In persons hypersensitive to horse serum, the test conjunctiva will show reddening, itching and lacrimation within 15–30 minutes.

Unfavorable Reactions following Serum Administration

Immediate Anaphylaxis
Exceedingly rare if skin tests are negative. Consists of difficulty in breathing, nausea, vascular collapse, and shock within 5–60 minutes after serum administration. Give 1 mL of 1:1000 epinephrine subcutaneously at the first sign of reaction and other supportive measures. Unless the patient responds promptly, give hydrocortisone 100 mg I/V.

Febrile Reaction
Occurs even when skin test was negative. Chills, fever, nausea within 1–6 hours after serum was given intravenously. Use supportive measures, no epinephrine.

Serum Sickness
Occurs often, even when skin test was negative. Fever, pruritus, edema, urticaria, lymphadenopathy, arthralgia, and occasionally arthritis with effusion develop 7–11 days after serum injection. Rarely, an accelerated reaction of the same type develops 1–6 days after injection of serum or drug (penicillin).

Treatment
Epinephrine is helpful. Corticotropin adrenocorticotrophin hormone (ACTH) or corticosteroids tend to suppress the symptoms of hypersensitivity reactions.

If the Skin Test is Positive

a. Obtain antiserum from another animal species (e.g. rabbit or goat) and repeat skin test with it.
   Avoid implicated drug.
b. Desensitize the patient to horse serum.

Desensitization is Done as follows

Inject gradually increasing doses of horse serum diluted 1:10. Begin with 0.1 mL, then 0.2 mL and 0.5 mL subcutaneously at 30 minutes intervals. If no significant reaction is observed, give 0.1 mL undiluted horse serum followed by 0.2 mL and 0.5 mL at 30 minutes intervals. If a given dose is followed by a slight reaction, give the same or a smaller dose after 30–60 minutes together with 1 mL of 1:1000 epinephrine. Increase doses steadily until 2 or 3 mL can be given without reaction. Repeat every 30–60 minutes until the full dose of serum has been given. Similar desensitization is possible with drugs, e.g. streptomycin.

Treatment of Severe Reactions

After a severe reaction, it is unwise to continue serum administration and desensitization. If severe reaction should occur during desensitization, treat with:

a. Epinephrine, 1:1000, 1 mL SC or IV.
b. Atropine sulfate, 0.5 mg SC.
c. Antihistaminic drugs, e.g. diphenhydramine, IV.
d. Artificial respiration and oxygen if necessary; external warmth.
e. Hydrocortisone, 50–100 mg IV.

Direct Skin Tests for Allergens (Respiratory, Contact, Drug or Food)

Technique

In allergic work-up, the patient is ordinarily tested either with the scratch or the intradermal method for sensitivity to a variety of allergens. Test substances are available commercially or may be specially prepared. The usual positive response consists of a wheal and erythema appearing 5–30 minutes after contact with the allergen.

Interpretation

In interpreting such reactions, the following must be kept in mind:

a. A negative skin test does not rule out systemic sensitivity.
b. A positive skin test does not necessarily mean that allergic symptoms are related to the particular allergen.
c. Individuals may at times give positive reactions to many substances, at other times to none.

Prausnitz-Kustner Reactions

A method used to demonstrate presence of a substance by passive transfer of serum. Used in dermatology and investigations of allergy.

Technique

Obtain serum from the patient and inject 0.2–0.3 mL intradermally into a normal person. 12 to 18 hours later, inject the suspected antigen into the area thus prepared with the serum and into another comparable skin area, as control.

Interpretation

A positive reaction (erythema, wheal) at the test (but not the control site) indicates successful transfer of specific antibodies and, by inference, demonstrates that the patient possesses anaphylactic hypersensitivity to the allergen.

Penicillin Hypersensitivity

Use skin test of penicilloyl-polylysine, native penicillin, or its degradation products.

Foshay's Test

Rarely used in tularemia and brucellosis for the detection of circulating specific antigen. Immediate positive reaction to intradermal injection of specific antiserum is considered useful in diagnosis.

Extracts of Parasitic Worms (e.g. *Trichinella*)

It may give immediate and delayed reactions (see below).

DELAYED REACTION TYPE OF SKIN TESTS

Tuberculin Test (Mantoux Test, Pirquet Reaction)

An individual who has been infected with the *Tubercle bacillus* given a skin reaction of the delayed type when tuberculoprotein is administered. In addition to the local skin reaction, focal and general reactions may occur in very hypersensitive individual, e.g. persons with erythema nodosum or phlyctenular conjunctivitis. In such persons, therefore, only very minute quantities of test substances must be injected.

Test Materials

a. Old tuberculin (OT), a concentrated filtrate of broth in which tubercle bacilli have been grown.
b. Purified protein derivative (PPD), obtained by chemical fractionation of OT. Both OT and PPD are available commercially, standardized in terms of biologic reactivity as tuberculin units (TU). Equivalent values are given below.

Approximate Tuberculin Equivalents

TU	"Strength"	PPD mg/dose	OT dilution
1	First	0.00002	1:10,000
5	Intermediate	0.0001	1:2000
250	Second	0.005	1:100

Technique

The initial test dose most commonly used is 5 TU, but this should be reduced to 1 TU, if the individual is expected to be hypersensitive. Higher doses are injected when lower doses have given negative results. Test material of suitable strength, 0.1 mL, is injected into the cleansed skin of forearm. Readings are taken 48 and 72 hours later.

Reading and Interpretation

The tuberculin test is considered positive if induration of 10 mm diameter or more follows injection of 5 TU. Erythema has no specific meaning. In very strongly positive reactions, there may be central necrosis. A positive tuberculin test indicates only that the individual has been infected with tubercle bacilli in the past; it does not indicate present, active disease, resistance, or immunity. The test is most helpful, if there is evidence of recent "conversion", i.e. change from tuberculin-negative to tuberculin-positive in a few months. This clearly indicates recent infection and requires study to rule out active disease.

Tuberculin tests may be falsely negative during illnesses which induce "anergy", e.g. milliary tuberculosis, measles and other exanthems, Boeck's sarcoid, and Hodgkin's disease, or in persons receiving immunosuppressants, e.g. corticosteroids.

Candida

Skin test material used to test for reactivity to a universal antigen or the presence of anergy.

Filariasis

Dirofilaria immitus. Skin test (CDC, Atlanta) supports diagnosis.

Ducrey Test (Ito-Reenstierna test, Chancroid Skin Test, Dmelcos Test; for the Diagnosis of Chancroid)

Ducrey vaccine (a suspension of *H. ducreyi*) is injected intradermally. A positive reaction (erythema and induration) in 1–3 days denotes past infection with *H. ducreyi*; hypersensitivity may persist for years.

Brucellergen Test (For the Diagnosis of Brucellosis)

Intradermal injection of a suspension of *Brucella* organisms may give positive results in 12–48 hours. However, the test is not reliable and agglutination reactions are usually preferred if the organism cannot be isolated from the patient: Skin tests may raise agglutination titer.

Tularemia Skin Test

Highly specific. Remains positive longer than agglutination titer.

Frei Test (For the Diagnosis of Lymphogranuloma Venereum)

The antigen consists of suspension prepared from infected chick embryos and is injected intradermally. A control injection with uninfected chick embryo material must be included in the test. A positive reaction consists of induration and erythema 5–20 mm in diameter in 48 hours. Reading of the control injection must be negative. A positive reaction is suggestive of past infection with one of the agents of the lymphogranuloma—psittacosis group.

Mumps and Herpes Simplex Tests

Skin tests for infections are available, denoting past infections with these viruses. The reaction (after intracutaneous infection of virus material) is often faint and fleeting. It may reach its maximum erythema in 6–48 hours.

Echinococcus Skin Test (Casoni Reaction; for the Diagnosis of Hydatid Disease)

Antigen (hydatid fluid) obtained from human or animal sources gives immediate or delayed reactions after intradermal injection.

Trichinella Skin Test (For the Diagnosis for Trichinosis)

Commercial antigens can be used but an acid-soluble protein fraction (self-prepared) of *Trichina* larvae gives good specificity of delayed reaction.

Coccidioidin Test (For the Diagnosis of Past Infection with *Coccidioides Immitus*)

This test is essentially similar to the tuberculin test. The fungus is grown in synthetic medium for a long period and the broth is filtered and concentrated. Usual test strength is 1:100, giving a positive reaction with induration and erythema in 24–48 hours. Hypersensitivity is commonly acquired as a result of subclinical infection following transient and minimal exposure to infectious arthrospores. Hypersensitivity persists for life. The test is occasionally negative in disseminated coccidioidomycosis. Cross-reactions with other fungal infections are noted with lower dilutions of coccidioidin.

Histoplasmin Test (For the Diagnosis of Past Infection with Histoplasma Capsulatum)

Histoplasmin is prepared similarly to tuberculin and coccidioidin. Usual test strength is 1:100. Positive test indicates past infection. Cross-reactions with other fungal infections are apparently rather high.

Blastomycin Test (For the Diagnosis of Past Infection with *Blastomyces*)

Test material is a suspension of yeast phase of *Blastomyces*. Test interpretation and reading are similar to those of the coccidioidin test.

Kveim-Siltzbach Test (For the Diagnosis of Sarcoidosis)

An extract of sarcoid tissue (especially lymph node of spleen) injected into the skin of a person with sarcoidosis results in a small papule which persists for months. Excision of this papule after 4–8 weeks discloses a microscopic pattern resembling other sarcoid lesions. The basis of the reaction is uncertain, and the test not quite reliable.

Toxoplasma Skin Test

Injection of test material prepared from a suspension of killed organisms gives a delayed reaction in some persons with positive serologic tests. No reaction in others. The test is unreliable for diagnosis and is used mainly for epidemiology.

Nonbacterial Regional Lymphadenitis ("Cat Scratch Fever") *Test*

Pus from active cases is diluted 1:5, heated at 60°C for 10 hours, and stored at 4°C. Intradermal injection of 0.1 mL gives a delayed reaction in persons with this disorder, no reaction in others.

These investigations have now largely been replaced by more accurate (and not at all dangerous) enzyme-linked immunosorbent assay (ELISA) tests, NAT-PCR based test and Rapid immunochromatography tests.

CHAPTER 30

Cytogenetics

INTRODUCTION

Human cytogenetics is study of chromosomes (*chroma*-color; *soma*-body) of man. Human beings possess 46 chromosomes (or 23 pairs); of these, 44 are autosomes and 2 are sex chromosomes. Sex chromosomes are XX in females and XY in males. Approximately, 1% of newborns are now found to have chromosomal defects, which in many cases allow possible prevention of recurrence of the disease with counseling and prenatal diagnosis.

Many clinical entities are found to be associated with specific chromosomal abnormalities. Several chromosomal disorders like trisomy 21 in Down, XO in Turner's, XXY in Klinefelter's, etc. have been discovered. Some neoplasms are associated with nonrandom chromosomal changes like Philadelphia chromosome (Ph.) in chronic myeloid leukemia, an interstitial deletion on chromosome 13 in retinoblastoma, etc. Amniocentesis and culturing of chorionic villi are now common techniques for detection of abnormalities in early fetus. Recent advances in banding techniques allow detection of chromosomal abnormalities from a minute defect to complex derangements. Chromosomal study by in situ molecular hybridization has made it possible even to localize gene on the chromosomes and their transpositions. So much so that one thinks in terms of genetic engineering these days, i.e. replacement of defective genes by the correct ones—this will go a long way in eliminating the genetic disorders when it becomes possible.

Cell Division

There are two types of cell division: mitosis and meiosis.

Mitosis occurs in somatic cells (Fig. 30.1), while meiosis is seen in germ cells. Mitosis involves a single division, whereas, meiosis entails two divisions (1. heterotypical or reduction division and 2. homotypical or equational division). The number of chromosomes remains same in mitosis, but is halved in meiosis, leading to formation of two cells in the former and four in the latter (Fig. 30.2). The chromosomal behavior is independent of each other in mitosis but in meiosis homologous chromosomes get paired together.

BLOOD LYMPHOCYTE CULTURE

The preparative procedure for observation of chromosomes involves four important steps:

a. Arresting dividing cells at metaphase.
b. Treatment with hypotonic solution.
c. Fixation of cells in acetomethanol.
d. Spreading of chromosomes on slide surface.

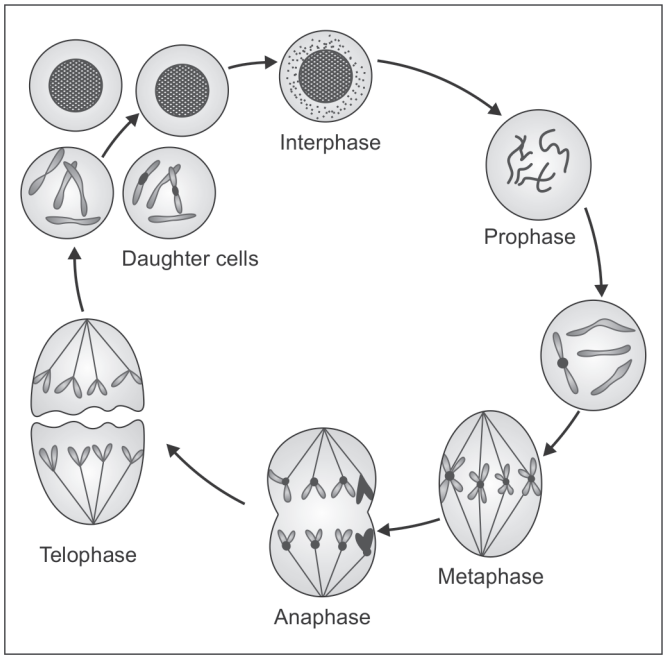


FIG. 30.1: Schematic diagram of mitotic cell division

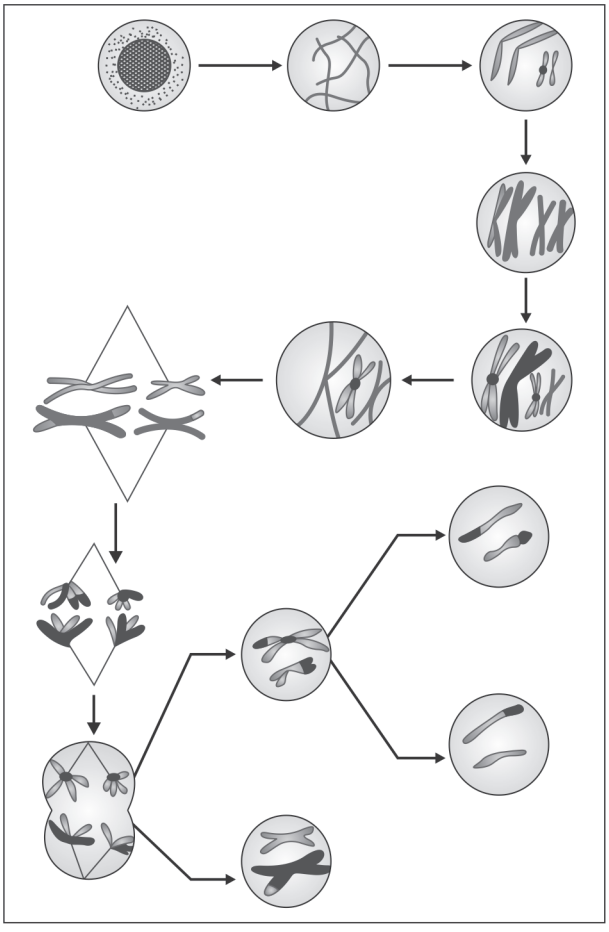


FIG. 30.2: Schematic diagram of Meiotic cell division

Most commonly, peripheral blood lymphocytes and bone marrow cells are utilized for preparation of mitotic chromosomes and gonadal tissues for meiotic chromosomes.

Chromosome Preparation from Bone Marrow

In a normal healthy adult, bone marrow contains sufficient number of dividing cells for direct observation of chromosomes.

1. Add 5 to 10 drops of bone marrow aspirate in prewarmed 5 ml of TC-199 media containing 40–50 units of heparin per ml and 0.02 µg/mL of colcemid.
2. Mix the material thoroughly by pipetting and incubate at 37°C for 1–2 hours.
3. Centrifuge the suspension for 5 minutes at 1000 rpm.
4. Suspend the cell button with 0.075M (0.56 g/100 mL) potassium chloride for hypotonic treatment for 10–15 minutes.
5. Centrifuge and discard the supernatant.
6. Add methanol: Glacial acetic and (3:1) fixative, fix gradually, mix thoroughly by pipetting and keep for 30 minutes in refrigerator.
7. Change fixative at least 3–4 times.
8. Prepare slides by air-dry method by dropping 2 or 3 drops of cell suspension on a clean slide.
9. Stain slides with Giemsa and mount with DPX.

CHROMOSOME PREPARATION FROM WHOLE BLOOD CULTURE

Peripheral blood lymphocytes are the most convenient source of mitotic chromosomes.

Chromosomes are prepared after the lymphocytes are stimulated to grow and divide by a suitable mitogen. Phytohemagglutinin (PHA) is the most commonly employed mitogen. All glassware and reagent including the media to be used for leukocyte culture must be sterilized.

1. Draw about 2 mL of venous blood under aseptic conditions. Transfer the blood into a tube containing heparin (40–50 units/mL) and mix gently to avoid clotting.
2. Add about 0.3 mL of whole blood into a sterile 15 mL screw-capped culture bottle containing 5 mL of TC-199 medium with Hanks base (pH 7.2–7.4) supplemented with 20% fetal calf serum or human serum, 0.1 mL of PHA and 100 µg/mL streptomycin and 200 units/mL of penicillin.
3. Set up at least two parallel cultures for each test sample.
4. Incubate the culture bottles at 37°C for 72 hours with caps tightly closed. Shake the bottles every 12 hours.

Harvesting of Cultures

5. Add colcemid 0.02 µg/mL to each culture bottle 2–3 hours before harvesting to arrest mitosis at metaphase.
6. Transfer the whole content of culture bottle into a centrifuge tube and centrifuge at 1000 rpm for 5 minutes.
7. Discard the supernatant and add 5 mL of prewarmed (37°C) KCl solution (0.075 M) and incubate for 10–15 minutes at 37°C.
8. Centrifuge at 1000 rpm for 5 minutes and discard the supernatant. Fix the cells by adding freshly prepared fixative (methyl alcohol and glacial acetic acid in a ratio of 3:1). Add chilled fixative drop by drop with gentle shaking. After adding 1 mL of fixative and mixing the cell button well, make the volume to 5–10 mL by adding more fixative.
9. Keep the tubes in refrigerator for 30 minutes to fix the cells. Resuspend the cells in fresh fixative and centrifuge as before. Repeat the process until a colorless cell pellet is obtained.

Preparation of Chromosome Slide

10. Discard the supernatant completely without disturbing the cell button. Finally add 0.5 to 1.0 mL of freshly prepared fixative. The final concentration of the cell suspension has to be adjusted depending on the cellular concentration.
11. Keep the precleaned slides in absolute alcohol.
12. Place 2–3 drops of the cell suspension with a pasteur pipette on the slide from a distance to facilitate better spreading.
13. Allow the slides for air drying on heat drying.
14. Stain with buffered Giemsa solution (1:10) at pH 6.8.
15. Dry and pass the slides through xylene and mount in DPX.

Cleaning and Preparation of Slides

1. New slides are kept in concentrated nitric acid for overnight.
2. Keep the slides in horizontal coplin jar under running tap water for 2–3 hours.
3. Rinse in distilled water and store in 90% ethyl alcohol.
4. Wipe off and dry it with a clean cloth or tissue paper.

Procedure of Giemsa Staining

1. Commercial Giemsa solution is diluted 1:10 in phosphate buffer (pH 6.8).
2. Place a few drops of freshly prepared stain on the slide sufficient to cover the entire surface for 5–10 minutes.
3. Rinse in distilled water.
4. Air dry at room temperature.
5. Pass the slide through xylene and mount in DPX.

Preparation of Phosphate Buffer Solution

Solution A: 0.067 M KH_2PO_4 (9.08 g/liter of distilled water)

Solution B: 0.067 M Na_2HPO_4 (11.88 g/liter of distilled water)

Mix the solutions as given below:

For pH 6.4—73.2 mL Sol. A + 26.8 mL Sol. B.

For pH 6.8—50.8 mL Sol. A + 49.2 mL Sol. B.

KARYOTYPING

After doing Giemsa staining, the chromosomes are photographed. The individual chromosomes are cut out from the photograph. The chromosomes are then arranged in an orderly fashion, in homologous pairs, to produce a standard arrangement called as karyotype.

The cut out photomicrographs of the 46 chromosomes of a spread are at first arranged in pairs. Thereafter, these pairs are arranged in descending order in relation to their total lengths, placing all their centromeres along the same horizontal line. When arm lengths are unequal, shorter arm is made to point upwards and the longer one downward. The 22 pairs can be arranged in 7 groups. These are as follows:

1. A group: 1 to 3 pairs—metacentric
2. B group: 4 to 5 pairs—submetacentric
3. C group: 6 to 12 pairs—submetacentric
4. D group: 13 to 15 pairs—acrocentric
5. E group: 16 to 18 pairs—submetacentric
6. F group: 19 to 20 pairs—metacentric
7. G group: 21 to 22 pairs—acrocentric.

Group A consists of the longest metacentric chromosomes. Group G consists of the shortest acrocentric chromosomes.

In males, group G includes Y chromosome. Y chromosome is an acrocentric chromosome like others in the group, but it showns two differences:

a. It is usually the longest in group G.
b. Its long arms are usually parallel to each other, which are divergent in the other members of group G (Fig. 30.3).

The X chromosome is a member of group C and can be distinguished from other members of the group by banding techniques and by using special stains.

Parameters used to characterize a chromosome in the karyotype:

1. Shape of the chromosome
2. Length of the chromosome

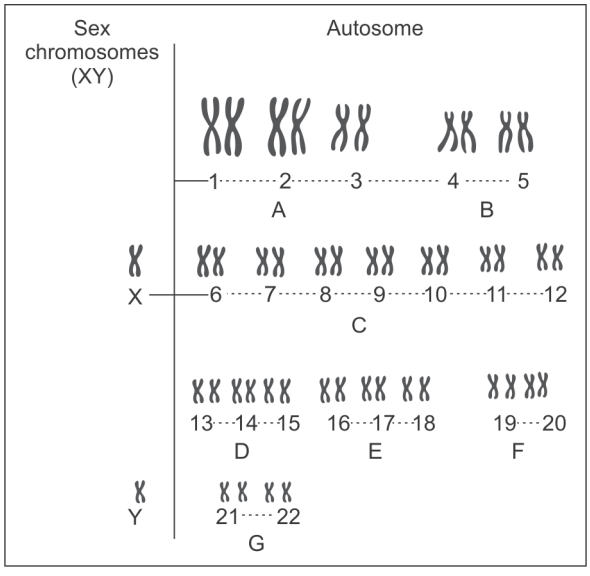


FIG. 30.3: The human male karytype

3. Centromeric index: This index is expressed in the form of the short arm length to the total chromosome length. So,

$$\text{Centromeric index} = \frac{\text{Short arm length}}{\text{Total chromosome length}}$$

For example, this, in a metacentric chromosome is 0.5.

4. Proportion of the arms: It is the ratio between the long and short arms of the chromosome. In a typical metacentric chromosome, this ratio is 1:1.

G AND Q BANDINGS

G and Q bandings are the commonly employed techniques usually asked for chromosomal studies.

G Banding

Trypsin digestion method (Modified, Seabright, 1971)

1. Slides are allowed to age for 4–5 days before banding technique is employed.
2. Slides are dipped in 15% hydrogen peroxide (H_2O_2) for 5 minutes.
3. Rinse the slides for about 10–15 seconds in normal saline.
4. Immerse the slides in 0.2% trypsin (pH 6.8) solution at 20°C for about 7–10 seconds (if the slides are older, trypsin treatment may be prolonged).
5. Rinse the slides for about 10–15 seconds in normal saline and distilled water and allow to dry.
6. Dry slides for about 10–15 minutes in 5% buffered Giemsa solution at pH 6.8.
7. Wash the slides in running tap water to remove excess stain and dry.
8. Pass the slides through xylene and mount with DPX.

G banding makes identification of each chromosome possible by its characteristic dark and light bands.

Q Banding (Quinacrine Banding Technique)

This reveals bright fluorescent bands throughout the entire length of chromosomes. The long arm of Y chromosomes shows bright fluorescence. Identification of each chromosome and its regions is possible by its characteristic fluorescence bands.

1. Slides are allowed to age for 2–3 days before banding technique is performed.
2. Pass the slides through down grades ethyl alcohol to distilled water (3 minutes in each).
3. Immerse the slide in phosphate buffer solution at pH 5.5.
4. Place the slide for 15 minutes in solution of Quinacrine dihydrochloride, or Quinacrine mustard (0.2%) in phophate buffer pH 5.5.
5. Rinse the slide in buffer solution (pH 5.5) for 5 minutes.
6. Mount the slide in buffer with a clean cover glass.
7. Seal the cover glass (after removing excess buffer) with rubber solution or nail polish.
8. Screen the slides under fluorescence microscope with appropriate combination of excitation and barrier filters.

C Banding Technique

This is used for demonstrating centromeric heterochromatin. The chromosomes show dark staining at centromeric heterochromatic regions.

1. Air dried or heat dried chromosomes slides are allowed to age for 7–10 days before banding technique is employed.
2. Put the slides in 0.2 N HCI for 1 hour at room temperature.
3. Rinse the slides thoroughly in distilled water.
4. Rinse the slides in 5% aqueous solution of barium hydroxide at 50°C for 5–7 minutes in water bath (before dipping the slides, keep aqueous solution of barium hydroxide in water bath at 50°C).
5. Wash the slides with several changes of distilled water to remove the precipitate of barium hydroxide.
6. Incubate the slide in 2 × SSC for about 1 hour in coplin jar at 60°C (2 × SSC should be kept at 60°C well in advance). 2 × SSC: 1.754 gram of sodium chloride and 0.882 gram of trisodium citrate in 100 mL of distilled water
7. Rinse the distilled water and pass the slides briefly in 70% and 90% ethanol and allow the slides to dry.
8. Stain with 5% buffered Giemsa solution at pH 6.8, for 10–15 minutes.
9. Rinse the slides in distilled water, dry and pass through xylene and mount in DPX.

IMPORTANCE OF CHROMOSOMAL STUDIES

Chromosomal studies are useful:

i. In diagnosis of various chromosomal abnormalities like Turner's syndrome, Down syndrome, Klinefelter's syndrome, etc.
ii. Clinically, in investigation of patients with abnormalities of sexual developments or infertility.
iii. In determination of sex of an unborn child to assess risk of gender related inherited diseases.
iv. In larger scale population surveys, e.g. to detect the effects of occupational hazards on chromosomes in relation to various environmental factors like cold, heat, chemicals, dust, etc.
v. In new fields involving separation of X or Y bearing sperms for preventing gender related inherited disorders.

Variations in the Chromosome Number

Polyploidy

It is used to denote presence of multiples of haploid number of chromosomes. For example, a tetraploid cell has four times the haploid number.

Aneuploidy

It is used to indicate absence of the property of being a normal multiple of haploid number of chromosomes. Thus, a human cell with 45, 47 or any other number of chromosomes that is not exact multiple of 23 is an aneuploid cell.

Aneuploidy can be of two types.

1. *Hyperploidy:* It is the condition in which there is addition of one or more chromosomes to the diploid number. It may be called:
   i. Trisomy (2n + 1) when one chromosome is added to the diploid number. Such a cell would have 47 chromosomes.
   ii. Tetrasomy (2n + 2) when two chromosomes of a homologous pair are added to the diploid number. Such a cell would have 48 chromosomes.
2. *Hypoploidy:* It involves the loss of one or more chromosomes to the diploid number. It may be called:
   i. Monosomy (2n–1): When there is loss of one chromosome from the diploid set. A cell exhibiting this condition would contain 45 chromosomes.
   ii. Nullisomy (2n–2): When both the chromosomes of a homologous pair are lost from the diploid set. Such a cell would have 44 chromosomes.

BARR BODY ANALYSIS AND BUCCAL SMEAR FOR STAINING OF SEX CHROMATIN MASS

Normal values	*Number of Barr Bodies*
Normal female (XX)	1
Normal male (XY)	0
Turner syndrome (female) (XO)	0
Klinefelter's syndrome (male) (XXY)	1
Klinefelter's syndrome (male) (48 XXXY)	2
Klinefelter's syndrome (male) (49 XXXYY)	2
Klinefelter's syndrome (male) (49 XXXXY)	3

Usage: Screening for sex chromosome abnormalities.

Description: A Barr body, or sex chromatin body, is a tightly coiled, X chromosome lying against the nuclear membrane of female cells or any cell with more than one X chromosome. It shows up as a dark-staining body in the shape of a half-moon and is absent in male cells. Barr bodies are believed to function in early embryonic development and later become inactivated to maintain gene balance of Xs to autosomes. The number of Barr bodies in a patient is one less than the number of Xs.

Preparations

a. Rinse the mouth with mouthwash.
b. Obtain a metal spatula, saline, two sides, and preservative.

Procedures

a. Gently scrape the buccal mucosa with the metal spatula dipped in saline.
b. Clean the spatula and repeat the procedure gently but firmly.
c. Smear the material on the two slides and place them in the preservative.

Postprocedure Care

a. Label the container of the slides with the patient's name, the date, and the contents.

Client and Family Teaching

a. Refer the patient with abnormal results for genetic counseling.

Factors that Affect Results

a. None known.

Other Data

a. Barr bodies do not give any information about Y chromosomes.
b. Human chromosome analysis, rather than buccal smears, should be used for evaluations of newborns with ambiguous genitalia.

CHAPTER 31

World's Latest and Best Technologies by Roche

Life writes the questions –

Roche provide the answers

Disease raise many questions. We empower doctors and patients with the information they need to answer these questions. And we help hospitals and laboratories to deliver that information efficiently and reliably.

For more than a century Roche has played a pioneering role in healthcare. As the global leader in in vitro diagnostics (IVD), Roche today supplies a wide range of diagnostic instruments and tests for timely, reliable disease detection, management and therapy monitoring. With expertize spanning all IVD areas, Roche is a full provider and key enabler of novel clinically differentiated diagnostic solutions.

Roche serves customers in hospitals and commercial laboratories, in doctors offices, at home and in research institutions tailoring its products to local market needs and the needs of laboratories of various types and sizes. Continuous investment in research and development and close collaboration with customers has kept Roche an industry leader in addressing unmet medical needs.

Roche empowers healthcare professionals with a wide range of innovative technologies, one of the industry's broadest testing portfolios and a large global installed base of diagnostic instruments. Roche continues to expand its test menu and currently operates in more than 130 countries around the globe.

BUSINESS AREAS

Roche's Diagnostics Division has several businesses, each serving a particular customer segment. Each business is active in research and development, manages its own product portfolio, defines its global strategic direction and has its own marketing and business development organizations.

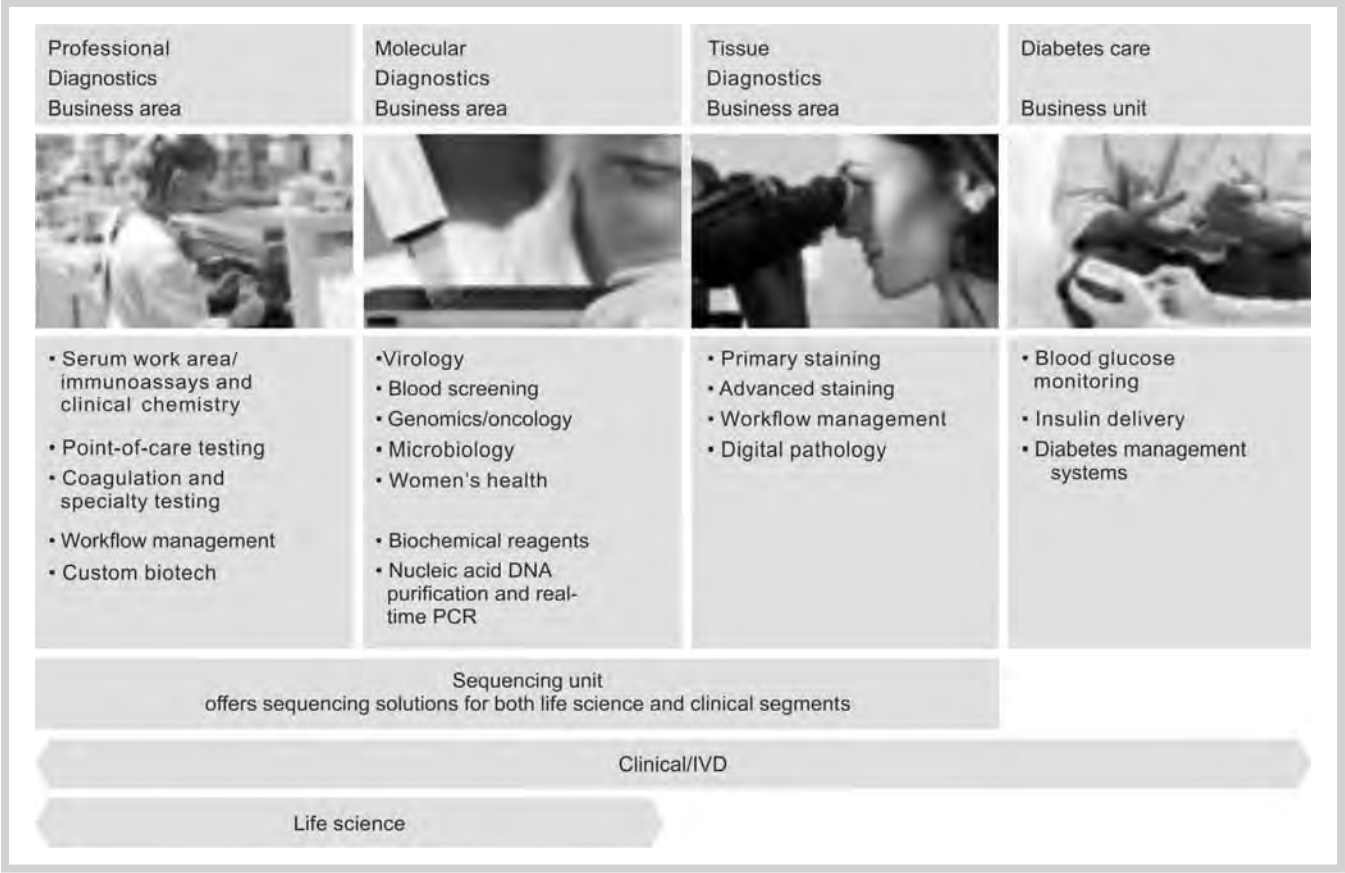
REVALUATING DIAGNOSTICS

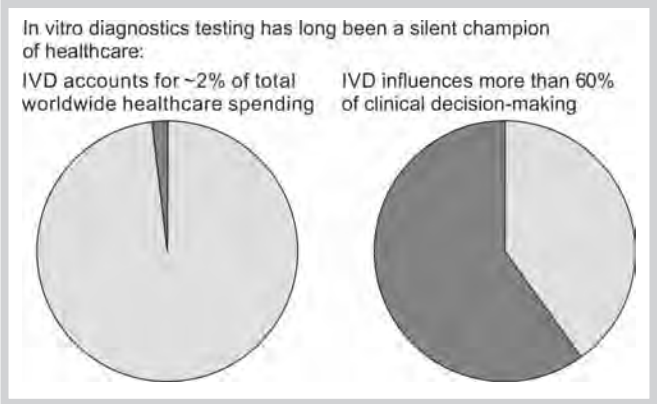
The IVD tests influence more than 60% of clinical decisions, yet account for only 2% of healthcare expenditure. Roche remains strongly committed to developing novel diagnostic solutions that provide healthcare professionals with high-value, actionable results they can use to prevent, manage and treat disease more effectively.

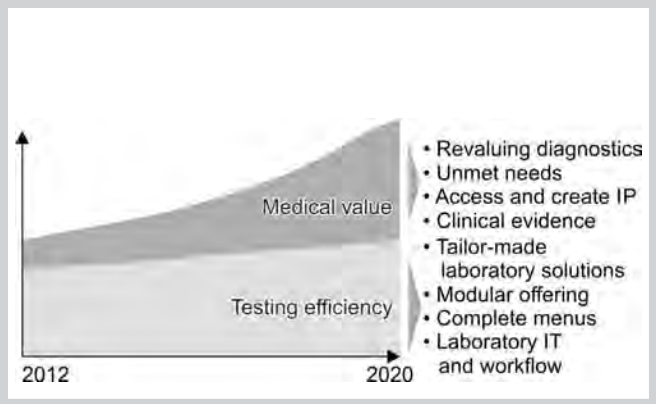
The importance of diagnostics will continue to grow with advances in biomarkers and technology. It is time to revalue diagnostics and recognize the contribution they make to people's health and to the healthcare system.

TESTING EFFICIENCY AND MEDICAL VALUE

Roche stands out in the industry through its combined strengths in pharmaceuticals and diagnostics. As the world leader in vitro diagnostics, Roche develops evidence-based diagnostic tests that respond to unmet medical needs. Together, our tests and highly efficient laboratory solutions help to improve patients' survival, health and quality of life.







Providing True Medical Value

Leading the industry through research-based innovation, we strive to provide diagnostic solutions of true medical value for healthcare professionals and patients. We prioritize areas with the greatest unmet medical need and devote substantial resources to acquiring the intellectual property needed to develop products delivering maximum clinical and health-economic benefit.

Personalized Healthcare

Personalized healthcare means fitting the right treatments to the right groups of patients. Our crucial edge in this field comes from combining the knowledge of our Pharmaceuticals and diagnostics divisions and drawing on it throughout the R and D process, from early research to approval of new diagnostic tests and medicines and their use by patients. Around 60 % of the compounds

in Roche's pipeline include a personalized healthcare approach. All Roche drug development programs include an associated biomarker program, and there are more than 240 personalized healthcare collaborations within the Roche Group.

In addition, Roche has more than 40 agreements with external biotech and pharmaceutical companies to develop companion diagnostic tests for their medicines. We aim to be the partner of choice in this field. Diagnostics are integral to the new paradigm of personalized healthcare, allowing healthcare professionals to identify the patients most likely to respond to a particular treatment.

For example, our **cobas**® 4800 BRAF V600 mutation test allows healthcare professionals to assess whether a melanoma patient is eligible for and will respond to treatment with Zelboraf®. This medicine is indicated for patients with inoperable or metastatic melanoma with BRAF V600 mutations. These mutations are thought to occur in about half of all melanomas and 8% of solid tumors.

Increasing Testing Efficiency

Faced with tightening healthcare budgets in a globally changing environment, today's laboratory managers are under pressure to streamline overall processes and workflow and make them more efficient. With its complete and integrated solutions, Roche enables laboratories to better manage expanding volumes of tests and data and to optimize testing efficiency. For physicians and patients this translates into timely, accurate and reliable results.

Infectious disease is one of the areas where Roche has demonstrated the clinical impact of combining medical value and testing efficiency. From screening to diagnosis to therapy selection and monitoring, Roche supplies laboratories with advanced technologies and the broadest test menu in the industry, providing them with a complete solution for many of today's most significant medical needs.

EFFECTIVE MANAGEMENT OF INFECTIOUS DISEASES

Covering the Continuum of Care in Infectious Diseases

Increasing blood safety

Testing blood donations for infectious diseases is essential to maintaining a safe blood supply. Roche offers one of the most comprehensive ranges of serology and molecular screening tests in the industry.

PORTFOLIO HIGHLIGHTS

NEW: Elecsys® Syphillis Immunoassay:

A treponemal test suitable for screening in the general population, pregnant women and blood donations. It uses the latest technology for superior sensitivity.

cobas® TaqScreen MPX test v-2.0: Covers five critical viral targets in one easy-to-use assay.

Results you can rely on

Virus variability poses a challenge in diagnosing infectious diseases. At Roche we are continuously developing innovative new products and solutions that give healthcare professionals results they can truly rely on.

PORTFOLIO HIGHLIGHT

Elecsys® HBsAg II quant assay:

Combines a high level of sensitivity, including excellent mutant detection, with high specificity.

COBAS® AmpliPrep/COBAS® TaqMan HIV®♥ 2.0: Expands coverage by targeting two highly conserved regions of the HIV-1 genome to compensate for the possibility of mutations or mismatches.

SCREENING (DISEASE CONTROL) DIAGNOSIS

Reliable Tools for Improved Clinical Decision-Making

Personalized treatment is becoming increasingly important as new drugs reach the market. Patients' responses to treatment vary, and no drug is suitable for all patients. With its extensive expertise in infectious disease management, Roche is helping to advance personalized healthcare with tests that identify patients likely to benefit from new treatments.

PORTFOLIO HIGHLIGHT

COBAS AmpliPrep/COBAS TaqMan

HLA-B 5701 screening test: Helps to identify patients with hypersensitivity to the anti-HIV drug Abacavir.

Optimized, Personalized Treatment for the Best Clinical Outcome

Monitoring a patient's response to therapy can be critical for treatment success. A broad range of tests from Roche enable healthcare professionals to assess and adapt treatments to ensure the best possible outcome.

PORTFOLIO HIGHLIGHT

Elecsys HBsAg II quant and COBAS

AmpliPrep/COBAS TaqMan HBV assay: These tests can be used together for response-guided peginterferon α-2a therapy for hepatitis B infection.

Serum Work Area, Laboratory Automation and IT Solutions

In medicine today the emphasis is on cost-efficiency as well as on effective, quality care. Roche's serum work area laboratory automation and IT solutions provide vital information for clinical decision-making. They help to maintain quality while keeping costs at a manageable level.

Laboratories have to manage critical workflow processes and provide uninterrupted service. Our **cobas**® platforms offer fully harmonized end-to-end solutions covering everything from sample entry to result reporting and archiving. With their scalable modular design, they can be customized to meet any laboratory's needs.

Intelligent process control is important not only during sample analysis but also during the pre- and post-analytical stages of testing. Throughout the entire testing cycle it contributes to safer, more efficient workflows and reduces complexity.

Roche's automated pre- and post-analytical solutions are integral to providing complete flexibility and process optimization. We offer a full array of stand-alone and networked solutions to meet all of your laboratory's needs. From laboratory layout to full implementation of systems and services, you can get everything from a single source.

An integrated solution combining IVD and IT reduces risk and complexity for your laboratory.

Roche's flexible **cobas IT** systems include middleware applications, laboratory information systems and hospital point-of-care solutions. They enable you to use your resources more effectively, while monitoring laboratory performance and increasing quality and confidence.

Our innovative and comprehensive test portfolio meets demands for workflow consolidation while also addressing previously unmet medical needs. With our ready-touse reagents and best-in-class Elecsys® immunoassay and DuREL homogeneous assay technologies, we guarantee outstanding sensitivity and the highest quality results combined with best-in-class convenience.

COBAS® MODULAR PLATFORM

Flexible Family Concept for Tailormade Solutions

Today, laboratories are challenged to deliver reliable and high-quality diagnostics while at the same time ensuring efficient analytical workflow. To meet these demands, Roche has developed the **cobas** modular platform. It is an intelligent and flexible solution based on a common architecture that delivers Tailor-made solutions for diverse workload and testing requirements. The **cobas** modular platform is designed to reduce the complexity of laboratory operation and provide efficient and compatible solutions for network cooperation.

YOUR BENEFIT

Increased Efficiency

- Consolidation of 98% or more of serum work area workload
- Consistent and predictable turnaround times for smooth laboratory operation
- Further enhanced automation through broad offering of pre- and post-analytic and **cobas IT** solutions from Roche.

Reduced Complexity

- Unique, ready-to-use reagents for maximum convenience of handling, minimal logistic effort and cost-effective operation
- Common look and feel of the user interface of on all systems for reduced training time and flexible staff allocation.

Consistent and Fast Patient Results

- Standardized results across the entire **cobas** modular platform ensured by using the same reagents
- 9 min. STAT assays for superior support of emergency samples.

Reliable and Future Proven

- Proven Hitachi instrument reliability ensures maximum uptime for economic operation and reliable service to physicians

Unique reagent concept for maximum handling convenience and minimal logistic efforts

- >21,500 **cobas** modular platform system installations worldwide.

Product Characteristics

- Flexible combinations of clinical chemistry (c) and immunochemistry (e) modules for serum work area or dedicated immunochemistry/clinical chemistry solutions
- More than 120 assays and applications on the clinical chemistry platform, ready to use in **cobas c** packs
- Almost 100 assays on the immunochemistry platform, ready to use in **cobas e** packs.

COBAS® 8000 MODULAR ANALYZER SERIES

Intelligent LabPower

The **cobas** 8000 modular analyzer series is designed for high workload laboratories with a throughput of 2.5 to 15 million tests per year. A modular configuration consists of a core unit, an optional ISE unit (**cobas** ISE module), and up to 4 analytical modules: the high throughput clinical chemistry modules (**cobas c** 702 and **cobas c** 701), the medium throughput clinical chemistry module (**cobas c** 502) and the immunochemistry module (**cobas e** 602).

Cobas 8000 modular analyzer series acts intelligently, empowering the laboratory to improve customer and patient services.

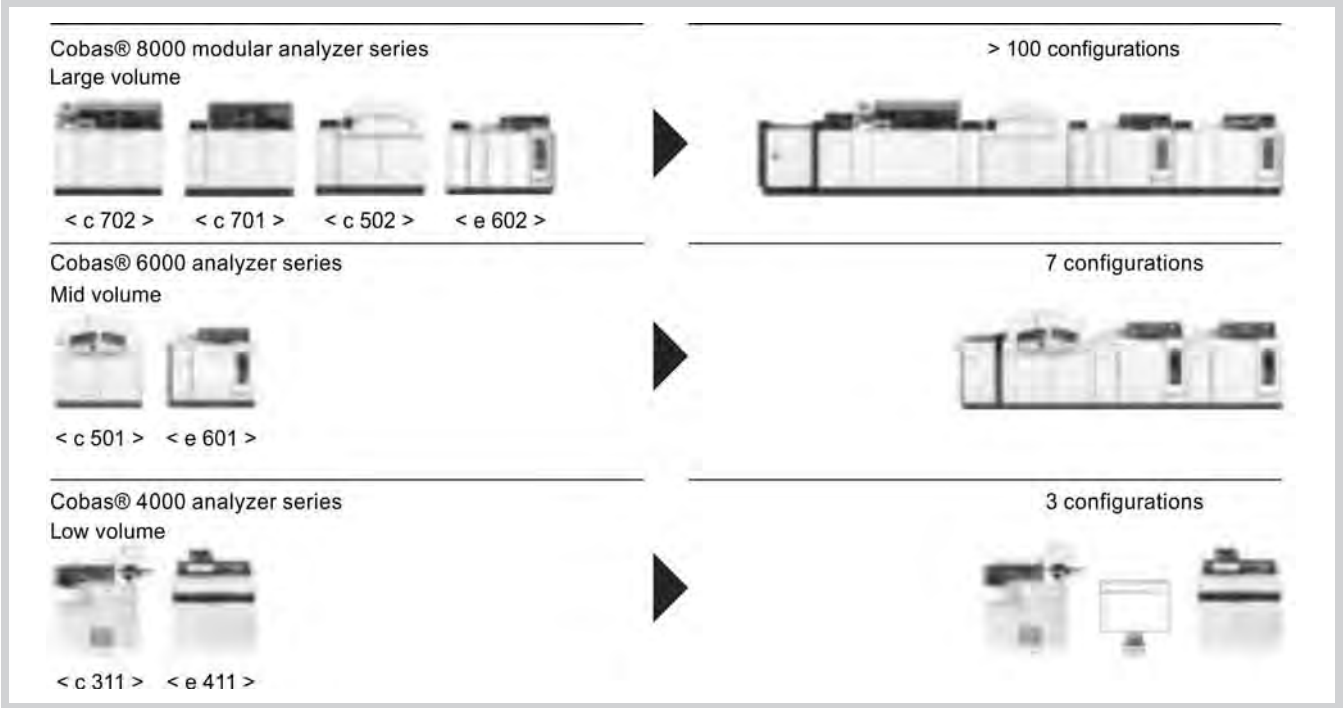
YOUR BENEFIT

Efficiency

- Maximizes walk-away time
- Optimizes cost management
- Improves sample turnaround time and availability.

Productivity

- Delivers throughput with maximum consolidation power



- Manages peak times efficiently
- Increases sample capacity on board.

Process Innovation

- Ensures unrestricted rack traffic flow for intelligent sample routing
- Optimizes workflow
- Provides confidence in results.

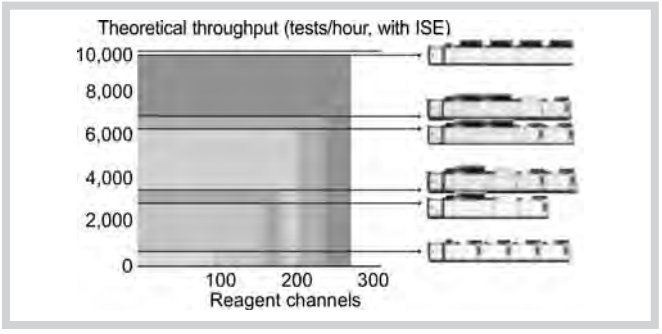
Consolidation

- Real tailor-made solutions for every lab and highly efficient change management
- Maximizes throughput and consolidation power without compromising workflow
- Consolidates very frequently requested tests with less frequently requested tests.

Product Characteristics

- High speed: From 170 to 680 immunoassay tests/hour and 2,000 to 9,800 clinical chemistry tests/hour depending on configurations
- Up to 280 reagent channels

MULTIDIMENSIONAL MODULARITY



- Multidimensional modularity: more than 100 configurations for tailored solutions with fast on-site expandability
- More than 120 clinical chemistry and almost 100 immunochemistry assays.

COBAS® 8000 MODULAR ANALYZER SERIES

1. Cobas 8000 Data Manager

- Traceability records, for easy tracking of calibration and reagent information, offers more transparency
- User-defined, fully automated, selective rerun and reflex testing.

2. Core Unit

- Loading capacity of 300 samples
- Unloading capacity of 300 samples
- Throughput of up to 1,000 samples/hour
- Dedicated STAT port
- Optional sample rotation unit.

3. Cobas ISE Module

- Sodium, potassium, and chloride
- 900 or 1,800 tests/hour
- ISE-specific sample probe with clot detection
- Independent processing line.

4. Cobas c 702 Module*

- More than 120 assays and applications on the clinical chemistry platform including substrates, enzymes, proteins, DATs, and TDMs
- Throughput of up to 2,000 tests/hour
- 70 reagent channels directly accessible for pipetting
- Specimen integrity via serum indices, clot and liquid level detection
- Contact-free ultrasonic mixing.

4a. Reagent Manager

- 10 reagent positions
- Reagent RFID reader
- Continuous reagent cassette loading and unloading during operation
- Reagent cassette decapping
- Reagent cassettes can be placed in the reagent manager at any time and as convenient.

5. Cobas c 502 Module

- More than 120 assays and applications on the clinical chemistry platform including substrates, enzymes, proteins, DATs, TDMs, and electrolytes
- HbA1c (whole-blood measurement)
- Throughput of up to 600 tests/hour
- 60 reagent channels directly accessible for pipetting
- Automatic reagent loading and unloading during operation
- Specimen integrity via serum indices, clot and liquid level detection
- Contact-free ultrasonic mixing.

6. Cobas e 602 Module

- Heterogeneous immunochemistry testing with almost 100 assays for anemia, bone, tumor markers, hormones, cardiac and infectious diseases
- 9 min. STAT applications for hsTnT, TnI, CK-MB, NT-proBNP, Myoglobin, PTH and hCG
- Throughput of up to 170 tests/hour
- 25 reagent channels directly accessible for pipetting
- Carryover-free disposable tips
- Clot, liquid level, and air bubbles detection.

7. Module Sample Buffer

- Capacity for 20 sample racks resulting in additional capacity of 100 samples per module
- Freely definable STAT positions
- Environmentally controlled compartment for 5 Auto QC racks
- Backup operation port
- Switch gates for shortcuts; gripper for moving the racks from line to line
- Random access to racks; racks can go from anywhere to everywhere.

COBAS® 6000 ANALYZER SERIES

The Success Story Continues

Just as every patient requires individualized care, every laboratory is unique. Striking a balance between high standards and efficient operation requires tailor-made solutions.

* Alternatively, cobas c 701 module can be used. It is based on the same technology and it offers the same number of channels as cobas c 702, but has no reagent manager function.

The **cobas** 6000 analyzer series is a member of the **cobas** modular platform family. It offers medium to high workload laboratories tailor-made solutions for clinical chemistry and immunochemistry testing. Depending on the configuration, the **cobas** 6000 analyzer series achieves a throughput of up to 2.5 million tests per year. The **cobas** 6000 analyzer series is the result of vast knowhow and decades of experience combined into one successful concept. With over 9,000 systems worldwide, the success story continues.

YOUR BENEFIT

Increased Efficiency

- Perfect fit of throughput and reagent channels achieved across the 7 different configurations
- Consolidation of 98% of the serum work area testing on Serum Work Area workloads
- Simplified lab processes and reduced costs.

Quality of Results

- That you can trust and are right the first time
- Predictable turnaround time
- Peace of mind.

Maximum Uptime

- Highly reliable system based on more than 35 years of experience
- Superior support provided by Roche organizations worldwide.

Optimized Workflow

- Wide range of pre- and post-analytical solutions and complete IT solutions
- Workflow efficiency and reduced complexity.

PRODUCT CHARACTERISTICS

High System Reliability

- More than 9,000 systems installed worldwide
- Proactive automated maintenance for over 96 % uptime including maintenance on a 24/7 basis.

Unique Reagent Concept

- No preparation and no mixing required, economic usage with high stabilities and convenient kit sizes.

First Class Performance

- State-of-the-art immunoassay testing using ECL technology
- High quality results by ensuring sample and result integrity.

Intelligent Sample Workflow

- Combines STAT with routine testing without disruption.

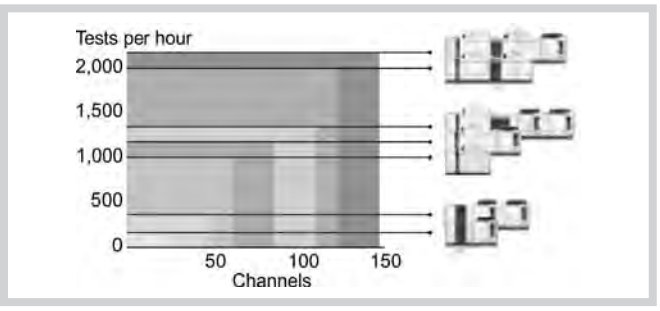
Professional Management of Lab Processes

- Wide range of complete pre- and post-analytical solutions from small task target automation to total lab automation.

1. Core unit

- Loading and unloading capacity of 150 samples
- Throughput of up to 600 samples/hour
- Dedicated STAT port
- Simple operation with continuous loading and unloading.

Delivers Customized Solutions for Various Work and Testing Requirements



True Workflow Consolidation

2. Rack rotor

- Capacity for 20 sample racks
- Freely definable STAT positions
- Option of 3 Auto QC racks
- Random access for the racks.

3 Cobas c 501 Module

- ISE measurements (K, Na, Cl)
- More than 120 assays and applications on the clinical chemistry platform including proteins, enzymes, DATs, TDMs, substrates and electrolytes
- HbA1c (whole-blood measurement)
- Throughput of up to 1,000 tests/hour
- 60 reagent channels directly accessible for pipetting
- Automatic reagent loading and unloading during operation
- Specimen integrity via serum indices, clot and liquid level detection
- Contact-free ultrasonic mixing.

4. Cobas e 601 Module

- More than 100 assays on the immunochemistry platform including anemia, bone, tumor markers, hormones, cardiac and infectious diseases
- 9 min. STAT applications for hsTnT, TnI, CK-MB, NT-proBNP, Myoglobin, PTH and hCG
- Throughput of up to 170 tests/hour
- 25 reagent channels, directly accessible for pipetting
- Carryover-free disposable tips
- Clot, liquid level, and air bubble detection.

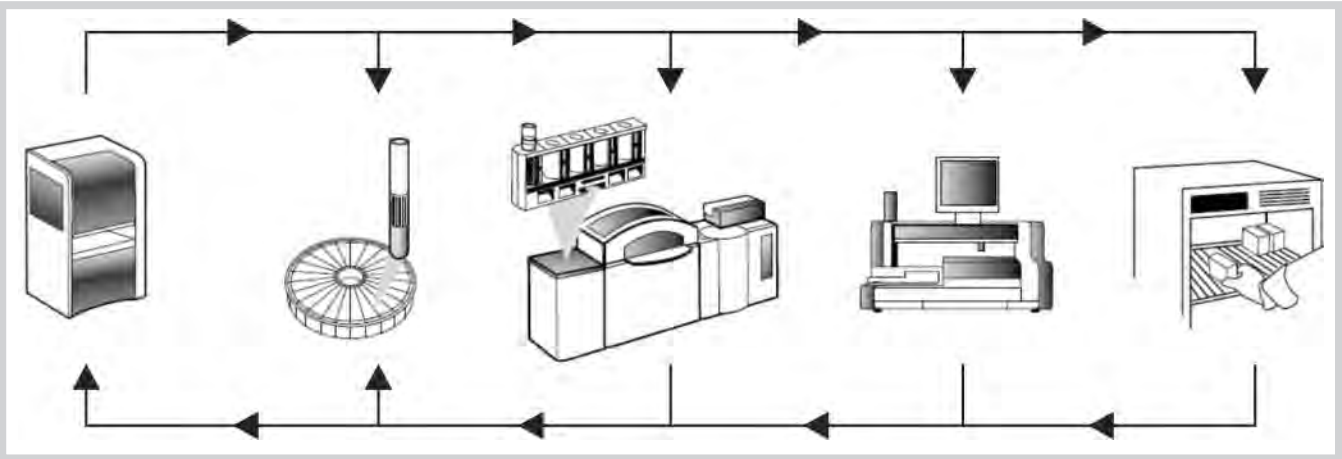
Cobas p 312 Pre-analytical System is the Ideal Companion for the cobas® 6000 Analyzer Series, for a Fully Harmonized and Complete Solution

Safe and efficient workflows with minimum complexity, using a single square meter footprint. The cobas p 312 pre-analytical system is Roche's answer to fulfill automation needs of many small to mid-sized laboratories. It includes the necessary functionality to significantly improve laboratory organization and increase workflow efficiency. This on a single square meter.

The simplicity of this solution and the small space requirements allow its easy implementation in almost any laboratory.

The cobas p 312 pre-analytical system will take over the following key tasks:

- Sample registration at a single entry point
- Sorting and distribution of samples
- Recursive workflow
- Archiving.



COBAS® 4000 ANALYZER SERIES

Freedom to Realize Your Lab's Potential

The **cobas** 4000 analyzer series is a member of the **cobas** modular platform family and designed for laboratories processing 25,000 to 500,000 tests per year or 50 to 250 samples per day. It consists of the **cobas c** 311 analyzer for clinical chemistry and the **cobas e** 411 analyzer for immunochemistry testing. Together with **cobas IT** solutions and the ability to integrate the **cobas p** 312 pre-analytical system, the **cobas** 4000 analyzer series provides a comprehensive serum work area solution that takes workflow efficiency to the next level.

YOUR BENEFIT

Increased Efficiency

- Consolidation of 98% or more of serum work area workloads.

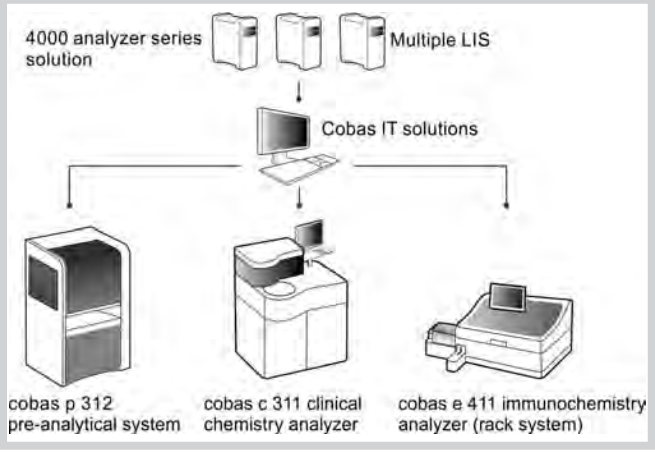
Maximum Uptime

- Highly reliable system based on more than 35 years of experience
- Superior support by Roche organisations worldwide.

Quality of Results

- Integrated safety features for results you can trust
- Predictable turnaround time.

Cobas® 4000 Analyzer Series Solution



PRODUCT CHARACTERISTICS COBAS C 311 ANALYZER

First Class Performance

- More than 120 assays and applications available including DATs, TDMs, specific proteins and whole blood HbA1c
- Throughput: up to 300 tests/h; ISE: 150 samples/h (corresponding to 450 tests/h).

Intelligent Sample Workflow

- 108 sample positions with continuous random access and flexible STAT priority settings.

Unique Reagent Concept

- Convenient and error-free handling of **cobas c** packs
- Economic usage with high stabilities and convenient kit sizes.

High System Reliability

- Programable automated maintenance functionalities.

PRODUCT CHARACTERISTICS COBAS E 411 ANALYZER

First Class Performance

- Almost 100 assays available
- Throughput: up to 86 tests/h
- Superior immunoassay testing using ECL technology
- 9 min. STAT applications including Troponin, CK-MB, Myoglobin, ß-hCG and PTH

- Disposable tips and cups for carryover-free sample pipetting.

Intelligent Sample Workflow

- 75 sample positions (rack system)
- 30 sample positions (disk system)
- Continuous random access and flexible STAT priority settings.

Unique Reagent Concept

- Convenient and error-free handling of **cobas e** packs
- Economic usage with high stabilities and convenient kit sizes.

High System Reliability

- More than 10,000 analyzers installed worldwide
- High uptime of 99.8%.

COBAS C 111 ANALYZER

Small Box, Big Performance

The **cobas c** 111 analyzer is the smallest member of the cobas serum work area platform family and the ideal solution for clinical chemistry testing in laboratories running 10 to 50 samples per day. With a comprehensive test menu and easy integration of STAT samples, it can support testing of both routine clinical chemistry panels and rapid turnaround critical care markers. In addition, the **cobas c** 111 analyzer uses the same reagent formulations as the larger **cobas** clinical chemistry analyzers. This standardizes patient results, which is vital for integrated laboratory networks serving outpatient services, emergency departments and clinics, as well as private laboratories serving primary care physicians.

YOUR BENEFIT

High Quality of Results

- Comprehensive testing capabilities
- Results you can trust the first time, every time.

Increased Efficiency

- Essential routine testing on a small footprint
- Simplified system operation.

Maximum Uptime

- Highly reliable system delivering >99 % uptime
- Superior support provided by Roche organisations worldwide.

Optimized Workflow

- Reducing complexity for a range of laboratories, both networked or standalone
- Consistent results across the **cobas** platform.

PRODUCT CHARACTERISTICS

World-class Performance

- More than 40 assays and applications available including whole blood HbA1c, hsCRP, and D-dimer
- Externally rated world-class performance[1]

Good Fit for Labs <50 samples/day

- Throughput of up to 100 tests/hour
- Compact benchtop system for labs with limited floor space
- Easy, intuitive software handling

High System Reliability

- Robust system design
- Wizard-guided maintenance procedures.

Network Compatibility

- Ability to connect to local IT surroundings
- Common reagent chemistry across the **cobas** platform.

COBAS INTEGRA® 400 PLUS

The Specialist in the Routine Laboratory

The COBAS INTEGRA 400 plus analyzer is the perfect solution for laboratories running 50 to 400 samples per day. Its broad test menu comprises over 120 assays and applications that consolidate clinical chemistry with specific proteins, therapeutic drug monitoring and drug of abuse testing. This compact tabletop analyzer offers maximum versatility to improve efficiency and reduce costs. It uses the convenient **cobas c** pack reagent format, which standardizes patient results across integrated laboratory networks.

YOUR BENEFIT

High Quality of Results

• Results you can trust the first time, and every time.

Increased Efficiency

- Comprehensive testing capabilities on a compact footprint
- Simplified processes and reduced costs.

Optimized Workflow

- Consistent results across the cobas platform.

PRODUCT CHARACTERISTICS

First Class Performance

- More than 120 assays and applications available including clinical chemistry, specific proteins, TDMs, DATs and whole blood HbA1c.

Good Fit for Labs Processing 50 to 400 Samples/day

- Throughput of up to 400 tests/hour
- Compact benchtop system for labs with limited floor space.

High System Reliability

- Robust system design
- Clot detection and pipetting safeguards.

greater consolidation by integrating analytical systems with Roche pre-analytical and post-analytical systems.

Roche flexible and harmonized solutions allow every laboratory to increase its efficiency without adding complexity.

Stand-alone and connected solutions meet differing needs, from sample entry to archiving. High quality and predictable turnaround times are always ensured, together with proven system reliability.

Unique Reagent Concept

- Convenient and error-free handling of cobas c packs
- Economic usage with high stabilities and convenient kit sizes.

PERSONALIZED LAB AUTOMATION

Scalable Functionality that's Just Right for Your Lab

The personalized lab automation portfolio offers turnkey solutions specifically designed for laboratories which seek

Personalized Lab Automation—Stand-alone Solutions

	Tube type identification Sample volume detection Sample quality Recapping Integrated centrifugation Bulk loader module	Tube type identification Sample volume detection Sample quality Recapping Integrated centrifugation Bulk loader module
Sorting Decapping Archiving Recursive workflow	Sorting Decapping Archiving Recursive workflow	Sorting Decapping Aliquoting with barcode labeler Archiving Recursive workflow
		
cobas p 312 pre-analytical system	*cobas p 512 pre-analytical system*	*cobas p 612 pre-analytical system*

Personalized Lab Automation—Connected Solutions

MODULAR® *PRE-ANALYTICS* **EVO**	**MODULAR** *PRE-ANALYTICS* **EVO** offers integration by automation solutions, consolidation of analytics and process organization.
Cobas connection modules	**Cobas** connection modules use flexible conveyor units to connect **cobas p** 512 and **cobas p** 612 pre-analytical systems to laboratory analyzers and post-analytics.
Cobas® 8100 automated workflow series	**Cobas** 8100 automated workflow series features an intelligent sample routing workflow and prioritization system.

Personalized Lab Automation—Post-analytics Solution

cobas p 501/**p** 701 post-analytical units	**cobas p** 501/**p** 701 post-analytical units are refrigerated archiving systems enabling sample retrieval and add-on test management

COBAS P 312 PRE-ANALYTICAL SYSTEM

Pre-analytics on Small Footprint

Cobas p 312 pre-analytical system is a small footprint standalone system for decapping, sorting and archiving of sample tubes for SWA, hematology, coagulation, urinalysis and blood screening.

Product Characteristics

- Throughput: up to 450 tubes/hour
- Registration of primary samples
- Selective decapping
- Flexible sorting with tube barcode alignment:
  - Out of centrifuge buckets
  - Into and out of analyzer's target racks

- Remote access for error handling and service
- Archiving of samples (recursive workflow).

COBAS P 512 PRE-ANALYTICAL SYSTEM

Pre-analytics to Increase Efficiency

Cobas p 512 is a standalone or connectable, fully automated pre-analytical system which offers a highspeed solution for high-throughput laboratories.

PRODUCT CHARACTERISTICS

- Throughput: up to 1,100 samples/hour
- Registration of primary samples
- Selective decapping of sample tubes
- Orientation of barcode in a "good-to-read" position
- Sorting of tubes directly into analyzer's target racks
- Sorting of tubes with unreadable barcode, without test requests or with insufficient sample to default.

COBAS P 512 PRE-ANALYTICAL SYSTEM WITH SINGLE CENTRIFUGE COBAS P 471 AND BULK LOADER MODULE

- Archiving of processed samples with optional recapping
- Optional: recapper, connection to single or double centrifuge, connection to bulk loader module (BLM), tube type identification, volume detection, sample quality assessment, connection to analyzers via cobas connection modules
- Four insort drawers provide capacity for up to 600 samples, eight outsort drawers can be configured for up to 1,100 tubes and 41 sorting targets.

COBAS P 612 PRE-ANALYTICAL SYSTEM

A Complete Solution for Your Pre-analytics

Cobas p 612 pre-analytical system includes an aliquoting section with barcode labeling of the secondary tubes and a throughput of approx. 330 primary tubes/hour (depending on number and volume of aliquots).

cobas p 512 and 612 pre-analytical systems could be used as stand-alone systems or as a core part of a capable pre-analytic solution. Therefore the following optional features are available:

Connection to Centrifuges

The **cobas p** 471 and **cobas p** 671 centrifuge units offer a comprehensive and flexible front-end automation solution.

- Spinning with high g-forces

- Auto-balance function
- Flexible parameters
- Start timer.

Connection to Bulk Loader Module (BLM)

Convenient sample loading with a capacity of 600 samples reduces manual sample handling and guarantees continuous sample flow. BLM can be connected in front of the single centrifuge **cobas p** 471 (throughput up to 830 tubes per hour without centrifugation) or in front of the pre-analytical system (throughput up to 1,100 tubes/hour).

Camera Options

Smart features to detect the test volume
by infrared (IR-LLD) and to get information about sample quality to identify hemolytic, icteric or lipemic samples are available on **cobas p** 512 and **p** 612. This ensures automatic quality assessment as well as early identification of insufficient material.

Liquid level detection supports the aliquoting process – no conductive tips are needed.

COBAS P 501 AND COBAS P 701 POST-ANALYTICAL UNITS

The automated archive

PRODUCT CHARACTERISTICS

- Storage throughput: up to 400 tubes/hour (storage)
- Retrieval throughput: up to 40 tubes/hour (retrieval, without influence on storage throughput)
- Storage capacity:
  13,000 tubes <p 501>
  27,000 tubes <p 701>
- Retrieval of samples within 2 minutes after ordering

- Identification of primary sample tubes
- Automated storage, disposal and retrieval of sample tubes
- Selective recapping of tubes for storage
- Selective decapping of tubes for retrieval.

MODULAR® *PRE-ANALYTICS* EVO

Connecting Solutions of Excellence

MODULAR *PRE-ANALYTICS* EVO is a modular system for the fully automated processing of primary samples from centrifugation to archiving, including automated delivery of samples to **cobas** 6000 analyzer series and **cobas** 8000 modular analyzer series. There are 3 models, plus options and upgrades to provide the greatest flexibility. Thus, MPA EVO meets a wide range of demands with regard to sample throughput, laboratory layout, instruments connected and functionalities.

YOUR BENEFIT

Full Automation

- Sample entry, result reporting and archiving
- Reduced biohazard risks for personnel.

Consolidation of Analytics

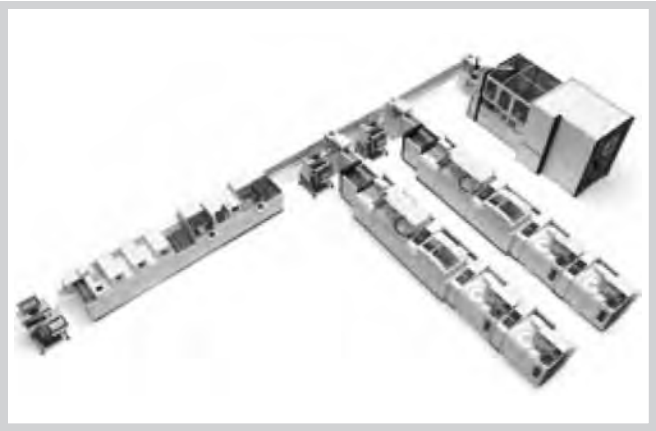
- Reduced complexity with fewer analyzers and fewer process steps.

Process Organization

- Streamlining of processes by providing IT networking of all components along with complete data and workflow management.

Integration by Automation

- Shorter, predictable TAT
- Reduction of labor-intensive processes.



Solution with all pre- and post-analytical steps including barcoded aliquots for offline analyzer

PRODUCT CHARACTERISTICS

	MPA EVO model A	*MPA EVO model B*	*MPA EVO model C*
Centrifugation	–	–	–
Decapping	–	–	–
Online aliquoting	–	–	–
Offline aliquoting	–	–	–
Barcode labelling	–	–	–
Recapping	–	–	–
Sorting/archiving	–	–	–
Primary tube workflow only	–	–	–
Online connection to: • **MODULAR**® *ANALYTICS* **EVO** • **cobas** 8000 modular analyzer series • **cobas** 6000 analyzer series • **cobas p** 501/701 post-analytical units	–	–	–
MPA options and upgrades: • Primary sample sorter (PSS) • Additional flexible sample sorter (FSS) • 2nd centrifuge	–	–	–

3 different MPA models; A,B,C plus options and upgrades for the greatest flexibility.

COBAS® CONNECTION MODULES (CCM)

The New Perspective on Sample Flow Solutions

Cobas connection modules (CCM) enable the on-line connection of **cobas** pre-analytical systems such as **cobas p** 512 and **cobas p** 612 to different analyzers, **MODULAR**® *PRE-ANALYTICS* **EVO** and post-analytical units.

CCM include pre-analytical systems as **cobas p** 512 or **cobas p** 612 with a modified outsort and various conveyors and turn-units to provide flexibility in design and on-site upgradability of the conveyor layout.

Available in different versions*, CCM offer streamlined solutions for high-throughput laboratories as well as upgrade options for systems that are already connected.

YOUR BENEFIT

Workflow Efficiency

- All features of Roche PVT pre-analytical systems available
- Connectivity to improve throughput and reduce manual steps

Reduced Complexity

- Single point of entry
- Predictable turnaround times

Flexibility

- Freely definable outsort drawers
- Easy installation and expansion

Control and Security

- Less manual handling of samples and racks
- Optional liquid level detection and sample quality assessment

PRODUCT CHARACTERISTICS

Cobas Connection Modules for High-throughput Laboratories

For high-throughput laboratories handling over 1,000 samples per hour and over 4,000 samples per day, CCM offer the possibility to have two stand-alone pre-analytical systems on-line connected to the final sample destinations.

Cobas Connection Modules the Flexible Combination with MPA EVO

Connecting **cobas p** 512 or **cobas p** 612 to MPA EVO, CCM combine the advantages of MPA EVO to the speed, functionality and flexibility of stand-alone solutions, through new pre-analytical functionalities.

COBAS® 8100 AUTOMATED WORKFLOW SERIES

3-D Intelligence in Lab Automation

Cobas 8100 automated workflow series is Roche's new modular automation solution that autonomously manages all the operational pre-analytical steps of laboratory and covers the needs of a high throughput laboratory.

Cobas 8100 features some industry-first innovations with such as sophisticated 3-D transportation system in

order to avoid bottlenecks and therefore keep turnaround times predictable.

Designed with options for connectivity to a range of instruments, **cobas** 8100 allows increased traceability throughout sample processing.

YOUR BENEFIT

High Throughput, Small Footprint

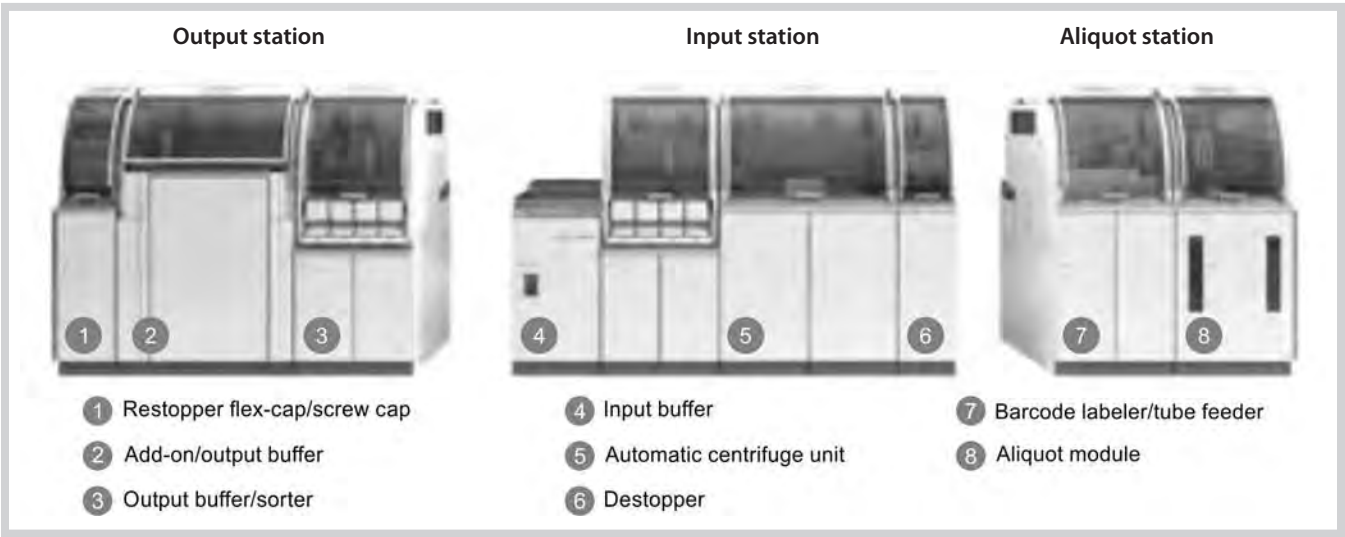
- Throughput of 1,100 samples/hour (800 including all the pre-analytical steps, 300 for additional sorting)
- Highly consolidated and connected **cobas** pre-analytical, analytical and post-analytical solution.

Intelligent Workflow

- Intelligent 3-D bidirectional transport system, to ensure predictable and short TAT
- Emergency STAT workflow.

Maximum Consolidation

- Options for connectivity to hematology, coagulation and third-party analyzers in development
- Integrated post-analytical solutions: add-on buffer module for add-on requests.



Flexible Workflow

- Primary sample workflow—if the focus is on less waste
- Aliquot workflow—if the focus is on sample integrity and parallel testing
- Mixed workflow—to combine the benefits of both.

PRODUCT CHARACTERISTICS

cobas 8100 is made up of three stations: output, input and aliquot stations. Each station can be configured according to the number of samples laboratories need to handle and the kind of workflow requested.

1. Restopper flex-cap/screw cap
2. Add-on/output buffer
3. Output buffer/sorter
4. Input buffer
5. Automatic centrifuge unit
6. Destopper
7. Barcode labeler/tube feeder
8. Aliquot module.

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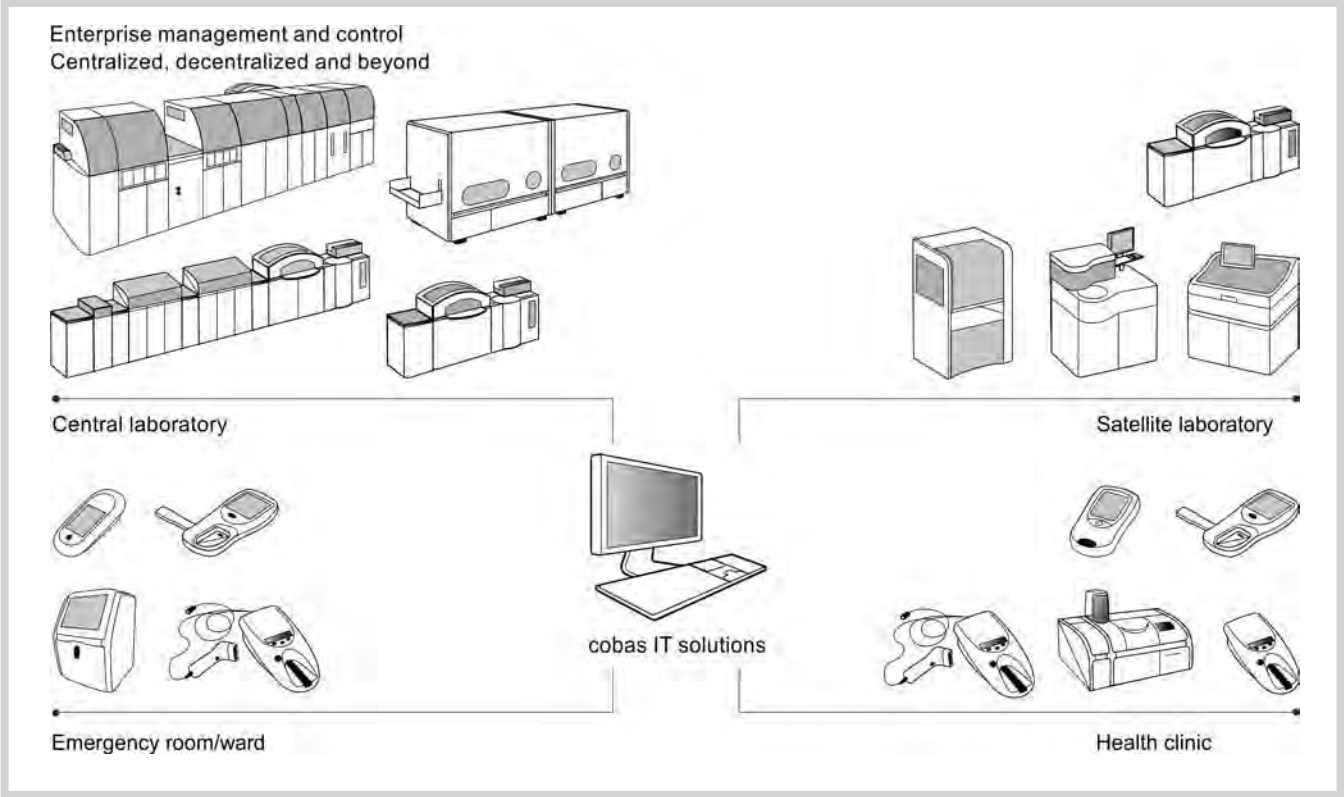
COBAS® IT SOLUTIONS

Simplicity, Flexibility and Confidence

At Roche, IT is the nucleus of our diagnostics solutions. **cobas IT** solutions give you the control you need to ensure quality and efficiency across your IVD testing enterprise. For the laboratory, an integrated IT solution reduces complexibility, improves efficiency and helps to streamline information to the respective recipients.

cobas IT solutions offer the flexibility to cover the specific needs of a healthcare enterprise today and in the future. Solutions range from workflow management in the core lab to complex, multi-discipline, multi-instrument and multi-site set-ups covering both workflow as well as LIS functionality where needed. Our POC IT solutions facilitate efficient and secure management of hospital point of care.



cobas IT Solutions

cobas IT middleware	Laboratory workflow management IT solutions that are scalable to provide full process control across multiple instruments and sites
cobas infinity IT solutions	A modular laboratory IT solution that is scalable beyond workflow management in the core lab, driving information and sample flows across various lab discliplines and sites, and capable of covering LIS functions
cobas POC IT solution	IT solution for comprehensive management of the POC program at the hospital

cobas IT solutions enable laboratories to meet increasing quality and regulatory demands while efficiently managing complexity in a fast-changing environment.

COBAS® MIDDLEWARE SOLUTION

IT Solutions from Roche for Your Core Laboratory

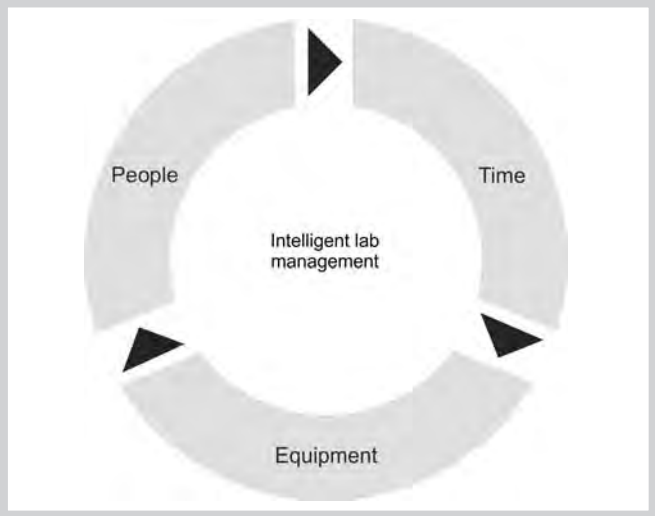
Cobas IT middleware is the workflow manager for your laboratory, consolidating **cobas** instruments, third-party instruments and host systems to enable efficient sample workflows. Different IT solutions from Roche are available to meet regional customer needs (**cobas IT** middleware, **cobas infinity** IT solutions and **cobas IT** 3000 application).*

The intuitive automated validation and quality control tools reduce operator intervention, while allowing laboratory production to be monitored through real-time dash-boards.

YOUR BENEFIT

Effective Use of Your Resources

- Manage your laboratory instruments and the people that use them from a single application
- Expert system allows you to focus on critical information.



Mapping your lab organization in a flexible way

Improved Quality Performance

- High level of traceability and transparency through audit trail for each sample
- Support to achieve compliance with regulations.

Easy Accessible Management Information

- Task-oriented for proactive exception management
- Sample archive management for automated or manual post-analytical phase.

Saving Time and Reducing Duplication of Effort

- Configurable automated validation with multiple levels of expertise ensuring reproducible outcome
- Task-oriented and easy-to-use user interface.

Efficient Workflows for Today and the Future

- Connects multiple instruments and softwares, multiple LIS from multiple sites
- Scalable to follow the growth of your organization
- Automated or manual pre-analytics and post-analytics with complete traceability.

Helping to Improve Your Quality Processes

- Quality control management including multi-rules and drift control.

COBAS® LABORATORY INFORMATION SYSTEM

Streamline Patient Data and Information Flows

The **cobas** laboratory information system goes beyond the core laboratory workflow management, streamlining patient data and information flows across various clinical disciplines. Different Roche IT solutions are available to meet regional customer needs (**cobas IT** 5000 application, SWISSLAB system and **cobas infinity** IT solutions).*

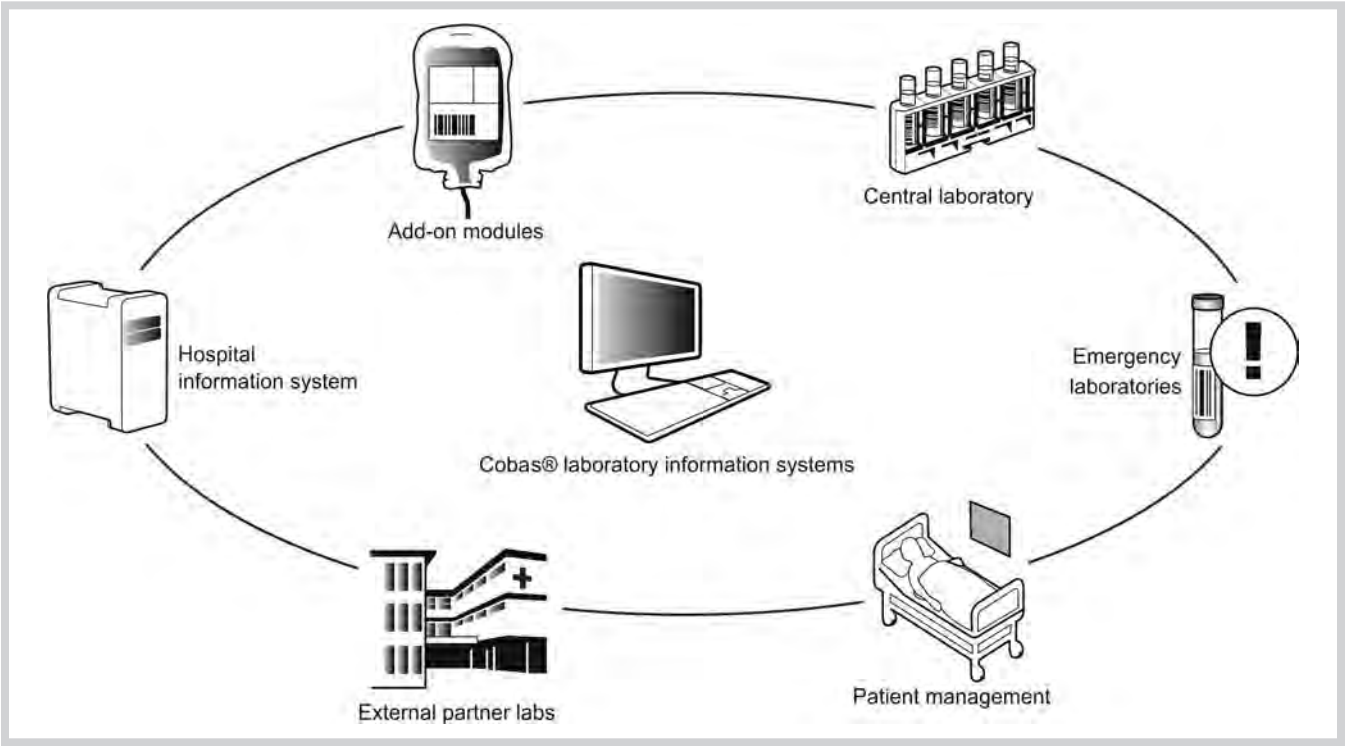
The software enhances laboratory operations by providing an end-to-end solution from orders to reports.

Data-mining capabilities allow you to explore your operational information to its maximize medical value.

YOUR BENEFIT

Allows a Patient-centric Approach

- Consolidated patient data across different clinical disciplines: chemistry, hematology, microbiology



- Access to results in any location
- Patient-based presentation of all results, including previous values
- Display of individual and cumulative findings
- Configurable plausibility data check for test results.

Provide Decision Support

- Guidance to enable clinical decision-making beyond just delivering results
- Support in-depth statistical analysis to manage laboratory efficiency in terms of KPI, such as turnaround times
- Dynamic access to data stored in the database in real time.

Demonstrate Working Excellence

- Empowers the lab as a trusted partner for the doctors
- Consistency of management across the elements of your Roche platform.

Communication with Hospital Information Systems

- Automated, real-time download of patient orders and demographics
- Wide-ranging, flexible search and sort options
- Multi-site support.

Modular Design

- Dedicated modules designed for specific workflows in specific clinical disciplines
- Allows dedicated modular usage based on a common database.

COBAS® INFINITY IT SOLUTIONS

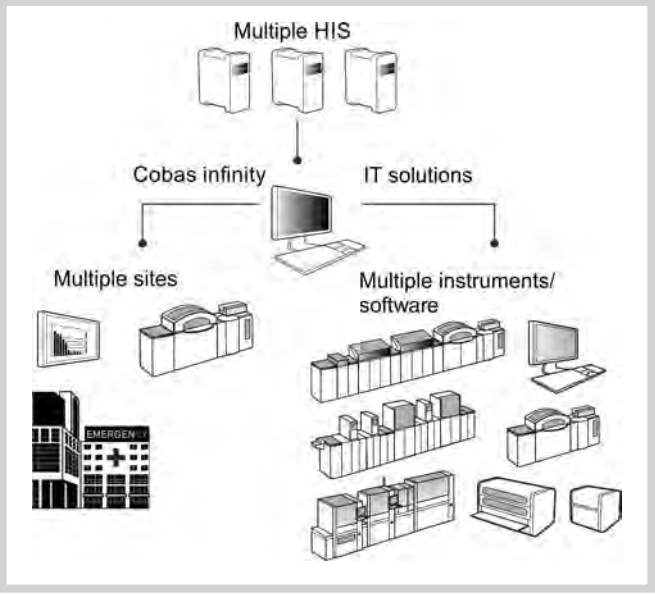
More Powerful than You can Imagine

Cobas infinity IT solutions are laboratory information solutions that go beyond workflow management of the core laboratory and cover information flows across various clinical disciplines. A modular architecture can serve as middleware, or add LIS (laboratory information system) functionality—depending on customers' needs. The fully web-based system along with modular architecture is designed to meet the specific needs of each institution flexibly and can grow together with the laboratory. To further enhance laboratory efficiency, this system integrates a consistent look-and-feel user interface and personalized work areas in which you can tailor information availability to selected user types.

YOUR BENEFIT

Simplicity—See What is Needed

- Consistent look and feel across all user interfaces helps staff learn quickly and promotes better communication in and across your laboratories.



- Personalized work area concept that enables information availability to be tailored to selected user groups, enhances efficiency and streamlines routine works in your laboratory.
- Fully web-based technology supports easy operation that keeps your laboratory up and running.

Flexibility—See What is Possible

- Modular architecture supported by fully web-based technology gives you a scalable solution that meets your current and future needs
- Comprehensive coverage of multiple laboratory disciplines and expandability from a single site to multisite networks gives you great flexibility

Confidence—See What is Important

- Dashboard shows you the key performance of the laboratory almost in real time. The visual display supports performance monitoring of your laboratory team
- Consistency through managed validation and workflow supported by intelligent rule engine aids quality management in your laboratory.

PRODUCT CHARACTERISTICS

cobas infinity IT solutions is a new powerful lab IT solution consisting of six modules. It provides a scalable solution that will meet the needs of broad market segments and has the ability to grow into further areas.

Six modules are available providing interoperability and expandability.

- **Cobas infinity** general lab module—Caters for the needs of core laboratory disciplines with personalized

work areas offering specialized functionalities in biochemistry, immunology, hematology, serology and urinalysis. Includes performance dashboards to monitor TAT clearly and directly.

- **Cobas infinity** lab flow module—Dedicated sample workflow module designed to create efficient testing across integrated solutions.
- **Cobas infinity** emergency lab module—Sub module of general lab focused on the management of emergency samples.
- **Cobas infinity** microbiology module—Paperless work environment with ease of use in mind utilizing touch-screen technology.
- **Cobas infinity** lab link module—From ordering to results across wards, physician offices, collection centers and satellite labs
- **Cobas infinity** total quality management—Suite to maintain laboratory accreditation through document, audit, issue, indicator and equipment management, along with non-conformities and subsequent corrective actions.

OVERVIEW OF SERUM WORK AREA TESTS

	cobas c *111 analyzer*	***cobas*** *® modular platform: c module*	***cobas*** *modular platform: e module*	*COBAS INTEGRA®* ***400 plus***
Anemia				
Ferritin		•	•	•
Folate			•	
Folate RBC			•	
Iron	•	•		•
Iron binding capacity—Unsaturated		•		•
Soluble transferrin receptor		•		•
Transferrin		•		•
Vitamin B_{12}			•	
Lactate Dehydrogenase	•	•		•
Bone				
Calcium	•	•		•
N-MID Osteocalcin			•	
P1NP			•	
Phosphorus	•	•		•
PTH			•	
PTH (1-84)			•	
b-CrossLaps			•	
Vitamin D total			•	

Contd...

Contd...

Cardiac				
Apolipoprotein A1		•		•
Apolipoprotein B		•		•
Cholesterol	•	•		•
CK	•	•		•
CK-MB	•	•		•
CK-MB (mass)			•	
CK-MB (mass) STAT			•	
CRP hs	•	•		•
Cystatin C		•		•
D-Dimer	•	•		•
Digitoxin		•	•	•
Digoxin		•	•	•
HDL Cholesterol direct	•	•		•
Homocysteine	•	•		•
Hydroxybutyrate Dehydrogenase		•		•
LDL Cholesterol direct	•	•		•
Lipoprotein (a)		•		•
Myoglobin		•	•	•
Myoglobin STAT			•	
NT-proBNP			•	
NT-proBNP STAT			•	
Troponin I			•	
Troponin I STAT			•	

[1] not on cobas e 411
[2] not on cobas c 311
[3] not on cobas c 701 and c 702
[4] in development
[5] launch in 2014
[6] only on cobas c 501 and c 502

Contd...

Troponin T hs			•	
Troponin T hs STAT			•	
Coagulation				
AT III		•		•
D-Dimer	•	•		•
Drugs of Abuse Testing				
Amphetamines (Ecstasy)		•		•
Barbiturates		•		•
Barbiturates (Serum)		•		•
Benzodiazepines		•		•
Benzodiazepines (Serum)		•		•
Cannabinoids		•		•
Cocaine		•		•
Ethanol		•		•
LSD		•		•
Methadone		•		•
Methadone metabolites (EDDP)		•		•
Methaqualone		•		•
Opiates		•		•
Oxycodone		•		•
Phencyclidine		•		•
Propoxyphene		•		•
Endocrinology				
Amylase—pancreatic	•	•		•
Amylase—total	•	•		•
ACTH			•	
Anti-Tg			•	
Anti-TPO			•	
Anti-TSH-R			•	
Calcitonin			•	
Cortisol			•	
C-Peptide			•	
FT3			•	
FT4			•	
hGH			•	
Hydroxybutyrate Dehydrogenase		•	•	•
Insulin			•	
Lipase	•	•	•	•
PTH STAT			•	
T3			•	
T4			•	•

Contd...

Contd...

Thyreoglobulin (TG II)			•	
Thyreoglobulin confirmatory			•	
TSH			•	
T-uptake			•	•
Fertility				
Anti Muellerian Hormone[4]			•	
DHEA-S			•	
Estradiol			•	
FSH			•	
hCG			•	
hCG plus beta			•	
LH			•	
Progesterone			•	
Prolactin			•	
SHBG			•	
Testosterone			•	
Hepatology				
Alkaline phosphatase (IFCC)	•	•		•
Alkaline phosphatase (opt.)		•		•
ALT/GPT with Pyp		•		•
ALT/GPT without Pyp	•	•		•
Ammonia	•	•		•
Anti-HCV			•	
AST/GOT with Pyp	•	•		•
AST/GOT without Pyp	•	•		•
Bilirubin—direct	•	•		•
Bilirubin—total	•	•		
Cholinesterase Acetyl		•		
Cholinesterase Butyryl		•		•
Gamma Glutamyl Transferase	•	•		•
Glutamate Dehydrogenase		•		•
HBeAg			•	
HBsAg			•	
Lactate Dehydrogenase	•	•		•
Infectious diseases				
Anti-HAV			•	
Anti-HAV IgM			•	
Anti-HBc			•	
Anti-HBc IgM			•	
Anti-HBe			•	
HBeAg			•	

Contd...

Contd...

Anti-HBsAg			•	
HBsAg			•	
HBsAg confirmatory			•	
HBsAg quantitative			•	
Anti-HCV			•	
Chagas[4]			•	
CMV IgG			•	
CMV IgG Avidity			•	
CMV IgM			•	
HIV combi PT			•	
HIV-Ag			•	
HIV-Ag confirmatory			•	
HSV-1 IgG			•	
HSV-2 IgG			•	
HTLV 1 and 2[4]			•	
Rubella IgG			•	
Rubella IgM			•	
Syphillis[5]			•	
Toxo IgG			•	
Toxo IgG Avidity			•	
Toxo IgM			•	
TPLA (Syphilis)		•[6]		
Inflammation				
Anti-CCP			•	
ASLO		•		•
C3c		•		•
C4		•		•
Ceruloplasmin		•		•
CRP (Latex)	•	•		•
Haptoglobin		•		•
IgA		•		•
IgE			•	
IgG		•		•
IgM		•		•
Immunglobulin A CSF		•		
Immunglobulin M CSF		•		
Interleukin 6		•		
Kappa light chains		•		•
Kappa light chains free		•[3]		•
Lambda light chains		•		•
Lambda light chains free		•[3]		•

Contd...

Contd...

Prealbumin		•		•
Procalcitonin		•	•	
Rheumatoid factor		•		•
a1-Acid Glycoprotein		•		•
a1-Antitrypsin		•		•
Metabolic				
Bicarbonate (CO_2)	•	•		•
Calcium	•	•		•
Chloride	•	•		•
Fructosamine		•		•
Glucose	•	•		•
HbA1c (hemolysate)	•	•[3]		•
HbA1c (whole blood)	•	•		•
Insulin		•	•	•
Lactate	•	•		•
Magnesium	•	•		•
Potassium	•	•		•
Sodium	•	•		•
Total Protein	•	•		•
Triglycerides	•	•		•
Triglycerides Glycerol blanked		•		
Vitamin D			•	
Oncology				
Acid phosphatase		•		•
AFP			•	
CA 125			•	
CA 15-3			•	
CA 19-9			•	
CA 72-4			•	
Calcitonin			•	
CEA			•	
Cyfra 21-1			•	
hCG plus beta			•	
HE4			•	
Kappa light chains free		•[3]		•
Lambda light chains free		•[3]		•
NSE			•	
proGRP			•	
Oncology				
PSA free			•	
PSA total			•	

Contd...

Contd...

SCC[5]			•	
S-100			•	
Thyreoglobulin (TG II)			•	
Thyreoglobulin confirmatory			•	
β2-Microglobulin		•		
Renal				
Albumin (BCG)	•	•		•
Albumin (BCP)		•		•
Albumin immunologic	•	•		•
Creatinine (enzymatic)	•	•		•
Creatinine (Jaffe)	•	•		•
Cystatin C		•		•
Potassium	•	•		•
PTH			•	
PTH (1-84)			•	
Total Protein	•	•		•
Total Protein, Urine/CSF		•		•
Urea/BUN	•	•		•
Uric acid	•	•		•
α1-Microglobulin		•		•
β2-Microglobulin		•		
Therapeutic drug monitoring				
Acetaminophen (Paracetamol)		•		•
Amikacin		•		•
Carbamazepine		•		•
Cyclosporine A		•	•	•
Digitoxin		•	•	•
Digoxin		•	•	•
Everolimus[4]			•	
Gentamicin	•	•		•
Lidocaine	•			•
Lithium	•	•		ISE
ISE	•	•		•
Mycophenolic acid	•	•		•
NAPA	•	•		•
Phenobarbital	•	•		•
Phenytoin	•	•		•
Primidone	•	•		•
Procainamide	•	•		•
Quinidine	•	•		•
Salicylate	•	•		•

Contd...

Sirolimus[4]	•		•	
Tacrolimus	•		•	
Theophylline	•	•		•
Tobramycin	•	•		•
Valproic acid	•	•		•
Vancomycin	•	•		•
Women's health				
Anti Muellerian Hormone[5]			•	
AFP			•	
cobas c 111 analyzer			•	
cobas® modular			•	
platform: c module			•	
cobas modular			•	
platform: e module			•	
COBAS INTEGRA® 400 plus			•	
b-Crosslaps			•	
Estradiol			•	
FSH			•	
free ßhCG			•	
hCG			•	
hCG plus beta			•	
hCG STAT			•	
HE4			•	
LH			•	
N-MID Osteocalcin			•	
PAPP-A			•	
PlGF			•	
sFlt-1			•	
P1NP			•	
Progesterone			•	
Prolactin			•	
SHBG			•	
Testosterone			•	
CMV IgG			•	
CMV IgG Avidity			•	
CMV IgM			•	
Rubella IgG			•	
Rubella IgM			•	
Toxo IgG			•	
Toxo IgG Avidity			•	
Toxo IgM			•	

Contd...

ECL—UNIQUE IMMUNOASSAY TECHNOLOGY

Still Light Years Ahead

ECL (ElectroChemiLuminescence) is Roche's technology for immunoassay detection. Based on this technology and combined with well-designed, specific and sensitive immunoassays, Elecsys® delivers reliable results. The development of ECL immunoassays is based on the use of a ruthenium complex and tripropylamine.

The chemiluminescence reaction for detection of the reaction complex is initiated by applying a voltage to the sample solution resulting in a precisely controlled reaction. ECL technology can accommodate many immunoassay principles while providing superior performance.

YOUR BENEFIT

Rapid Response Times

- 93 % of assays with 18 min. assay time or less
- 9 min. STAT applications for emergency samples.

Wide Measuring Range

- Linear signal response over six orders of magnitude.

Low Sample Volume

- High analytical sensitivity allows low sample volumes
- Patient-friendly 10–50 µL per test.

Controlled Reaction

- High on-board stability and long shelf-life due to highly stable constituents.

Precision and Sensitivity

- Superior low-end detection limits
- Excellent precision over the entire measuring range.

PRODUCT CHARACTERISTICS

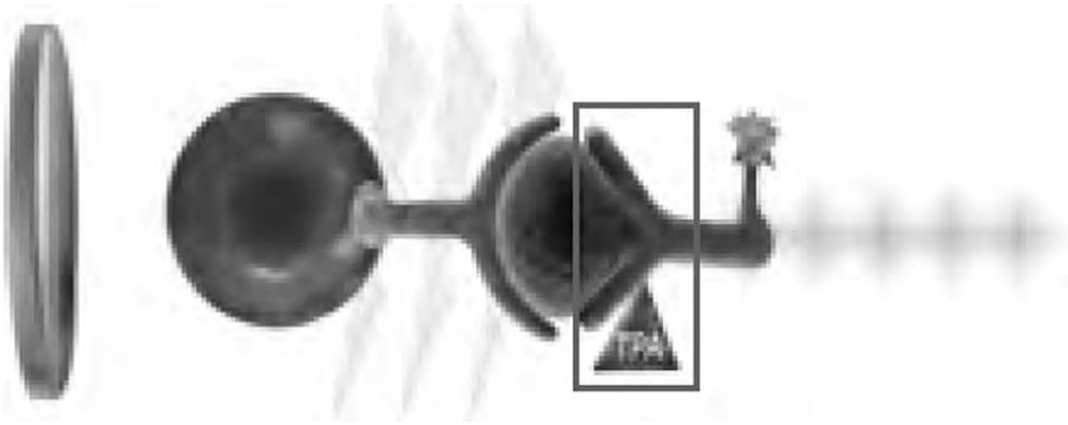
ECL is a highly innovative technology with distinct advantages

- Extremely stable non-isotopic label for long onboard stability and economic use of reagents
- High sensitivity for patient-friendly low sample volumes and fast results due to short turnaround times
- Broad measuring range for fewer repeats and a streamlined workflow
- High precision over the entire measuring range for reliable results
- Applicable for the detection of all analytes for a broad assay menu including innovative markers.

Elecsys® Diagnostic Markers with Advanced Assay Design

- Robustness against interference (e.g. HAMA) due to a multidimensional approach: blocking proteins,

ElectroChemiLuminescence (ECL) Technology



fragmented catcher or tracer antibodies or chimeric antibodies
- Reference-traceable results with high lot-to-lot stability allow accurate long-term monitoring
- Unique reagent concept with ready-to-use, fail-safe and convenient reagent packs (**cobas e** pack) for consistent handling
- Consistently precise results across **cobas**® immunochemistry platforms based on standardized reagents and low inbuilt variability.

TECHNOLOGY FOR HOMOGENEOUS IMMUNOASSAY DETECTION

Integrate Specific Protein Testing into Your Routine

Turbidimetry setting new standards: Consolidation without compromise

The testing of "specific proteins" continues to be one of the key routines in laboratories due to their wide-ranging clinical utility.

In the past, specific proteins were analyzed using a variety of specialized methods, such as radial immunodiffusion, immunoelectrophoresis or using dedicated nephelometers. This incremental investment and the resulting additional costs, handling complexity and reductions in throughput were accepted due to the perceived benefits in performance offered by these methods.

Today, specific protein determinations are frequently carried out on consolidated, random-access clinical chemistry systems using turbidimetric technology. Routine efficiencies such as reduced turnaround times are thereby achieved for these parameters.

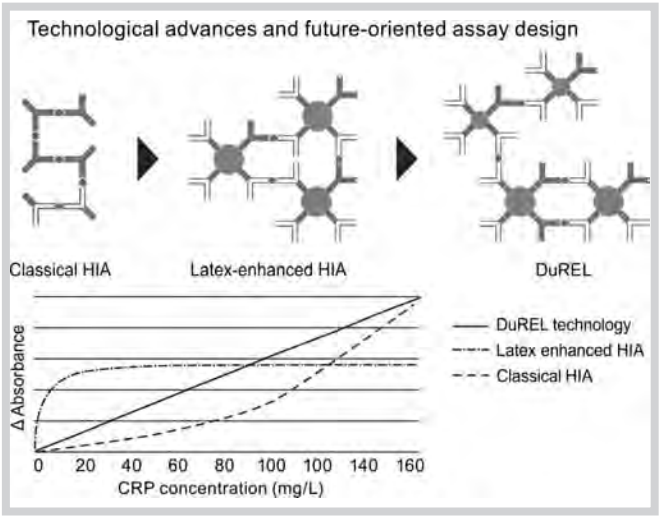
YOUR BENEFIT

Efficiency and Accelerated Result Reporting

- High throughput without the associated cost of a dedicated instrument for protein assays
- High sample throughput capability and no sample split
- Most efficient assay usage with high onboard stability and low calibration frequency.

Consolidation without Compromise

- Broadest specific protein menu on a fully consolidated platform including open channel offering
- Broad system platform portfolio for every lab size with standardized reagents across the platforms.



Product Characteristics

- Turbidimetry is Roche's technology for homogeneous immunoassay detection. Continuous development of the classical antigen-antibody assay design to the patented DuREL technology forms the basis for high sensitivity and broad dynamic range detection.
- The use of bichromatic wavelengths in spectrophotometry in conjunction with the measurement of a sample blank minimizes interference effects.

ELECSYS® HBsAg II QUANT

A Powerful Tool for Therapy Monitoring

Hepatitis B virus (HBV) accounts annually for 1 million deaths worldwide. After HBV infection, the surface antigen (HBsAg) is the first immunological marker detectable in serum. An important goal in therapy of HBV infections is the clearance of HBsAg, which is associated

with complete and definitive remission of the activity of chronic hepatitis B and an improved long-term outcome. HBsAg levels decline under treatment with peginterferon α-2a in sustained viral responders but not in relapsers or nonresponders.

YOUR BENEFIT

Optimized Management of Chronic Hepatitis B Patients

- Via the combination of HBV DNA and HBsAg quantification (see also Chapter Molecular Diagnostics).

Allows a Response-guided Therapy

- For interferon-based treatment (e.g. PEGASYS®) of chronic hepatitis B patients.

Markers for Risk Prediction

- Of cirrhosis and hepatocellular carcinoma and accurate identification of inactive carriers.

Enhanced Convenience

- Minimization of retesting due to broad linear measuring range, onboard dilution, "8 weeks" onboard stability

Maximal Reliability

- Accurate results, elimination of pipetting errors, validated with all genotypes.

Optimized for Clinical Decision Making

- Linear range reflecting relevant HBsAg titers, excellent precision, traceable to WHO second international standard for HBsAg.

Monitoring PEG-IFN
• **HBeAg-positive:** No decline in HBsAg level or levels >20,000 IU/mL at week 12 are associated with low probability of anti-HBe seroconversion (stopping rule) • **HBeAg-negative:** No HBsAg decline and <2 log10 IU/mL decline in HBV DNA level at week 12 predicts non-response (stopping rule)
Untreated inactive carriers
• HBV inactive carriers identified by persistently normal ALT levels, HBV DNA <2,000 IU/mL and HBsAg levels <1,000 IU/mL
Monitoring NAs
• A decline of HBsAg in HBeAg-positive patients may predict subsequent HBeAg or HBsAg clearance

PRODUCT CHARACTERISTICS

- Assay time: 18 min.
- Measuring range: 0.05—52,000 IU/mL
- Sample volume: 50 µL
- Intermediate imprecision: **cobas e** 411, E2010: 5.6 % **cobas e** 601/**cobas e** 602, "E170:4.9–9.6%"
- Onboard stability: 8 weeks

EASL HBV Management Guidelines Update 2012[1]

For the first time clinical practice guidelines have incorporated recommendations on HBsAg quantification in treated and non-treated chronic HBV patients:

ELECSYS® HIV COMBI PT 4TH GENERATION (Ag+Ab TEST)

Designed for Early Detection of HIV Infection

The human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome

The HBV Portfolio: Covering all Stages of Hepatitis B

				Response-guided therapy	
Prevention	*Diagnosis*	*Risk assessment*	*Therapy initiation*	*Therapy*	*Monitoring and prognosis*
Elecsys Anti-HBs (vaccine response)	• Elecsys HBsAg II	• Elecsys HBsAg II quant	• Elecsys® HBeAg	• PEGASYS	• **Elecsys HBsAg II quant**
	• ALT	• ALT	• ALT		• COBAS AmpliPrep/COBAS TaqMan HBV DNA
	• Elecsys® Anti-HBc (IgM and total)	• COBAS® AmpliPrep/COBAS® TaqMan® HBV DNA	• COBAS AmpliPrep/ COBAS TaqMan HBV DNA		• Elecsys HBeAg and Anti-HBe
	• Elecsys Anti-HBs				

[1] European Association for the Study of the Liver. J Hepatol. 2012;57: 167–185.

(AIDS), belongs to the family of retroviruses. HIV can be transmitted through contaminated blood and blood products, through sexual contact or from a HIV infected mother to her child before, during and after birth. Reliable screening and diagnosis constitutes a crucial aspect of the global strategy for reducing the human and financial burden of HIV transmission.

With the Elecsys HIV combi PT assay, the HIV-1 p24 antigen and antibodies to HIV-1 and HIV-2 can be detected simultaneously in one determination. This leads to improved sensitivity and, therefore, a shorter diagnostic window as compared to anti-HIV assays. The assay uses recombinant antigens derived from the env- and pol-region of HIV-1 (including group O) and HIV-2 to determine HIV-specific antibodies. Specific monoclonal antibodies are used for the detection of HIV-1 p24 antigen. This includes an automated sample pretreatment step with incubation with a detergent agent in order to lyse HIV virions and maximize exposure of the HIV p24 antigen to increase sensitivity.

YOUR BENEFIT

Earlier Detection of Infection

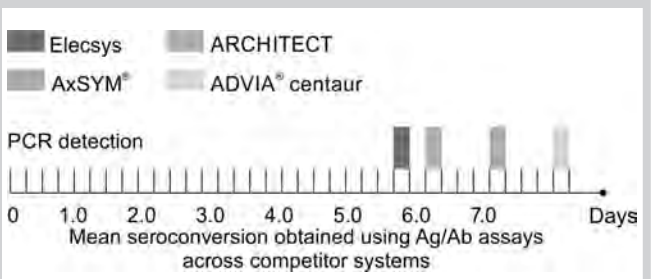
- Due to improved sensitivity by lysis of the virus using a pre-treatment (PT) step.

Compliant with Recent International Guidelines

- Analytical sensitivity below <2.0 IU/mL.

Robust to Viral Change

- Multiple target concept to ensure excellent inclusivity: special detection of subtypes and group HIV2 antibodies



Comparison of the time required until acute infection can be detected using different HIV antigen/antibody combination immunoassays.[1,2]

Cost Efficiency

- High clinical specificity reduces the need for repeat testing

PRODUCT CHARACTERISTICS

Elecsys® HIV Combi PT Test Characteristics

- Indications: Diagnostic use and for screening of blood donations
- Fast results: 27 min.
- Analytical sensitivity: 2.0 IU/mL
  Human immunodeficiency virus type 1 (HIV-1 p24 antigen)—1st International Reference Reagent 1992, code 90/636.

[1] Schmitt U, van Helden J, Hebell T, Schennach H, Mühlbacher A, Bürgisser Pet al. Poster presented at 6th International AIDS Society Conference, Rome, Italy; 2014. Available at: http://pag.ias2011.org/EPosterHandler.axd?aid=2370

[2] Mühlbacher A et al. Performance evaluation of a new fourth gen. HIV combination antigen-antibody assay. Med. Microbiol. Immunol. DOI: 2012;10.1007/s00430-012-0250-5.

- Sample material:
  - Serum, standard or separating gel tubes
  - Plasma, Li-heparin, K2 EDTA, K3 EDTA, sodium citrate, CPDA or Li-heparin plasma tubes containing separating gel
- Low sample volume: 40 µL
- Clinical sensitivity: 100 % (n = 1,532) HIV-1 group M, O and HIV-2
- Clinical specificity
  - Blood donors: 99.88% (95 % CI: 99.77 – 99.94) (n=7,343)
  - Samples from unselected daily routine, dialysis patients and pregnant women: 99.81 % (95 % CI: 99.47 – 99.90) (n=4,103).

THE SYPHILIS ASSAYS

Fully Automated Testing Panel for Complete Assessment of the Disease Syphilis

Syphilis is mainly transmitted sexually caused by the intracellular Gram-negative spirochete bacterium Treponema pallidum subspecies pallidum. It can also be transmitted from mother to fetus during pregnancy or at birth, resulting in congenital syphilis. Syphilis facilitates the acquisition of HIV.

Syphilis has been called the great imitator. The disease can be very difficult to diagnose early in its presentation. It can be confused with other dermal diseases and continue unnoticed for many years as latent syphilis. However, if diagnosed in the early stages, syphilis can be successfully treated and congenital syphilis prevented. Roche offers an automated panel of 3 assays for efficient and reliable determination of syphilis infections.

YOUR BENEFIT

- Consolidation of STD assays on the Elecsys® platform
- Fully automated and integrated with other tests in the TORCH and blood safety solutions portfolios
- Treponemal test suitable for screening in the general population, pregnant women and blood donations
- Complements the Mediace Rapid Plasma Regin (RPR) and *T. pallidum* Latex Agglutination (TPLA) assays also available from Roche, allowing reliable detection using recommended diagnostic algorithms for syphilis

Screening	*Diagnosis*	*Treatment*	*Treatment monitoring*
• Syphilis	• Syphilis		• RPR
• TPLA	• TPLA		
• RPR	• RPR		

Panel for the complete assessment of the syphilis patient.
Screening, diagnosis, and activity monitoring of the disease.

ELECSYS® SYPHILIS IMMUNOASSAY

Confidence in all Stages of Treponemal Infection

The Syphilis immunoassay has been designed using the latest recombinant thermostable-antigen technology, to achieve unprecedented high sensitivity and sensibility performance.

YOUR BENEFIT

Designed for High Sensitivity

- High sensitivity minimizes the probability of missing new infections.

Cost Efficiency

- High specificity reduces the need for re-testing.

Clear Results Interpretation

- Due to clear, cut-off separation of positive and negative results.

Efficient Use of Sample Volume

- Maximizes the chance to order all the tests required from the same sample.

PRODUCT CHARACTERISTICS

- Sample material: Serum and plasma, Li-heparin, K2 EDTA, K3 EDTA, sodium citrate, CPDA or Li-heparin plasma tubes containing separating gel
- Sample volume:10 µL
- Assay time: 18 minutes.

Test Format

- IgM/IgG (Three antigens: TpN15, TpN17, TpN47)
- Clinical sensitivity: 100 % (n=924)
- Clinical specificity: 99.88 % (n=8079)
- Blood donors: 99.93 % (n=4579)
- Routine samples: 99.80 % (n=3500).

ELECSYS® TORCH PANEL

Reliable Screening for Early Diagnosis

Infections with Toxoplasma gondii, rubella virus, cytomegalovirus (CMV) and herpes simplex virus (HSV) are especially risky during pregnancy. Prenatal diagnosis of such infections is important and demands assays of outstanding quality and reliability.

Opportunistic infections with Toxo and CMV can also have severe consequences for immunodeficient patients. A combination of high clinical sensitivity and specificity is therefore essential.

YOUR BENEFIT

High Efficiency

- Consolidation of TORCH panel on cobas immunology analyzers.

Early Detection

- Allows early management of acute congenital infections.

Fewer Confirmation Tests and Fewer Reruns

- Due to highly specific assays.

Fast Reporting

• Results in less than 20 minutes.

Product Characteristics

Roche has been continuously developing innovative TORCH assays. Based on recombinant antigens and specific assay formats such as μ-capture and DAGS (double antigen sandwich), these assays combine high clinical sensitivity and specificity.

Elecsys® CMV IgM, IgG and IgG Avidity

- Designed to detect all suspect primary infections
- Less sensitive to persistent IgM antibodies
- Prevents cross reactivity with other herpes viruses.

Elecsys® HSV-1 IgG and HSV-2 IgG

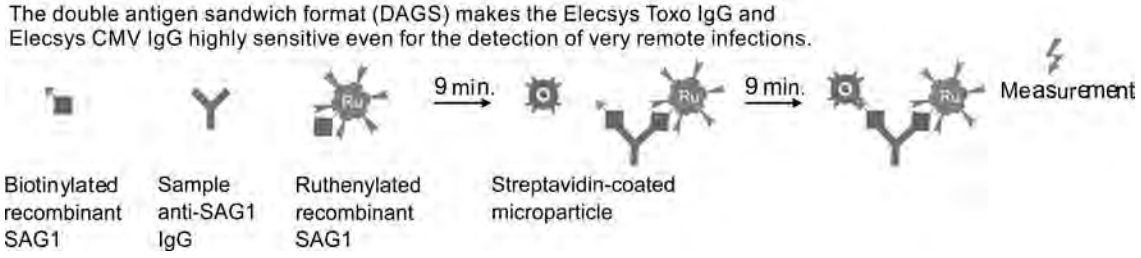
- Identification of silent carriers of Herpes simplex virus infection
- Type-specific assays for reliable differentiation between HSV-1 and HSV-2 (two Elecsys® HSV IgG assays available).

Rubella IgM and IgG

Clearly discriminates between an acute
and a remote infection

- Rubella IgG test ultrasensitive to remote infections
- Complemented with early detection of acute infections by the Rubella IgM test
- The combination of these assays provides an excellent tool for identifying and characterizing Rubella infections.

Test Principle: One-step double antigen sandwich (DAGS) assay (testing time 18 minutes)



- Elecsys Toxo IgM, IgG and IgG Avidity
- The Elecsys Toxo IgM assay design and respective cut-off minimize the probability of missing any new infection
- The Toxo IgG detects past infections with superior accuracy therefore immediately ruling out non-relevant cases
- Combined use of the 3 assays allows accurate determination of primary infections.

ELECSYS® TROPONIN T HIGH SENSITIVE (TnT Hs)

Improved Performance—Better Clinical Decisions

In a clinical setting consistent with myocardial ischemia, detection of a rise and/or fall in troponin is the cornerstone of myocardial infarction diagnosis. The Elecsys Troponin T hs test complies with the guidelines of ACC/ESC* and NACB/AACC** in achieving less than 10 % coefficient of variation (CV) at the 99 percentile upper reference limit of the reference population.

These requirements result in significant advantages in the diagnosis of acute coronary syndrome (ACS):

- Significantly earlier detection of a cTn increase during an acute myocardial infarction (AMI)
- Earlier rule-out and rule-in of AMI
- Increasing the number of patients correctly diagnosed with AMI, thanks to the greater sensitivity and better analytical precision
- Improving risk stratification of patients with elevated cTn levels without acute cardiac event.

YOUR BENEFIT

Guideline Compliant

- Test complies with the guidelines of ACC/ESC* and NACB/AACC**

Safe and Reliable Results

- Particularly at lower levels.

Earlier Diagnosis

- Greater sensitivity allowing the detection of more patients at risk.

High Prognostic Value for Cardiac Events

- In patients with renal failure.

Early Identification

- Of acute and chronic myocardial damage that would be not discovered at all or only later with conventional cTn assays.

Consistent Correlation

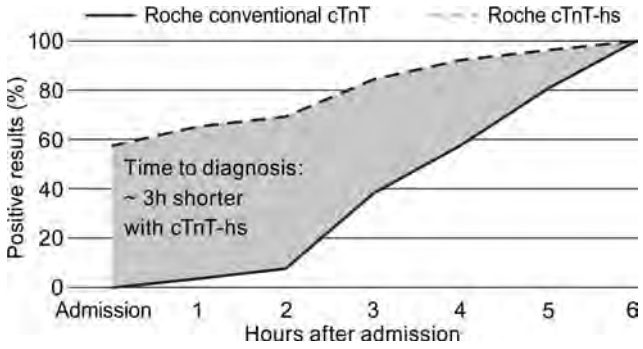
- Between POC devices for emergency testing and all **cobas**® immunoassay analyzers in the central laboratory

PRODUCT CHARACTERISTICS

- Fully automated test
- Sample material: Heparin, EDTA plasma and serum.

* ACC/ESC: American College of Cardiology/European Society of Cardiology
** NACB/AACC: National Academy of Clinical Biochemistry/Academy of the American Association for Clinical Chemistry
* Elecsys Troponin T high sensitive package insert.

Key Benefit: Earlier Diagnosis of AMI



Using the cTnT-hs assay, results in NSTEMI compared with the conventional cTnT test report:

- Time to diagnosis shorter by almost 3 hours
- 20 % more patients identified with a final diagnosis of NSTEMI.

- STAT test: 9 min.
- 99th percentile upper reference limit*: 14 ng/L (pg/mL)
- 10% CV precision: 13 ng/L (pg/mL).

Product Characteristics

- Fully automated test
- Sample material: Heparin, EDTA plasma and serum
- STAT test: 9 min.
- 99th percentile upper reference limit*: 14 ng/L (pg/mL)
- 10% CV precision: 13 ng/L (pg/mL).

KEY BENEFIT: EARLIER DIAGNOSIS OF AMI

Using the cTnT-hs assay, results in NSTEMI compared with the conventional cTnT test report:

- Time to diagnosis shorter by almost 3 hours
- 20% more patients identified with a final diagnosis of NSTEMI.

ELECSYS® NT-proBNP

A Leap Forward in the Diagnosis and Stratification of Cardiovascular Disease

Heart failure (HF) is a global health problem associated with high morbidity and mortality. Detection in its early stages and appropriate treatment are key objectives in improving quality of life. Patients with HF – especially with mild symptoms – are often not diagnosed. On the other hand, many patients with suspected heart failure are unnecessarily referred to echocardiography.

NT-proBNP is an innovative marker to improve clinical decisions. It delivers accurate data to help rule-out, rule-in, risk-stratify or monitor patients.

YOUR BENEFIT

Simplified Testing Process and Improved Efficiency of Testing

- NT-proBNP provides 72 hour room temperature stability without additional processing
- Test tube requirements allow one tube solution for all cardiac markers.

Consistent Correlation

- Between all **cobas**® immunoassay analyzers and POC devices.

Fast Diagnosis

- In cases of dyspnea; differentiation between cardiac or pulmonary causes.

Early Diagnosis of HF

- Even in early stages without symptoms.

Objectivity

- NT-proBNP concentration correlates with severity of disease.

Strong Prognosis

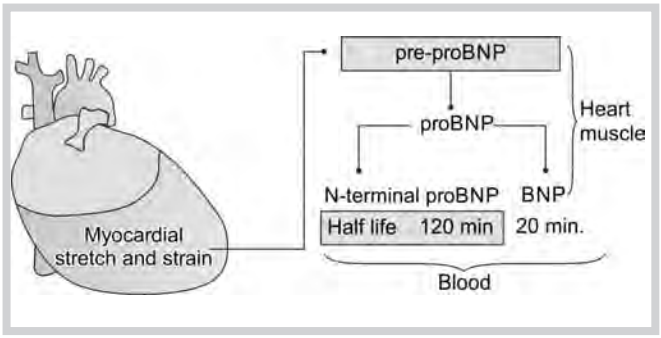
- High predictive value in cardiology risk patients.

Improved Therapy

- Aids in the evaluation of the clinical situation and optimization of therapy.

Suspicion of acute heart failure because of symptoms and signs			
Examination, ECG, X-ray and NT-proBNP			
Patient age (Years)	*NT-proBNP values (pg/mL)*		
<50	<300	300–450	>450
50–75	300–900	>900	
>75	300–1800	>1800	
Interpretation	Acute HF unlikely	Acute HF less likely, alternative causes must be considered	Acute HF likely, consider confounding factors
	NPV = 98 %		PPV = 92 %

NT-proBNP is formed by cleavage of proBNP



Product Characteristics

- Fully automated quantitative assay
- Low sample volume: 50 µL
- Fast results: 9 min. as STAT assay
- Longer sample stability: 3 days at room temperature and even longer at 4°C
- High test precision (CV 2.9 to 6.1%) coupled with a wide dynamic measuring range (5–35,000 ng/L)
- Sample material: standard serum and heparin/EDTA plasma.

ELECSYS® TUMOR MARKER PORTFOLIO

Supporting Improvements in Cancer Diagnosis and Monitoring

In the last decade, the sensible use of tumor markers and the careful interpretation of their results have led to the continual enhancement of their clinical significance. The inclusion of tumor markers in clinical management can help to provide more information for improved clinical decision-making and therefore maximize the quality of care. Nowadays, therapy management of cancer patients is guided by tumor marker monitoring based on the individual base levels before and after primary treatment. An excellent long-term assay accuracy and precision is crucial for the reliable evaluation of significant differences in tumor marker levels in cancer patients.

YOUR BENEFIT

Longitudinal Accuracy for Reliable Long-term Patient Monitoring

- High reproducibility and analytical precision over the entire measuring range, especially in lower concentration ranges
- High lot-to-lot consistency across all cobas® platforms.

Reliable Results

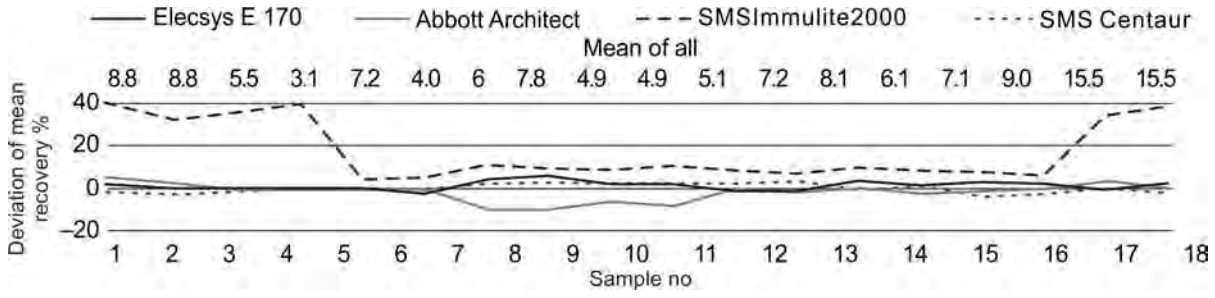
- Robustness against interference (e.g. HAMA) by blocking proteins, fragmented catcher or tracer antibodies or chimeric antibodies.

Roche Reagent and Application Portfolio for Consolidated Tumor Marker Testing

Test	*Cancer indications (primary and secondary)*	*Roche/Hitachi systems*	*COBAS INTEGRA*	*cobas e systems*	*MODULAR ANALYTICS EVO*	*cobas c systems*
AFP	Liver, testicles			•	•	
Calcitonin	Medullary, thyroid carcinoma			•	•	
CA 125	Ovary			•	•	
HE4	Ovary			•	•	
CA 15-3	Breast			•	•	
CA 19-9	Pancreatic, colorectal			•	•	
CA 72-4	Gastric, colorectal			•	•	
CEA	Colorectal, lung			•	•	
CYFRA 21-1	Non small cell lung, bladder			•	•	
Ferritin	Tumor related anemia	•	•	•	•	•
HCG	Chorion			•	•	
ß2 Microglobulin	Multiple myeloma (non-Hodgkin)	•	•		•	•
NSE	Small cell lung			•	•	
proGRP	Small cell lung			•	•	
Free PSA	Prostate			•	•	
Total PSA	Prostate			•	•	
S100	Malignant melanoma			•	•	
Anti-TG	Medullary, thyroid carcinoma			•	•	
Tg II (hs)	Medullary, thyroid carcinoma			•	•	

AFP = Alpha-fetoprotein, CA = Carcinoembryogenic antigen, CEA = Carcino-embryonic antigen,
CYFRA 21-1 = Cytokeratin fragment 19, HCG = Chorionic gonadotropin, HE4 = Human epididymal protein 4,
NSE = Neuron-specific enolase, PSA = Prostate-specific antigen

External Longitudinal Recovery Monitoring shows HIgh Lot-to-lot Consistency



Performance of Elecsys TPSA in external surveys 2010. Data compiled from external quality scheme. (2010). Data on file.
Recovery monitoring data for other markers are available on request.

- Standardized to international standards or, if no standard available, traceable to a commonly accepted methodology.

Operational Efficiency

- High degree of system automation
- Less retesting due to high precision and wide measuring ranges
- Broad tumor marker menu with specialties such as CA72-4, S100, NSE, CYFRA 21-1, HE4, and ProGRP
- Outstanding degree of SWA consolidation with >210 parameters for clinical chemistry and immunochemistry.

Complete Diagnostic Picture with Personalized Healthcare

- Coverage of the whole chain from diagnostics, therapy decision and monitoring by Roche's broad menu in Tissue Diagnostics, Elecsys tumor markers and the oncology portfolio in molecular diagnostics.

ELECSYS® HE4

An Oncological Biomarker Improving Ovarian Cancer Care

Worldwide, ovarian cancer is the second leading cancer in women and the fourth most common cause of death from cancer. It is a gynecological disease with one of the highest mortality rates.

The more the disease has progressed, the lower the survival rate is and unfortunately most cases of ovarian cancer are detected in later stages where the chances of cure are rather low.

In the early stages of ovarian cancer, symptoms are unspecific and cause little, if any, discomfort. Therefore, new methods and biomarkers which can help in diagnosing this disease at an earlier stage are highly desirable. The biomarker HE4 (human epididymal protein 4) together with the marker CA125 can play a very important role here.

YOUR BENEFIT

Early Marker with Increased Sensitivity for Supporting the Diagnosis of Epithelial Ovarian Cancer (EOC) Diagnosis

- As a single tumor marker, HE4 had the greatest sensitivity (at a specificity of 75 %) in detecting of EOC, especially in the early non-symptomatic stage.

High Discrimination Between Benign Ovarian Masses/Cysts and Ovarian Cancer

- The combination of HE4 and CA 125 shows the greatest accuracy in differentiating between patients with EOC vs. those with benign pelvic masses.

Improved Monitoring of Ovarian Cancer Recurrence and Progression

HE4 correlates with the recurrence status in women with a diagnosis of EOC and is an earlier marker for recurrence than CA 125.

Reliable Results with Efficiency

- Excellent precision and lot-to-lot consistency
- Comprehensive tumor marker menu available on all **cobas** platforms.

ROMA Increases the Diagnostic Value of the Dual Marker Combination HE4 and CA 125

- Measured values of HE4 and CA 125 can be combined in an algorithm called ROMA—which takes into account the menopausal status of the woman. Several published studies show that ROMA helps in the triage of pre- and postmenopausal women suspected of having ovarian cancer. Moore et al. (2009) found that the algorithm correctly classified 94% of women with epithelial ovarian cancer.[1] This high accuracy in stratifying women with low or high risk for EOC contributes to better diagnosis, treatment and outcome.

[1] Moore RG, et al. (2009). A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass. Gynecologic Oncology. 2009;112: 40-46.

Pelvic mass: Risk of Ovarian Malignancy Algorithm (ROMA)			
Pre-menopausal		*Post-menopausal*	
PI = –12.0 + **2.38***LN[HE4] + **0.0626***LN[CA125] PI = predictive index		PI = -8.09 + **1.04***LN[HE4] + **0.732***LN[CA125]	
*ROMA-value [%] = exp(PI) / [1 + exp(PI)] * 100*			
(exp(PI) = ePI)			
<11.1% low risk	*11.4% high risk*	*<29.9% low risk*	*29.9% high risk*
Calculation of the ROMA-values for pre-and postmenopausal women and individual cut-points for the Elecsys assays to separate between low and high risk patients.			

PRODUCT CHARACTERISTICS

- Assay time: 18 minutes.
- Sample material: Serum collected using standard sampling tubes or tubes containing separating gel Li-heparin plasma, K2-EDTA and K3-EDTA plasma
- Sample volume: 10 µL
- Limit of detection: 15 pmol/L
- Measuring range: 15–1,500 pmol/L
- Intermediate imprecision **cobas e** 411 analyzer, Elecsys 2010 analyzer: 2.7–4.3%
- **Cobas e** 601/**e** 602 modules, E170: 2.6–3.4%
- Repeatability **cobas e** 411 analyzer, Elecsys 2010 analyzer: 1.3–1.8%
- **Cobas e** 601/**e** 602 modules, E170: 1.5–1.9%

ELECSYS® PROGRP

Crucial Information for Differential Diagnosis in Lung Cancer

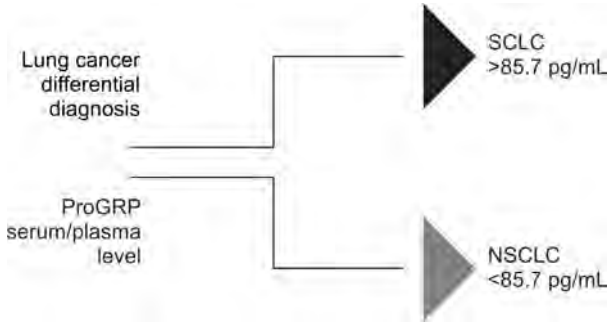
Pro-gastrin releasing peptide (ProGRP) is a tumor marker with benefits for the management of lung cancer patients.

Lung cancer is one of the most common cancers in the world with 1.35 million new cases diagnosed every year. The two main histological types of the disease are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). It is important to distinguish between these two subtypes as they have different treatments and prognoses. NSCLC (approx. 80 % of cases), when in the early stages, is curable with surgery. SCLC, however, is an aggressively spreading neoplasm of rapid growth that is usually only treatable with chemo- and radiotherapy.

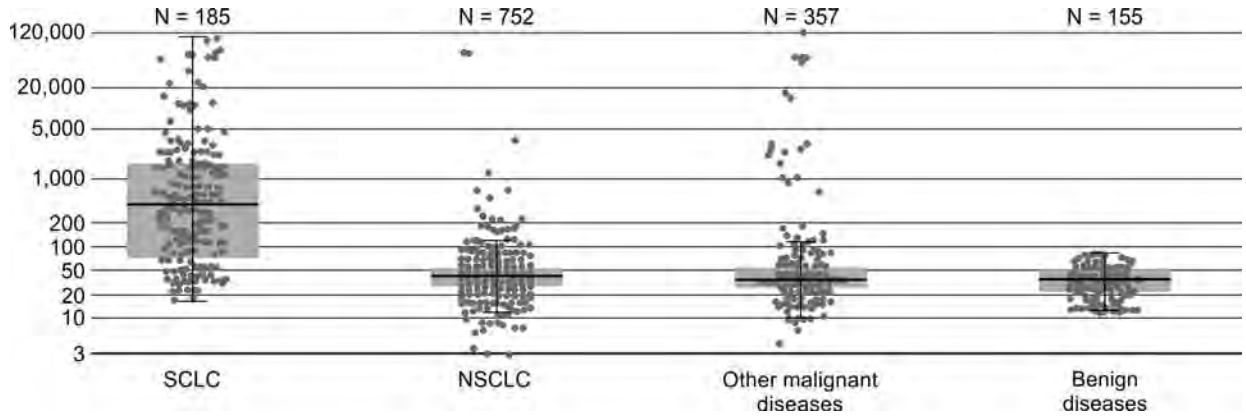
ProGRP is the tumor marker of choice for SCLC as it aids quick and decisive discrimination between SCLC and NSCLC for for faster decisions on patient treatment. ProGRP is also a tumor marker that can be used to assess response to therapy as well as to monitor recurrence of the disease.*

YOUR BENEFIT

- High sensitivity and discrimination aiding the accurate differential diagnosis of SCLC
- Excellent precision across the entire measuring range for reliable results
- Lung cancer biomarkers available on a single automated platform – CEA, CYFRA 21-1, NSE, and ProGRP
- Equivalent performance between plasma and serum for flexibility and convenience.



The 85.7 pg/mL cut-off value is based on the 95 % specificity of the NSCLC collective



Other malignant diseases include breast, ovary, prostate, renal, liver, pancreas, colorectal, gastrointestinal, carcinoid, cervical, medullary carcinoma of the thyroid, mesothelioma, neuroendocrine tumors, lymphoma, and stomach cancer. Benign diseases contain liver-, metabolic-, autoimmune and inflammatory diseases, as well as the benign lung diseases pneumonia, asthma, chronic obstructive pulmonary disease and tuberculosis.

PRODUCT CHARACTERISTICS

- Assay time: 18 minutes
- Sample material:
  - Serum collected using standard sampling tubes or tubes containing separating gel
  - Li-heparin plasma, K2-EDTA and K3-EDTA plasma
- Sample volume: 30 µL
- Limit of detection (LoD): 3 pg/mL
- Measuring range (lower end defined by LoD): 3–5,000 pg/mL.

ELECSYS® CALCITONIN

A Powerful Tool for the Diagnosis and Monitoring of Medullary Thyroid Carcinoma (MTC)

- Thyroid carcinoma is the most common malignancy of the endocrine system. In up to 10 % of all thyroid carcinoma patients a medullary thyroid carcinoma (MTC) is identified. These carcinoma produce elevated serum concentrations of calcitonin and therefore can be diagnosed with an exceptional degree of accuracy and specificity by immunoassays measuring serum calcitonin.
- The diagnostic marker calcitonin is a sensitive and specific tumor marker for the diagnosis as well as for the life-long monitoring of MTC patients after thyroid surgery.

YOUR BENEFIT

A Marker with High Specificity for MTC

- Sensitive tool for diagnosis and follow-up of MTC
- High correlation with tumor burden, supporting early detection of new or residual disease.

Elecsys Calcitonin with High Precision

- High sensitivity and precision at low end concentrations ensure improved follow-up and monitoring
- Excellent precision across the entire measuring range support accurate results.

Workflow Efficiency with the Most Complete Automated Thyroid Portfolio

- All tests required for differential diagnosis of thyroid diseases are consolidated on one platform, including routine thyroid assays and specialties such as Elecsys TgII, Elecsys Anti-Tg, Elecsys Anti-TPO and Elecsys Anti-TSHR.

Product Characteristics

- Assay time: 18 min.
- Sample material: Serum, Li-heparin plasma, K_2-EDTA plasma, K_3-EDTA plasma
- Sample volume: 50 µL
- LoB, LoD, LoQ*: 0.3 pg/mL, 0.5 pg/mL, 1 pg/mL
- Measuring range: 0.5–2,000 pg/mL

- Traceability: IRP WHO 89/620
- Total imprecision:
  - **Cobas e** 411 analyzer, E2010: 2.6–5.2%
  - **Cobas e** 601/**e** 602 modules, E170: 1.6–2.3%

ELECSYS® ANTI-TSHR

Complex Testing Simplified and Automated

Elecsys Anti-TSHR (TRAK) is a fully automated test for detection of autoantibodies to the TSH receptor.

Clinical Utility

- Detection or exclusion of Graves' auto-immune hyperthyroidism and differentiation from disseminated autonomy of the thyroid gland
- Monitoring therapy and prediction of relapse
- Assessing the risk of developing fetal hyperthyroidism in the last trimester of pregnancy.

YOUR BENEFIT

Improved Efficiency

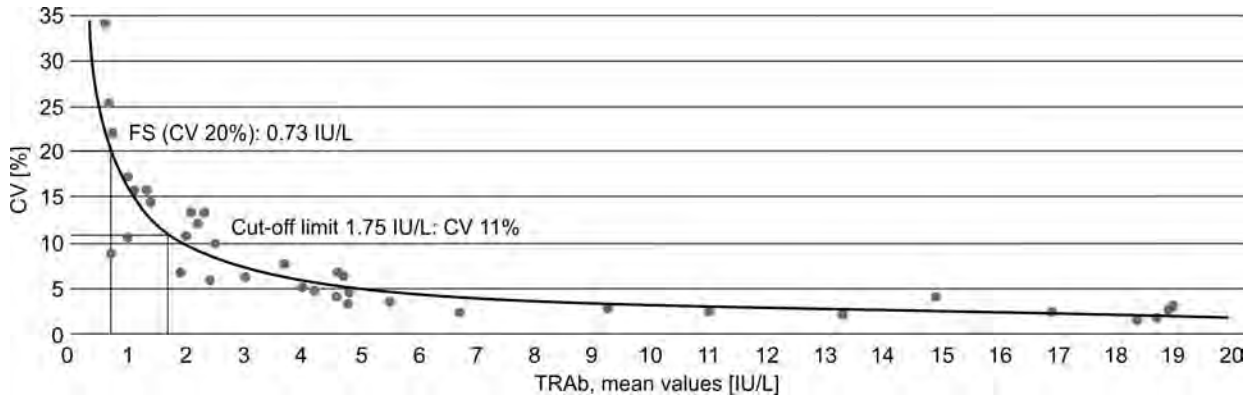
- Fully automated test for more workflow efficiency, allows for consolidation of tests required for differential diagnosis of thyroid diseases
- Rapid availability of Anti-TSHR results supports cost- and time-efficient differential diagnosis of thyroid diseases and early treatment.

High Quality Results

- Advanced assay quality based on proven and leading ECL technology
- Excellent precision across the entire measuring range
- High diagnostic value based on high sensitivity paired with high specificity.

PRODUCT CHARACTERISTICS

- Assay time: 27 minutes
- Sample volume: 50 µL
- Measuring range: 0.3–40 IU/L
- Functional sensitivity: 0.9 IU/L
- Cut-off: 1.75 IU/L
- Precision: <6 %
- Strong discrimination between positive and negative results
- Standardization: NIBSC 1st IS 90/672.



The functional sensitivity of Elecsys Anti-TSHR at approx. 0.9 IU/L is significantly below the cut-off (≥1.75 IU/L), allowing clear differentiation of pathological results.

ELECSYS® TG II

The Power to Offer More for Differentiated Thyroid Cancer (DTC) Management

The main application for thyroglobulin (Tg) testing is the post-operative follow-up of patients with differentiated thyroid carcinoma (DTC). Detectable levels of serum Tg after total thyroidectomy are indicative of persistent or recurrent DTC.

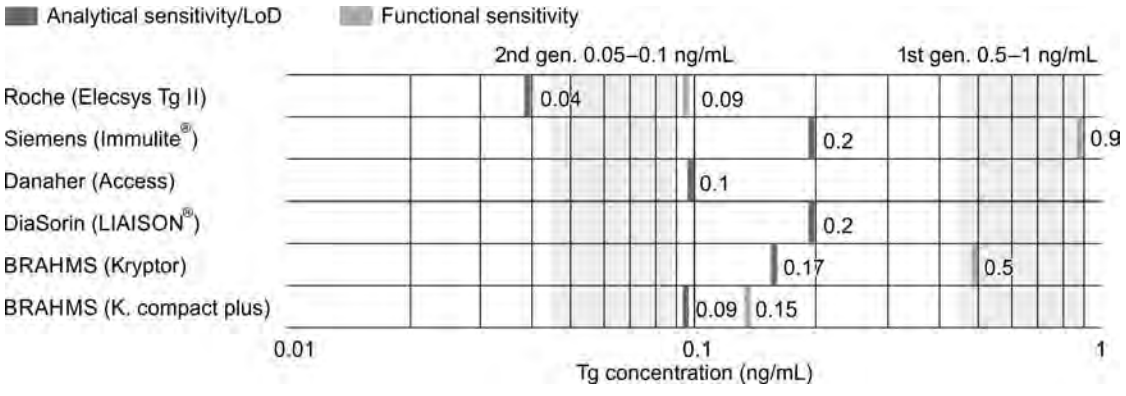
YOUR BENEFIT

Excellent Functional Sensitivity and Precision

- Improved sensitivity comes with better precision in the range around the clinical cut-off and improved negative predictive value
- Sensitive Tg assays can avoid TSH-stimulated Tg testing during follow-up in low-risk patients
- Patients with a basal Tg below the functional sensitivity of a sensitive Tg assay have a high chance of being free of disease.

High Quality Patient Results and Accurate Long-term Monitoring

- Excellent precision across the entire measuring range supports accurate results
- Lot-to-lot consistency across all cobas® platforms allows a reliable long-term patient monitoring
- Elecsys Tg II shows lower TgAb interference compared to other assays
- Higher sensitivity allows for potentially earlier detection of persistence or recurrence



The functional sensitivity of Elecsys Anti-TSHR at approx. 0.9 IU/L is significantly below the cut-off (≥1.75 IU/L), allowing clear differentiation of pathological results.

LoB = Limit of Blank; LoD = Limit of Detection;
LoQ = Limit of Quantitation with a total allowable error of ≤20 %

- Increasing concentrations of Tg (even at low concentrations) are an early and reliable indicator of recurrent disease
- Treatment is usually more successful with early detection as the tumor burden is lower.

Product characteristics

- Assay time: 18 minutes.
- Sample material: Serum, K2-EDTA plasma, K3-EDTA plasma
- Sample volume: 35 µL
- LoB, LoD, LoQ*: 0.02 ng/mL, 0.04 ng/mL, 0.1 ng/mL
- Measuring range: 0.04–500 ng/mL
- Traceability: BCR-CRM 457
- Total imprecision:
  - **Cobas e** 411 analyzer, E2010: 2.6–9.2%
  - **Cobas e** 601/e 602 modules: 4.0–5.9%

TINA-QUANT® LIPOPROTEIN (A) GEN. 2 TEST

For Accurate and Reliable Assessment of Cardiovascular risk

Cardiovascular disease (CVD) is a major health concern that continues to grow. 30 % of mortality associated with CVD occurs in individuals without increased conventional risk factors. There is thus a clinical need to expand the number of available diagnostic tools for evaluating an individual's risk of developing CVD. Numerous large-scale studies have demonstrated that the concentration of lipoprotein (a) (Lp(a)), but not the mass of Lp(a), can serve as an excellent and clinically useful risk factor for CVD.

Measuring Lp(a) levels in terms of concentration nmol/L rather than mass mg/dL provides results that are independent of the size of individual particles, which leads to a more accurate and reliable assessment of CVD risk.

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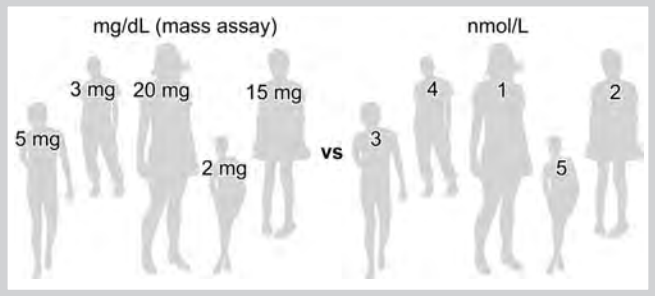


The risk of CVD correlates with the molarity of Lp(a) particles (nmol/L) and not the combined mass (mg/dl) of Lp(a) particles. Classifying patients based on the results from mass assays may lead to an incorrect assessment of CVD risk. For example, individuals with low numbers of large Lp(a) particles can display similar Lp(a) levels to individuals with high numbers of small Lp(a) particles when analyzed using mass assays, but have a lower risk of CVD

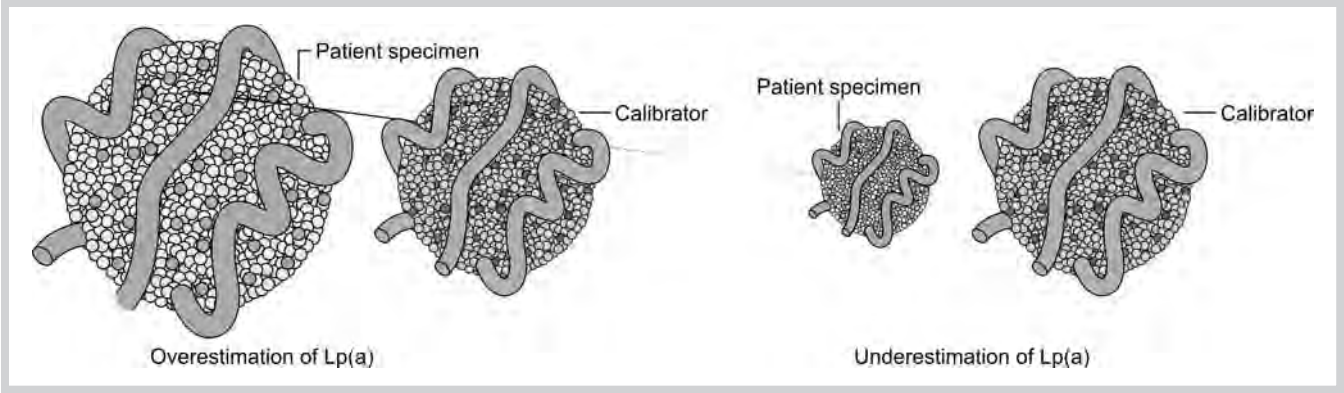
YOUR BENEFIT

- The nmol/L standardization—which is recommended by the European Atherosclerosis Society (EAS)—allows laboratories to measure the right value, which leads to a more accurate and reliable assessment of CVD risk
- Tina-quant® Lipoprotein (a) Gen. 2 shows excellent correlation to the reference method ELISA
- Cost-effective, fast, robust, easy to perform, stable over time with excellent accuracy and precision.

PRODUCT CHARACTERISTICS

- Roche follows the recommendation of the EAS to determine Lp(a) in nmol/L
- Sample material: Serum, plasma
- Measuring range: 7–240 nmol/L
- Precision (cobas c 501 module):

  Intraassay: 18.2 nmol/L = CV 5.6 %
  88.7 nmol/L = CV 2.5 %
  226 nmol/L = CV 0.8 %



Lp(a) concentrations will tend to be overestimated in samples containing particles larger than the assay calibrator and will tend to be underestimated in samples containing particles smaller than the assay calibrator

Interassay: 18.2 nmol/L = CV 8.0 %
88.7 nmol/L = CV 3.0 %
226 nmol/L = CV 1.1 %

TINA-QUANT® IMMUNOGLOBULIN A AND M CSF

For Comprehensive CSF Protein Differentiation

The measurement of intrathecal IgA/M synthesis in combination with corresponding serum levels is used as an aid to the diagnosis of different neurological diseases. Increased CSF IgA and IgM concentrations may occur because of either increased permeability of the blood-brain barrier or local/intrathecal production of IgA/IgM, or both. An elevated albumin CSF/serum ratio is an indication of disorders of the blood-brain barrier.

The results of the CSF/serum ratio for IgA/IgM and albumin, in conjunction with Reiber quotient scheme provide an aid in the diagnosis of functional blood-brain barrier disorders and/or intrathecal IgA/IgM synthesis.

YOUR BENEFIT

- Full basic CSF diagnostic panel on a consolidated platform.
- Tina-quant® IgA and IgM CSF offers excellent precision, onboard stability and calibration frequency
- Possibility of using automated software-aided Reibergram analysis (third-party product tested with Roche analyzers)
- Cost, labour and time savings through optimized workflow by offering ready-to-use reagents.

PRODUCT CHARACTERISTICS

Tina-quant Immunoglobulin A CSF

- Sample material: CSF, serum, plasma
- Measuring range:
  CSF: 0.4–25 mg/L,
  Serum, plasma: 0.1–6 g/L

- Precision (cobas c 501 module):
  Intraassay: CSF: CV 1.0–3.1 %,
  Serum, plasma: CV 1.2–3.1 %
  Interassay: CSF: CV 1.9–4.3 %,
  Serum, plasma: CV 1.3–4.2 %
- Expected values:
  CSF: 1– 3 mg/L.

These values are for guidance only.
The only relevant values are the CSF/serum ratios
Serum, plasma: 0.7–4 g/L.

For values for children and juveniles please refer to the package insert.

TINA-QUANT® HEMOGLOBIN A1C

Efficiency for the Diagnosis and Monitoring of Diabetes

HbA1c is viewed as a significant and accepted diabetic marker. For most people with diabetes, the target HbA1c is below 48 mmol/mol (6.5% HbA1c), since evidence shows that this can reduce the risk of developing diabetic complications.

In 2009 an international expert committee recommended HbA1c as a test for the diagnosis of type 2 diabetes and prediabetes. The Tina-quant assay provides a fast and precise routine HbA1c measurement for the comprehensive care of your diabetes patient.

YOUR BENEFIT

One Test for Diagnosis and Monitoring

- First HbA1c assay on the market that can be used for the diagnosis of diabetes and to identify persons at risk of developing diabetes, and for monitoring (FDA/CE).

Reliable Diabetes Management

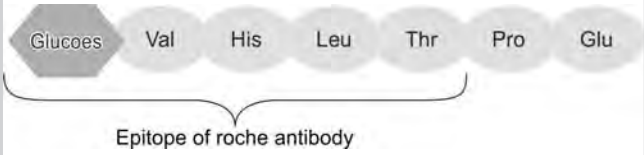
- With excellent precision and accuracy.

Uncompromised Performance

- With no interference from HbAS, HbAD, HbAD and HbAE or acetylated, carbamylated Hb and labile HbA1c.

Efficiency, Cost and Workflow Improvements

- Easy integration into routine testing for efficiency, cost and workflow improvements. Without post-analytical data review (e.g. interpretation of chromatograms).



Glycated (HbA1c) N-terminal hexapeptide and epitope recognition of the Roche HbA1c antibody for measuring the "true" HbA1c as defined by the IFCC reference system.

PRODUCT CHARACTERISTICS

- Twin test reaction technology
- Reagent lot-specific calibration
- NGSP certified and traceable to the IFCC and DCCT reference method
- Dual reporting in mmol/mol and %
- Intermediate precision (CV) <1.5%
- Whole blood and hemolysate application
- 70 % immersion depth into the primary tube for correct and reproducible recovery of fast settling whole blood samples
- FDA approved/CE.

TINA-QUANT® CYSTATIN C GEN. 2

Assess Renal Function Earlier and more Reliably

Chronic kidney disease (CKD) is an insidious disease with a dramatically increasing prevalence across the globe accompanied by a huge impact on healthcare budgets. Detecting chronic kidney disease at early stages allows for early intervention and thus has the potential to delay or even prevent the development of end-stage renal disease and related complications.

Creatinine, which has been widely used to date to assess renal function, is subject to variation due to a number of factors including age, gender, race, chronic illness, diet, and muscle mass. In addition, it doesn't detect mild kidney insufficiency since serum levels only begin to rise in CKD stage 3 when approximately 50 % of renal function is already lost ("creatinine-blind area").

Cystatin C is a marker with the ability to detect mild kidney insufficiency through subtle changes in the glomerular filtration rate (GFR). Cystatin C therefore offers additional medical value versus the use of creatinine, contributing to better patient care.

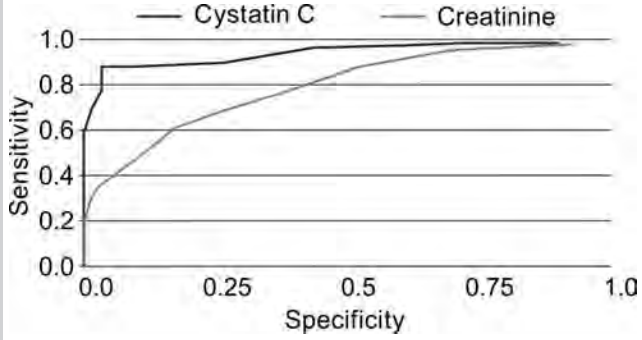
YOUR BENEFIT

- Early detection of CKD by determination of subtle changes in GFR due to high sensitivity and specificity
- Tina-quant Cystatin C is not influenced by gender, muscle mass or inflammation and therefore provides reliable results
- Tina-quant Cystatin C, together with creatinine measurement, provides detection of CKD across the complete range of renal function
- In patients with limited renal function, it allows exact dosing of medications eliminated by the kidneys
- Easy and efficient testing due to fully automated testing on all clinical chemistry analyzers from Roche and availability of a comprehensive renal diagnostics marker menu
- Traceable to ERM-DA71/IFCC.

PRODUCT CHARACTERISTICS

- Cystatin C can detect impairment of renal function in a GFR range of approx. 40–80 mL/min./1.73 m^2
- Sample material: Serum and plasma
- Measuring range: 0.4–6.8 mg/L

Highly Sensitive and Specific, Unaffected by Physical Factors



ROC analysis of cystatin C and creatinine.[1]

- Precision (cobas c 501 module):
- Intraassay: CV 0.6–1.0 %
- Interassay: CV 0.7–1.2 %
- Expected values: 20–70 years: 0.57 mg/L–1.53 mg/L

ELECSYS® PREECLAMPSIA

Advances in Diagnostics

Preeclampsia is a serious complication in pregnancy which affects both the mother and the unborn child. According to the WHO, preeclampsia is one of the leading causes of maternal and perinatal morbidity and mortality worldwide. Preeclampsia is a progressive and unpredictable disease that can only be resolved by delivery. The clinical presentation of preeclampsia and subsequent clinical course of the disease can vary tremendously, making diagnosis and assessment of disease progression difficult.

Determination of Subtle Changes in GFR is Crucial in the Early Detection of CKD

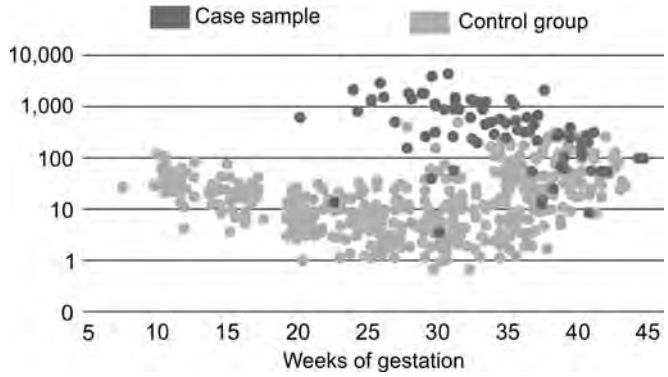
Cystatin C		*Creatinine*		
Creatinine-blind area				
GFR mL/in/1.73m^2 >89	60–89	30–59	15–29	<15
Stage 1 Kidney damage with normal/elevated GFR	Stage 2 Mild kidney insufficiency	Stage 3 Moderate kidney insufficiency	Stage 4 Severe kidney insufficiency	Stage 5 End stage renal disease (ESRD)

Stages of chronic kidney disease according to NKF KDOQI.[2]

[1] Stevens LA, Coresh J, Greene T, Levey AS. Assessing kidney function—measured and estimated glomerular filtration rate. N Engl J Med. 2006;354: 2473-83.

[2] National Kidney Foundation Kidney Disease Outcomes Quality Initiative, www.kidney.org/professionals/kdoqi – access date July 2012.

Elecsys sFlt-1/PlGF Ratio



In a multicenter case-control study including 351 pregnant women sFlt-1 levels were found to be higher and PlGF levels have been found to be lower in preeclampsia cases than in normal pregnancies. The sFlt-1/ PlGF ratio allows confirmation of preeclampsia with a sensitivity of 82% and a specificity of 95% at a cut-off of 85.

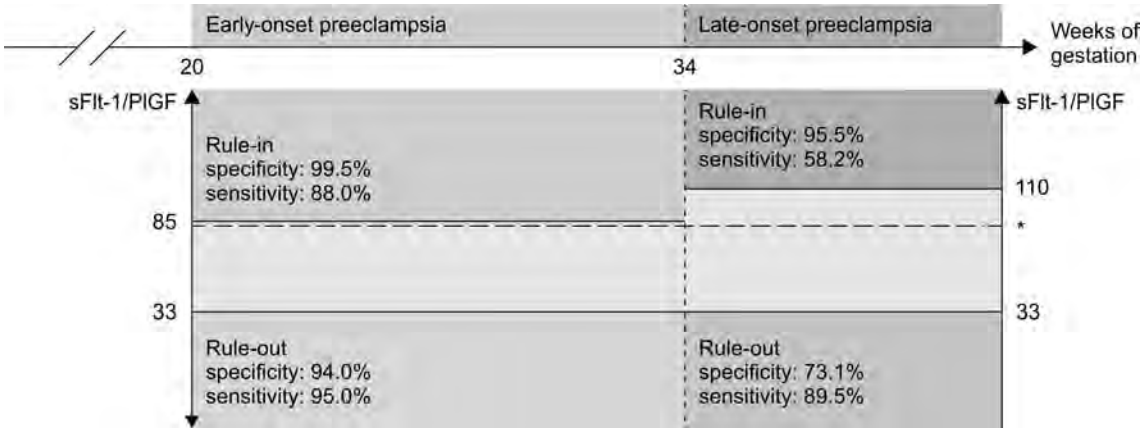
YOUR BENEFIT

- Elecsys sFlt-1 and PlGF immunoassays for preeclampsia are the first available and approved automated diagnostic tests for fast and easy assessment in a clinical context
- Early and precise diagnosis of preeclampsia leads to effective clinical management and improves the outcome for mother and child
- The sFlt-1/PIGF ratio is a reliable tool for discriminating between different types of pregnancy-related hypertensive disorders, assisting clinicians in the differential diagnosis of preeclampsia.

PRODUCT CHARACTERISTICS

	sFlt-1	*PlGF*
Total assay time	18 min.	
Sample material	serum	
Imprecision	<5 %	
Sample volume	20 µL	50 µL
Measuring range	50 µL	3–10,000 pg/mL
Analytical sensitivity	approximately 6 pg/mL	<2 pg/mL

sFlt-1/PlGF Ratio—Aid in the Diagnosis of Preeclampsia



* At a cut-off of 85 for the whole gestational period (from week 20+0 to delivery) sensitivity was calculated as 82 %, specificity as 95 %.

Verlohren S, Herraiz I, Lapaire O, et al. The sFlt-1/PlGF ratio in different types of hypertensive pregnancy disorders and its prognostic potential in preeclamptic patients. Am J Obstet Gynecol. 2012; 206:58.e1-8
Verlohren S, Galindo A, Schlembach D, Zeisler H, Herraiz I, Moertl MG, et al. An automated method for the determination of the sFlt-1/PIGF ratio in the assessment of preeclampsia. Am J Obstet Gynecol. 2010;202(2); 161.e1-161.e11.

ELECSYS® VITAMIN D TOTAL

Allowing Better Patient Care with Results You can Trust

Vitamin D has a proven impact on bone mineral density and bone quality. Desirable levels of 30 ng/mL have been shown to reduce the risk of falls and fractures.

There is also growing scientific evidence linking the level of vitamin D (25-OH) to an increased risk of other indications such as diabetes, cardiovascular disease, autoimmune diseases, and different forms of cancer.

The Elecsys® Vitamin D total assay aids in the assessment of vitamin D sufficiency.

YOUR BENEFIT

- Excellent functional sensitivity and superior precision for reliable results and improved patient management
- Standardized against LC-MS/MS (traceable to NIST) for confidence in patient results
- High lot-to-lot consistency for optimal therapy monitoring
- Efficiency due to consolidation of Vitamin D total, β-CrossLaps, P1NP, Osteocalcin and PTH testing on one fully automated platform.

PRODUCT CHARACTERISTICS

- Assay time: 27 minutes
- Sample material: Serum and plasma
- Sample volume: 15 µL
- Detection limit: 3.00 ng/mL (7.50 nmol/L)
- Functional sensitivity: 4.01 ng/mL (10.0 nmol/L) (CV 18.5 %)
- Measuring range: 3.00–70.0 ng/mL (7.50–175 nmol/L)
- Repeatability: Within-run precision:
  <15 ng/mL: SD ≤1 ng/mL,
  >15 ng/mL: ≤6.5 %
- Reproducibility: Intermediate precision:
  <15 ng/mL: SD ≤1.7 ng/mL,
  >15 ng/mL: ≤11.5%

Reagent onboard stability: 21 days on Elecsys® 2010 and **cobas e** 411 analyzer, and 28 days on **cobas e** 601 module, **cobas e** 602 module and E170.

ELECSYS® IL-6, PCT AND TINA-QUANT® CRP

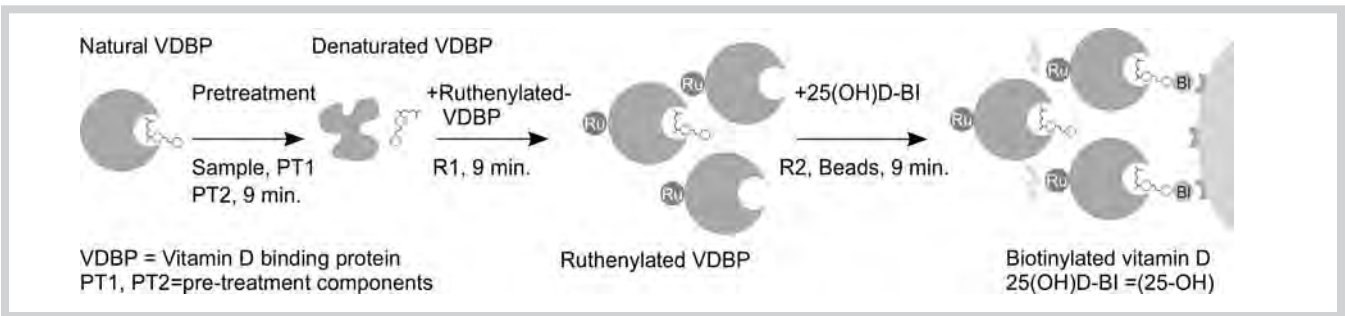
For Early and Effective Sepsis Management— because time matters

Sepsis, the systemic inflammatory response to infection, is a leading cause of death. With 18 million global cases annually, it is a major burden on healthcare.

Early recognition is critically important for patient survival, but clinical signs and symptoms are often ambiguous.

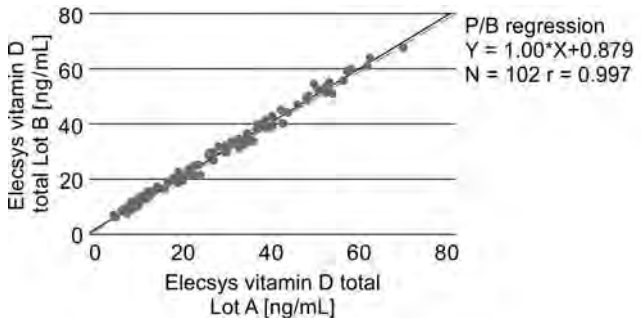
Elecsys IL-6, Elecsys BRAHMS PCT, in combination with CRP, deliver rapid, reliable information about the patient's immediate inflammatory status and likelihood

Competitive Protein Binding Assay Detecting 25-OH Vitamin D2 and D3

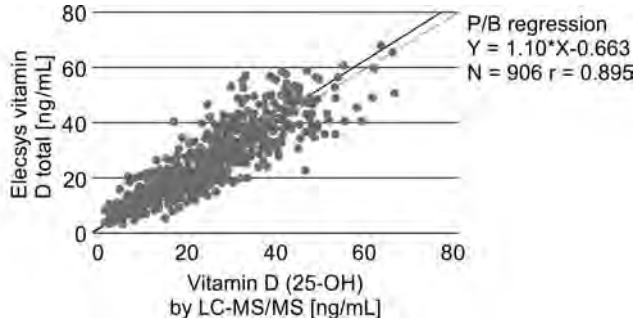


Precision, Accuracy and Convenience

Elecsys vitamin D total shows consistent results between different reagent lots



Elecsys vitamin D assay shows a robust correlation with LC-MS/MS



Data taken from the multi-center evaluation of Elecsys Vitamin D total

of bacterial sepsis, which is important for antimicrobial therapy management.

YOUR BENEFIT

Rapid Diagnostics

- Short total assay time.

Testing Efficiency

- All parameters from one sample tube.

Economical Sample Handling

- Low sample volumes, especially important for pediatrics

PCT, IL-6 and CRP: a biomarker panel to support early recognition and management of sepsis

IL-6: Early warning sign of (systemic) inflammation and sepsis

PCT: Follows IL-6 and indicates high probability of bacterial sepsis

CRP: Released from the liver as a later marker of inflammation.

Acute inflammatory episode	*Clinical indication of sepsis*	*Differential diagnosis*	*Severe sepsis/shock*
	Suspicion/treatment	*Characterization of infection**	*Therapy stewardship*
• **IL-6**	• Temperature	• Blood culture	• **PCT**
	• Heart rate	**– PCT**	• **IL-6**
	• Breathing rate	**– IL-6**	
	• Leukocytes	**– CRP**	
	• **CRP**		

* Rapid identification of sepsis pathogens is possible with LightCycler® SeptiFast Test.
Please see on page 182 for more details.

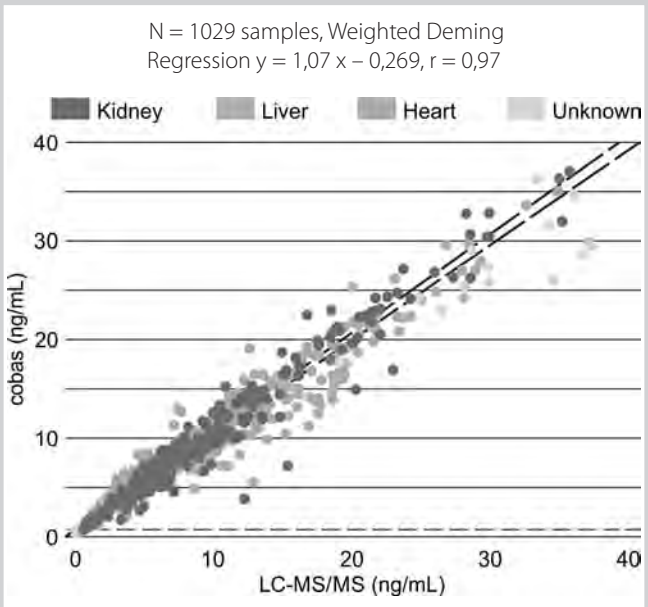
PRODUCT CHARACTERISTICS

Assay	*Elecsys BRAHMS PCT*	*Elecsys IL-6*	*CRPL3 on cobas c analyzers*
Sample material	Serum, Li-heparin and K3-EDTA plasma	Serum, Li-heparin and K2- and K3-EDTA plasma	Serum, Li-heparin and K2- and K3-EDTA plasma
Sample volume	30 µL	30 µL	2 µL
Assay time	18 minutes	18 minutes	10 minutes
Measuring range	0.02–100 ng/mL	1.5–5,000 pg/mL	0.3–350 mg/L
Analytical sensitivity	<0.02 ng/mL	1.5 pg/mL	0.3 mg/L
Functional sensitivity	<0.06 ng/mL	5 pg/mL	0.6 mg/L
Traceability	Standardized against BRAHMS PCT LIA	WHO Standard NIBSC 1st IS 89/548	IRMM reference preparation CRM470 (RPPHS)

ELECSYS® TACROLIMUS AND CYCLOSPORINE

Trusted and Consistent Results for Organ Transplant Patients

Optimal immunosuppressive therapy, defined clinically and by therapeutic drug monitoring (TDM), is essential to prevent acute rejection and ensure long-term survival of both the patient and the allograft. Characterized by a narrow therapeutic window, the use of immunosuppressive drugs (ISDs) requires both precise and consistent measurement of their concentration in whole blood during life-long monitoring.



Elecsys Tacrolimus: excellent correlation with a well evaluated LC-MS/MS. (Source: Multicenter evaluation study 2013)

YOUR BENEFIT

High Precision for Confidence in Results

- High precision at low drug concentrations and across a wide measuring range.

Consistent Results for Life-long Monitoring

- Consistent results across all cobas® platforms
- High comparability with well-established and validated LC-MS/MS methods.

Consolidation of Relevant Monitoring Needs

- Outstanding possibilities for consolidation of parameters, including those highly relevant for transplant patients (e.g. mycophenolic acid (MPA), infectious diseases, diabetes, kidney and liver function).

Universal Manual Sample Pretreatment for Elecsys ISDs

As the analytes are largely distributed in red blood cells and bound to proteins, a one-step manual pretreatment is performed to release them from the proteins. The pretreatment reagent and the one step procedure are universal for all Elecssys ISD assays.

PRODUCT CHARACTERISTICS

Tacrolimus

- Assay time: 18 min.
- Sample material: EDTA whole blood
- Sample volume: 300 µL
- LoB, LoD, LoQ*: 0.3 ng/mL, 0.5 ng/mL, 1.0 ng/mL
- Measuring range: 0.5–40 ng/mL
- Total imprecision:
  - **cobas e** 411 analyzer: 2.1–14.2 %
  - **cobas e** 601/**e** 602 modules: 2.4–10.4 %

Cyclosporine

- Assay time: 18 min.
- Sample material: EDTA whole blood
- Sample volume: 300 µL
- LoB, LoD, LoQ: 20 ng/mL, 30 ng/mL, 50 ng/mL
- Measuring range: 30–2,000 ng/mL
- Total imprecision:
  - **cobas e** 411 analyzer: 4.2–9.2 %
  - **cobas e** 601/**e** 602 modules: 3.1–6.4 %

* LoB = Limit of Blank; LoD = Limit of Detection; LoQ = Limit of Quantitation with a total allowable error of ≤20 %

HEMOSTASIS TESTING

Roche is rapidly moving towards a comprehensive new hemostasis testing portfolio with a number of industry firsts and innovative applications for early disease detection and monitoring. From easy-to-use, low-volume analyzers for self- and professional monitoring to systems meeting the high efficiency requirements of commercial laboratories, Roche's products offer outstanding productivity while reducing complexity.

Like Roche's current instruments, the new generation of testing solutions is driven by a commitment to delivering high-quality, cost-effective solutions capable of addressing the current and future testing needs of a wide range of customers.

The Multiplate® analyzer is a recent addition to our portfolio of self-monitoring and professional point-of-care solutions, which includes the Coagu-Chek® XS, XS Plus and XS Pro coagulation monitoring systems.

Used to assess patients' platelet function, the multiplate analyzer can help improve antiplatelet therapy and reduce the risk of thrombosis and bleeding. It provides hematologists, heart specialists and anesthesiologists with key information to support clinical decisions in cardiology, surgery and intensive care.

With its highly innovative testing technology, the Multiplate analyzer has the potential to set new standards in patient care. It is a perfect example of Roche's ambition to combine true innovation with proven medical and diagnostic expertise in creating a new hemostasis portfolio.

For more information please visit www.cobas.com and www.roche-multiplate.com

MULTIPLATE® ANALYZER

Platelet Function Testing with Best-in-Class Predictivity

Blood platelets play a pivotal role in physiological hemostasis, but also in the development of arterial thrombosis (myocardial infarction and stroke). Platelet

function testing is utilized in the analysis of inherited and acquired platelet function disorders that may cause a transient or permanent bleeding tendency. The Multiplate analyzer can detect platelet dysfunction and thus aid in the therapeutic management of such patients.

It can also be used for monitoring of anti-platelet drugs where both compliance and drug effectiveness are key issues. It was shown with Multiplate results[1] that up to 20% of patients do not respond adequately to clopidogrel treatment. These patients materialhave a 5–10 fold increased risk of stent thrombosis, stroke and myocardial infarction[1-4] following percutaneous coronary interventions. Multiplate delivers best-in-class predictivity[5] and evidence is available demonstrating that Multiplate guided anti-platelet therapy has the potential to improve patient outcome.[6-8]

The Multiplate analyzer also plays a role in the analysis of platelet function in anesthesia and intensive care, where platelet dysfunction can lead to severe bleeding complications. The detection or exclusion of platelet dysfunction before invasive procedures or in bleeding patients can aid the risk stratification and management in these situations.[9-10]

[1] Sibbing D et al. J Am Coll Cardiol. 2009;53(10):849-56.
[2] Sibbing D et al. Thromb Haemost. 2010;103(1):151-9.
[3] Schulz S et al. Am Heart J. 2010;160(2):355-61.
[4] Siller-Matula JM et al. (2010). J Thromb Haemost. 2010;8(2):351-9.
[5] Bonello L et al. J Am Coll Cardiol. 2010;56(12):919-33.
[6] Siller-Matula JM et al. Int J Cardiol. 2013;167(5): 2018-23.
[7] Sibbing D et al. J Am Coll Cardiol. 2012;59; E265.
[8] Aradi et al. J Am Coll Cardiol.2013;61(10): E1922.
[9] Ranucci M et al. Ann Thorac Surg. 2011;91(1):123-9.
[10] Weber CF et al. Anesthesiology, 2012;117(3):531-47.
[11] Straub N et al. Thromb Haemost. 2013;111(2). [Epub ahead of print]

YOUR BENEFIT

Cost-effective Therapies

- In cardiac surgery[10]
- In coronary interventions.[11]

Fast and Easy Assessment

- Of platelet function from small volumes of whole blood.

Best Predictivity

- For stratification of bleeding risk in surgical procedures
- For tailored anti-platelet therapy.

Consistent Results

- Using standardized reagents and procedures.

Medical Momentum

- More than 400 Medline publications, consensus papers with Multiplate and published guidelines for PFT.

Product Characteristics

- High throughput: 30 tests/hour
- Sample volume: only 300 µL per analysis
- Fast turn-around time: 10 minutes/test.

Comprehensive Reagent Menu of CE Marked Tests and Controls

Products	*Description*
ADPtest	ADP induced platelet activation sensitive to clopidogrel, prasugrel and other ADP receptor antagonists
ASPItest	Cyclooxygenase dependent aggregation (using arachidonic acid) sensitive to Aspirin®, NSAIDs and other inhibitors of platelet cyclooxygenase
COLtest	Collagen induced aggregation
RISTOtest	vWF and GpIb dependent aggregation (using ristocetin)
TRAPtest	Platelet stimulation via the thrombin receptor (using TRAP-6), sensitive to IIbIIIa receptor antagonists
Prostaglandin E1 reagent	For the assessment of ADPtest HS (high sensitivity). For the assessment of positive (i.e. abnormal) controls of the ADPtest
ASA reagent	Inhibitor of cyclooxygenase. Addition of ASA reagent to the blood sample leads to reduced aggregation responses in ASPItest and COLtest
GpIIb/IIIa antagonist reagent	Inhibitor of the platelet GpIIb/IIIa receptor. Addition to a blood sample leads to strongly reduced aggregation in the TRAPtest
Hirudin blood tubes	Anticoagulant for platelet function analysis with physiological calcium concentrations
Liquid control set	Quality control for electrical signal in impedance aggregometry based on the analysis of an artificial liquid control materialhave.

URINALYSIS

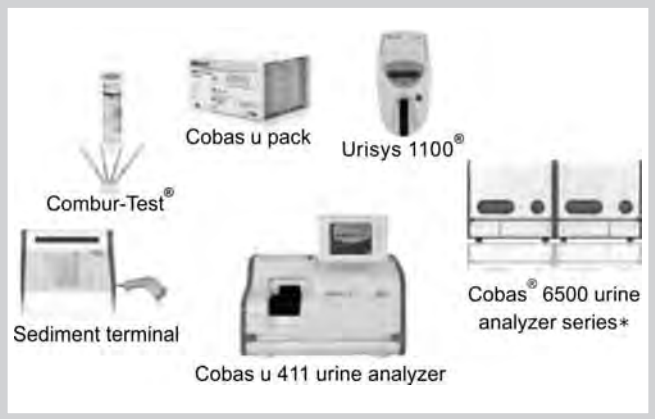
Urinalysis has always been an important diagnostic tool in medicine. Even today, urine is still a key health barometer for many diseases, mainly urinary tract infections, kidney disease and diabetes. The analysis of urine can reveal serious diseases that show no symptoms in their early stages but are treatable. These diseases can cause severe damage if they remain undetected. Urine test strips are a crucial diagnostic tool and easy to use, yielding quick and reliable information on pathological changes in the urine. Their diagnostic significance lies primarily in first-line diagnosis, screening during routine or preventive examinations, and treatment monitoring.

Today Roche offers a broad portfolio of urinalysis solutions for different customer needs. Drawing on our 50 years of experience in urinalysis, starting with the launch of the first Combur-Test® strip, we have continuously improved strip technology for clinical and general practice. In response to customer needs for increased efficiency and safety, we have developed a range of analyzers with differing degrees of automation and throughput capabilities. By combining the proven Combur-Test strip technology with Roche automation, we offer customized urinalysis solutions for physician office laboratories, hospital point of care and central laboratory settings.

For more information please visit www.cobas.com

URINALYSIS FROM ROCHE

Expertise Coming from a Long Tradition of More Than 50 Years



Urine Diagnostics Portfolio

	Combur-Test®	*Urisys 1100®*	*cobas u 411 urine analyzer*	*cobas® 6500 urine analyzer series**
Automation grade	Visual reading and for all UA platforms	Instrument intended for single measurements in wards or in physicians' offices	Semi-automated urinalysis system for small to medium sized laboratories	Fully automated urine work area solution for large-scale laboratories
Throughput	manual	20–50 samples per day	50–100 samples per day	100–1,000 samples per day
Test strips	Combur[2-7,9,10]	Test Combur[10] Test UX	Combur[10] Test M	**cobas u** pack
Consumables	◘			**cobas u** cuvette*

COMBUR-TEST® STRIP

A Quality Choice for Professional Use

Urine reagent strips are a useful tool for investigating, diagnosing and screening diseases immediately. Reliable and precise results are important, since adulterated results can lead to false negative results or re-testing of patients. Roche's unique test strip technology is used for visual test strips and for all instrument test strips.

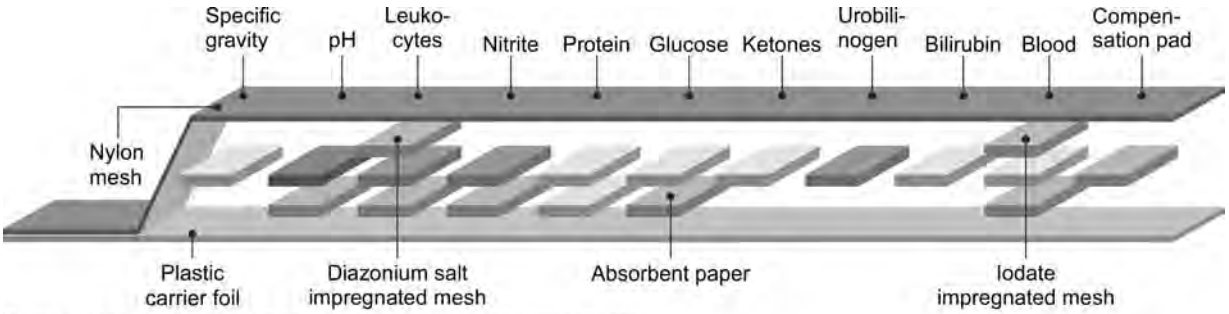
YOUR BENEFIT

Accuracy

- Combur-Test® strip* detects even low concentrations of glucose and erythrocytes/ hemoglobin (5–10 Ery/mL) in the presence of vitamin C.

Efficiency

- Avoidance of retesting and false-negative results in glucose and blood even with high levels of ascorbic



Combur-test urine test strips from roche have iodate impregnated mesh layers and are uninfluenced by ascorbic acid.
• For instrument tests only.

acid (up to 400 mg/L) with the application of an iodate impregnated mesh layer.

Safety

- Independence interference from of glued components as a result of a unique sealing technology
- Test area colors prevented from running with an absorbent paper
- Reduction of the risk of false results through compensation of strong intrinsic urine coloration with the availability of a color compensation pad*.

Easy Strip Handling

- Facilitation of analysis with a consistent reading time of 60 seconds for all parameters
- Advanced and hygienic strip handling with possibility of reading tip down.

URISYS 1100® ANALYZER

Connected, Compact and Intuitive Solution for Urinalysis

The Urisys 1100 analyzer is a small semi-automated benchtop instrument for a workload of 30 to 50 samples per day. It is optimal for small labs, doctor's offices or in decentralized settings.

The high quality Combur-Test® strips provide accurate results in one minute which can be optionally printed out for your convenient documentation.

YOUR BENEFIT

Compact

- Semi-automated urine analyzer for the small lab, ward or doctor's office.

Easy Handling

- Automatic printing of results.

Simplify Your Life

- Eliminate manual documentation through the export of data via host connection.

Safety

- Prevent unauthorized access and comply with accreditation requirements via an operator lock-out feature.

PRODUCT CHARACTERISTICS

- Combur-Test® is resistant to ascorbic acid interference
- Control-Test M for weekly calibration
- Throughput: approx. 50 test strips/hour
- Test strips*: Combur10 Test® UX

Strips										
Urine test strips	Combur-Test strips									
Parameters	SG	pH	LEU	NIT	PRO	GLU	KET	UBG	BIL	BL
Combur[10] Test UX	•	•	•	•	•	•	•	•	•	•
Calibration	Control-Test M calibration strip									

- Memory capacity: 100 results
- Printer: Thermal printer
- Connectivity to the **cobas** POC IT solution.

COBAS U 411 URINE ANALYZER

The Compact Solution for the Semi-automated Urine Work Area

The **cobas u** 411 semi-automated urine analyzer is designed for workloads of approximately 80 samples per day.

When connected to the optional barcode reader and sediment terminal, this analyzer designed optimized work and data flow.

YOUR BENEFIT

Fast and Efficient Workflow

- By connecting analyzer to sediment terminal and consolidating the results.

Ensure Reliable Results

- Ascorbic acid does not interfere with test strips.

Safe and Hygienic Handling of Strips

- Due to netsealing technology.

PRODUCT CHARACTERISTICS

- Throughput: 600 tests/h
- Continuous loading of test strips without requiring a measurement cycle
  - optional barcode reader simplifies manual worksteps
- Entry of tracking information including user identification and lot numbers for test strips, calibration strips and control material.

Consolidated Analysis

Parallel working on the cobas u 411 analyzer and its connected sediment terminal as a result of a consolidated work and data flow for strip analysis and microscopy. Easier documentation and improved overview of patient records with single print-out for strip and microscopic information.

COBAS® 6500 URINE ANALYZER SERIES*

Fully Automated Urine Work Area on a Modular Platform

The **cobas** 6500 urine analyzer series* is a fully automated urine work area solution for laboratories processing 100–1,000 urine samples per day.

Due to its modular design **cobas** 6500 urine analyzer series can be installed as a stand-alone urine analyzer or as a stand-alone microscopy analyzer or together as a fully automated urine work area.

YOUR BENEFIT

Automation of the Gold Standard

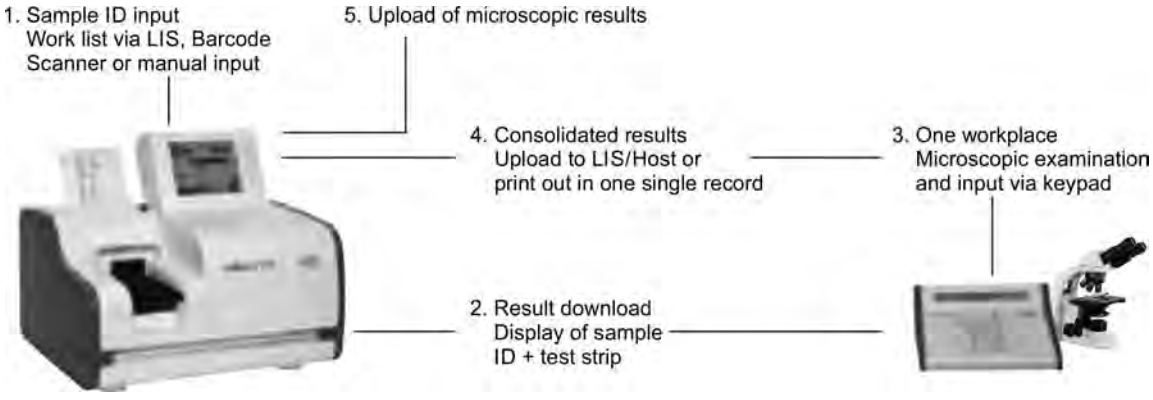
- Taking real microscopy images—eliminating operator variability and the need for manual review, improving TAT.

Precise and Safe Strip Results

- Safe reagent strip with ascorbic acid resistance
- Precise fully automated measurement, no contact with samples needed.

Consolidation of Urine Work Area

- Convenient validation—all results on one screen.



Semi-automated urine work area solution.

Workflow Optimization

- Reagent free cassette concept
- Automated sieve testing—sediment test only if needed, efficient cost management.

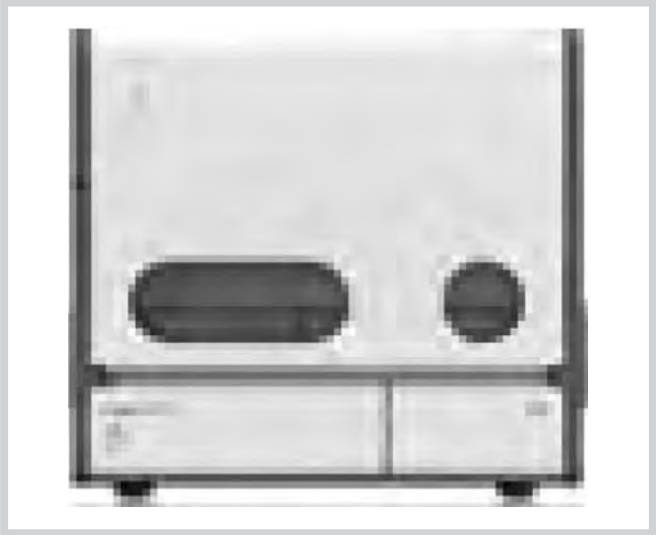
PRODUCT CHARACTERISTICS

Cobas u 601 Urine Analyzer

- fully automated urine strip system
- 12 on-board parameters
- cobas u pack;
  - cassette with 400 test strips
  - Combur-Test® strips
  - two weeks on-board stability (humidity protected).

Cobas u 701 Microscopy Analyzer*

- fully automated urine microscopy system
- reagent-free system
- only uses disposable cuvettes
- 400 cuvettes in one package (**cobas u** cuvette)
- Parameters:
  - Erythrocytes
  - Leukocytes
  - Bacteria
  - Non-squamous epithelial cells
  - Epithelial cells
  - Hyaline cells
  - Pathological casts
  - Crystals
  - Yeasts
  - Mucus
  - Sperm
  - Leukocytes.



POINT-OF-CARE TESTING

The goal of point of care from Roche is to help both healthcare professionals and patients achieve improved clinical and health-economic outcomes, by delivering robust, connected, easy to use point-of-care solutions outside the central laboratory providing immediate results and thus allowing treatment decisions to be made more quickly – inside or outside the hospital.

Point of care delivers those solutions meeting the clinical need for quick and accurate test results delivered where needed, when needed; on the device, in the electronic healthcare record on a patient/ward monitor, to the clinician on the move and directly to the patient.

Where the responsibility for providing the service is in the hands of professionals we also provide IT tools to be able to control all aspects of testing to ensure quality patient care:

- Provide accurate and timely analyses and match them to with the correct patient
- Ensure that operators are competent in the use of the system
- Provide reports that are useful to the clinician treating the patient
- Document testing and QC for audit purposes

For coagulation patient self-monitoring we also provide solutions for remote support and monitoring.

For more information please visit www.cobas.com

Overview of Point-of-Care Diagnostic Tests

	Combur (visual strips)	*TROP T sensitive (visual strip)*	***cobas h** 232*	*Accu-Chek® Inform II*	*CoaguChek® XS, XS Plus and XS Pro*	*Accutrend® Plus*	*Urisys 1100®*	***cobas b** 101*	*Reflotron® Plus and Reflotron® sprint*	***cobas b** 123**	***cobas b** 121**	***cobas b** 221**
Anemia												
Bilirubin							•		•	•		•
Bilirubin neonatal										•		•
Hemoglobin total	•						•		•	•	•	•
Hematocrit										•	•	•
Oxygen saturation (sO_2)										•	•	•
Blood gas												
pH										•	•	•
pCO_2										•	•	•
pO_2										•	•	•
Electrolytes												
Ca^{2+}										•	•	•
Cl^-										•	•	•
K^+										•	•	•
Na^+										•	•	•
CO-oximetry												
tHb-COOX										•		•
O_2Hb										•		•
HHb										•		•
COHb										•		•

Contd...

Contd...

MetHb										•		•
sO_2 COOX										•		•
Bilirubin neonatal										•		•
Barmetric pressure (Baro)										•		•
Cardiac												
Troponin T		•	•									
CK-MB			•									
Myoglobin			•									
D-dimer			•									
HDL						•		•	•			
LDL						•		•	•			
NT-proBNP			•									
Coagulation												
D-dimer			•									
PT (INR/% Quick/sec.)					•							
Metabolic												
Ca^{2+}										•	•	•
Cl^-									•	•	•	•
Glucose				•		•	•		•	•		•
HbA1c								•	•			
HDL cholesterol (or HDL-C)								•	•			
Ketone	•						•					
LDL cholesterol (or LDL-C)								•				
Lactate						•			•	•		•
Potassium									•	•	•	•
Sodium										•	•	•
Total cholesterol						•		•	•			
Triglycerides						•		•	•			
Hepatology												
Alkaline phosphatase									•			
Bilirubin									•			
Creatine kinase									•			
GGT									•			
GOT (AST)									•			
Hepatology												
GPT (ALT)									•			
Pancreatic amylase									•			
Urobilinogen	•											

Contd...

Contd...

Renal and urine											
Bilirubin	•						•		•		
Creatinine									•		
Erythrocytes (Hb)	•						•				
Glucose	•						•		•		
Ketone	•						•				
Leukocytes	•						•				
Nitrite	•						•				
pH	•						•				
Protein	•						•				
Specific gravity	•						•				
Urea (BUN)									•		•
Uric acid									•		
Urobilinogen	•						•				

*in addition several calculated parameters are available

COBAS POC IT SOLUTION

Bringing it All Together

cobas POC IT is responsible for collecting results from POC analyzers that are distributed across hospitals and primary care centres.

The **cobas** POC IT solution brings all POC information together to provide oversight via your POC program, provide you with insight required to ensure compliance and the long-range view to plan for improvements and expansion in the future.

Roche is committed to assisting POC coordinators with powerful tools required to effectively manage POC testing, improve workflows and meet accreditation and regulatory requirements around the world.

Proven open connectivity to a wide menu of POC devices gives you the freedom of choice to grow your POC program.

YOUR BENEFIT

Coordinated User Management

- A central point of control for all POC testing devices and users ensures result security
- Most efficient customizable online e-learning with automatic operator recertification saves a significant amount of time.

Innovative Functionality

- Over a decade of collecting user input and workflows has resulted in a high level of innovation that are firsts on the market such as true wireless communication and observed competency on-board POC devices, as well as positive patient ID—ensuring patient safety.

Local Service and Support

- Quick and easy access to Roche service personnel in your time zone and language provides efficient turnaround time for your questions and ensures maximum uptime for the systems.

Proven Commitment

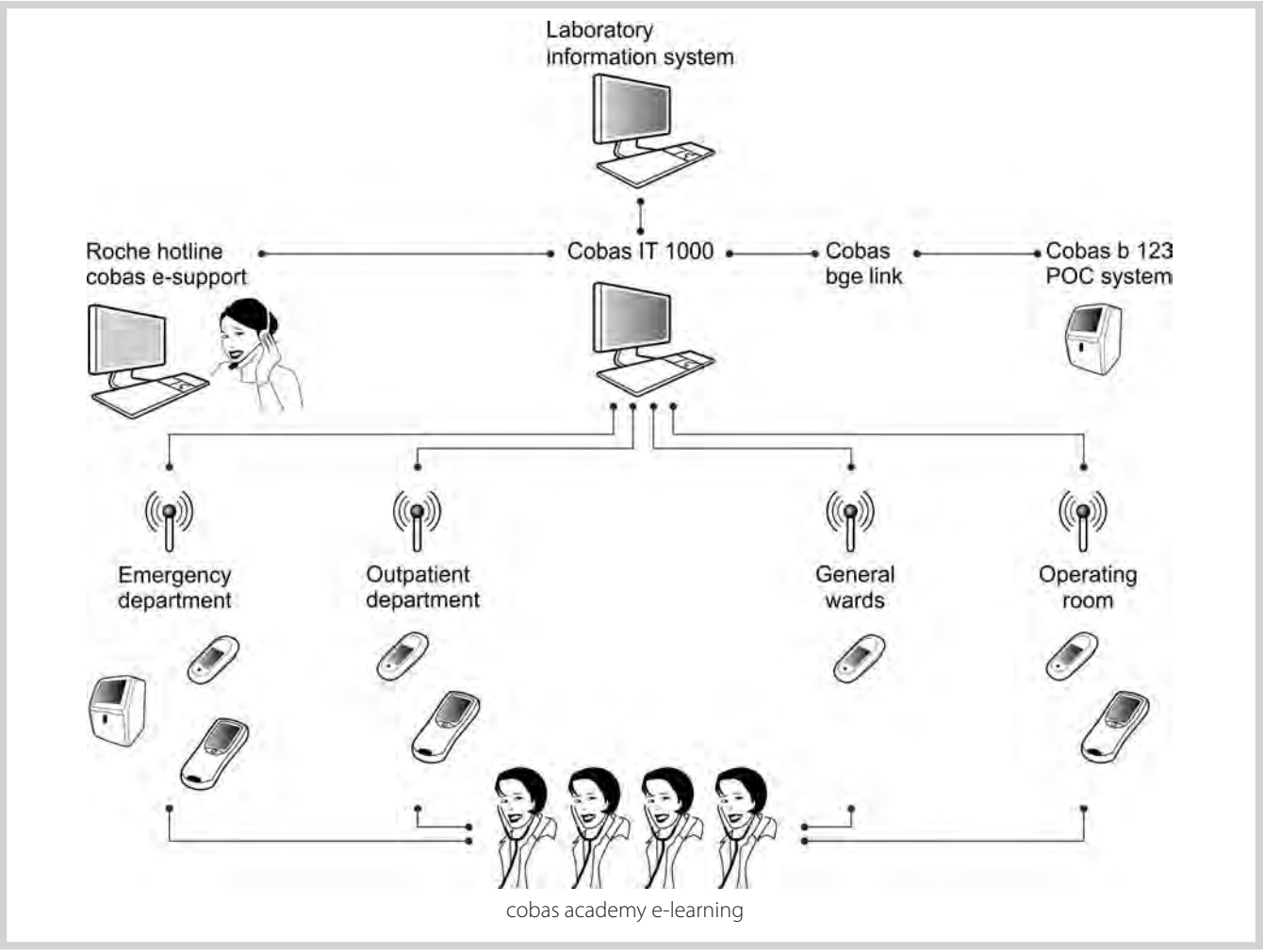
- The cobas POC IT solutions are proven to perform in over 1,450 systems in >50 countries with 70,000 connected devices.
- Including over >50 Roche and non-Roche POC devices – with a long term commitment to enhancing value for patients and POC coordinators.

PRODUCT CHARACTERISTICS

Cobas IT 1000 Application

- **Cobas IT** 1000 application gives you complete management of POC testing, including remote configuration and control of devices, user management and LIS/HIS interfacing from a single point of control

cobas POC IT Solution



cobas academy e-learning

- Connects the full Roche POC portfolio including Accu-Chek Inform II, CoaguChek XS Plus and Pro, cobas h 232, cobas b 101, Urysis 1100, cobas b 121, cobas b 123, cobas b 221.

Cobas Academy

- With cobas academy you can customize eLearning courses and deploy training content on your intranet, and also allow user re-certification automatically—the system will also automatically lock out users who are not certified until they have completed the required training.

Cobas bge Link

- The cobas bge link software gives you complete and easy remote management of POC blood gas analyzers, allowing you to view and control device operations simply and efficiently.

Cobas eServices

- Gives your local Roche experts remote access, enabling them to quickly and efficiently answer your questions in your time zone and language.

COBAS bge LINK SOFTWARE

Central Control of Your Roche Blood Gas and Electrolyte Analyzers

The **cobas bge link** software provides complete remote management and control of blood gas instruments from one workstation.

This valuable tool allows the complete management of all **cobas** blood gas analyzers that are connected to a hospital network. The **cobas bge link** software can improve workflow efficiency, freeing up valuable staff time and improving service to clinicians in critical care settings.

YOUR BENEFIT

Save time

- By not having to walk to each analyzer, with continuous remote status monitoring of your blood gas and electrolyte systems, from the laboratory.

Improve Analyzer Uptime

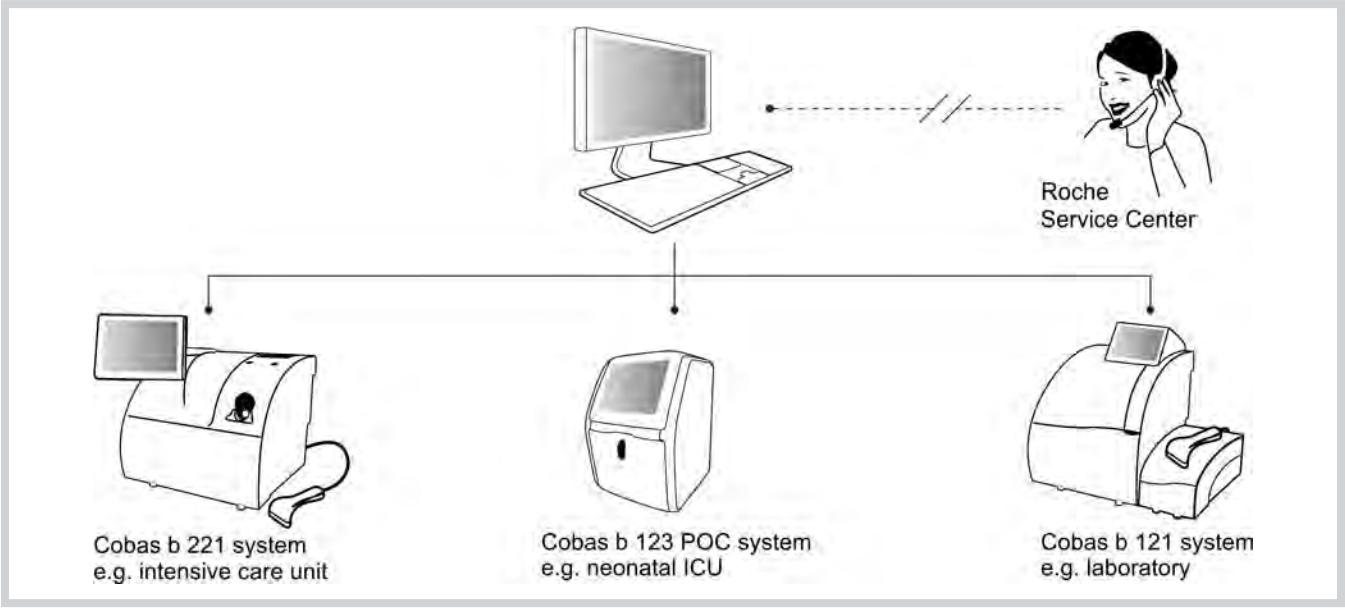
- With effective remote troubleshooting and remote control of analyzer functions (e.g. calibrations, QC, cleaning cycles, test functions).

Increase Confidence and Security

- With remote monitoring of analyzer performance and quality.

PRODUCT CHARACTERISTICS

- Information on analyzer status, parameters, reagents and reports in a clearly arranged layout
- Management of quality controls and calibration cycles
- Clear presentation of patient results measured with the blood gas and electrolyte systems from Roche
- Remote control of calibrations, cleaning cycles and test functions



- Initiation of quality control on the blood gas and electrolyte systems from Roche (AutoQC®), can be initiated from the laboratory
- Levy-Jennings overview of QC history and trends
- Extensive data management possible through integration into **cobas** POC IT solution.

COBAS B 121 SYSTEM

Quick and Efficient Testing in Critical Care

In critical care settings, fast test results mean rapid patient care. You can get valuable information on 10 of the most important parameters, all measured on the **cobas b** 121 system. The parameter profile can be customized to meet your individual requirements. In addition, this instrument offers easy handling and low maintenance, yet performs as well as larger, more complex systems.

YOUR BENEFIT

Meets Varied Testing Needs

- Of different departments through the broad parameter menu.

Increased Security and Confidence

- Providing you with laboratory-quality results at the point of care.

Highest Quality and Full Traceability

- Automated quality control with documentation software for certification requirements.

PRODUCT CHARACTERISTICS

- Throughput: 30 samples/hour
- Low sample volume: 60 µL, allows use in the neonatal setting
- Barcode scan prevents patient data mix-up
- Low maintenance electrodes
- Graphical user interface ensures ease of operation
- Liquid calibration for more convenience
- Connectable to network via the **cobas bge link** software for remote control and to the **cobas** POC IT solution for comprehensive data management.

Extended Blood Gas Profile

Parameters:

- Blood gases pH, PO_2, PCO_2
- Total hemoglobin tHb
- Oxygen saturation SO_2
- Hematocrit Hct.

Extended Emergency Profile

Parameters:

- Blood gases pH, PO_2, PCO_2
- Electrolytes Na^+, K^+, Ca^{2*}, Cl^-
- Total hemoglobin tHb
- Oxygen saturation SO_2
- Hematocrit Hct.

cobas b 121 system versions	*b 121*	*b 121 ‹bge›*
pH/blood gas (pO_2, pCO_2, pH)/Co-oximetry	•	•
Electrolytes (Na^+, K^+, Ca^{2+}, Cl^-)/Hematocrit	•	•
tHb/sO_2	•	
Auto QC	•	

COBAS B 221 SYSTEM

Convenience for Your Critical Care Testing

Blood gas analysis is considered the most important tool for diagnosis in critically ill patients. Analyzers should deliver rapid and reliable results, be easy to handle and require little maintenance. Our **cobas b** 221 system offers these features—and a flexible configuration which can meet your specific requirements for critical care testing in high throughput departments.

YOUR BENEFIT

Fast Diagnosis

- Results in less than 2 minutes to support timely clinical decision making.

Flexibility of Testing

- Comprehensive parameter menu to meet varying testing needs.

Confidence in Result Quality

- Lab-quality results where and when you need them.

Improved Uptime

- Due to long-life, maintenance-free electrodes and minimal preventative maintenance.

PRODUCT CHARACTERISTICS

- Throughput: up to 50 samples/hour
- Time to result: less than 2 minutes with whole-blood sampling
- Optional module for automatic quality control
- Three different parameter combinations (see table below) including glucose, lactate, urea and bilirubin
- Durable, low-maintenance sensors
- Easy-to-use touchscreen and intuitive user interface
- Trending acid-base maps to support clinical decisions
- Reagent tracking.

cobas b 221 system	*Versions*		
	2	*4*	*6*
pH/blood gas (PO_2, PCO_2, pH)/CO-oximetry	•	•	•
Electrolytes (Na^+, K^+, Ca^{2+}, Cl^-)/hematocrit		•	•
Metabolites Glu/Lac			•
Metabolites Glu/Lac/Urea (BUN)			•
Bilirubin	•	•	•

- Customizable features include a user-definable display and two types of sample application
- Connectable to network via the **cobas bge link** software for remote control and to the **cobas** POC IT solution for comprehensive data management.

COBAS B 123 POC SYSTEM

Allowing You to Focus on Patient Critical Care

The **cobas b** 123 POC system is a mobile, cartridge-based, critical care analyzer designed for POC testing. With flexible configurations and a throughput of up to 30 samples per hour, the **cobas b** 123 POC system can easily be customized to the clinical needs of the ICU, ER, NICU, OR*, dialysis units or the laboratory.

The operator-friendly system offers easy handling and requires no preventative maintenance, to reduce downtime.

reddot design award
winner 2011

MEDICAL DESIGN EXCELLENCE AWARDS® 2011 WINNER

YOUR BENEFIT

Easy to Use

- Intuitive graphical user interface, touchscreen and graphically guided instructions allow handling steps to be learned in minutes and simplify the training of POC users.

Safe

- Access control, clot prevention, data management including QC, remote control to increase analyzer uptime.

Rapid Results

- Near-patient, whole-blood sampling provides results in only 2 minutes to support timely clinical decision making.

Flexibility and Scalability

- Allows clinically relevant and cost-efficient POC testing including quality control.

* Intensive care unit, emergency room, neonatal intensive care unit, operating room.

PRODUCT CHARACTERISTICS

- Throughput: 30 samples/hour
- Integration of clot prevention features to ensure patient care without interruption and cost-efficient operation
- Optional mobile cart, battery operation and wireless connectivity enables instrument to be operated wherever needed
- Variety of sample types: whole blood,dialysis solution, QC solutions (both aqueous and blood-based)
- Connection to **cobas bge link** software and **cobas** POC IT solution
- Automated user management through **cobas** academy
- Trending acid-base maps to support clinical decisions
- Fluid pack—sizes 200, 400 or 700 samples.

Cobas b 123 POC system	*Versions*			
	1	*2*	*3*	*4*
pH/blood gas (pO_2, pCO_2, pH)	•	•	•	•
Electrolytes (Na^+, K^+, Ca^{2+}, Cl^-)/Hematocrit	•	•	•	•
Metabolites Glu/Lac	•	•	•	•
Bilirubin			•	•
Co-oximetry (tHb, O_2Hb, HHb, COHb, MetHb, SO_2)			•	•
Auto QC		•		•
Plus an extensive range of calculated parameters.				

ACCU-CHEK® INFORM II SYSTEM

Professional Glucose Testing for the Wireless Age

The Accu-Chek Inform II system helps nursing staff to do the right glucose test on the right patient at the right time.

It is a user-friendly hand-held system for point-of-care glucose testing and monitoring in hospitals. The **cobas** POC IT solution maintains all information, allowing central management of all meters and data.

YOUR BENEFIT

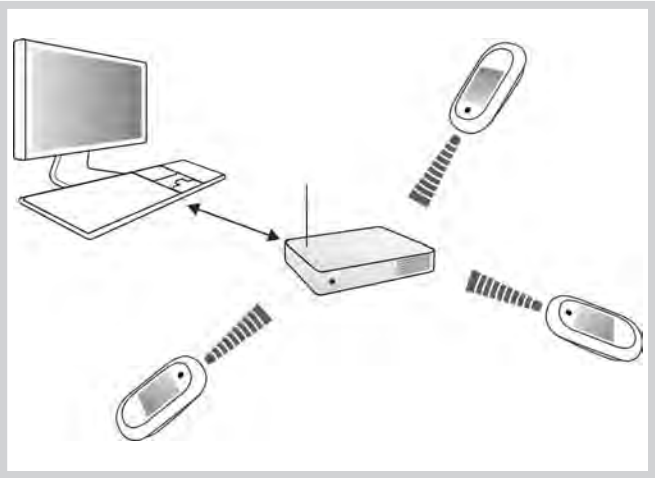
Improves workflow and regulatory compliance

- Real-time result transfer to hospital network with optional wireless connection (WLAN)
- Bidirectional data exchange with point-of-care networking software
- Enhanced patient identification using patient ID, name and date of birth
- Comprehensive quality control functions

- Easier and more hygienic blood sample application through improved Y-capillary at the tip of the test strip Precise, accurate, reliable results
- Strips with advanced chemistry to avoid maltose interference
- Calibration according to the newest standard (IFCC plasma).

PRODUCT CHARACTERISTICS

- Cutting-edge technology with a WLAN-enabled measuring device
- Data entry via touchscreen and/or 2D-barcode reader
  - User-ID and patient ID/case number
  - Password
  - Lot numbers for test strips and controls



- Watertight construction for easy cleaning and better infection control
- The Accu-Chek Inform II test strips
  - Speed and accuracy for professional use
  - Fast measuring time: only 5 seconds
  - Small sample volume: 0.6 µL
  - Approved for use with capillary, venous, arterial and neonatal blood
  - Not dependent on partial oxygen pressure.

ACCU-CHEK® SAFE-T-PRO PLUS

Single-use Lancing Device for Healthcare Professionals

When blood samples are obtained by healthcare professionals within the clinic and physician office lab, two aspects are of most importance: safety and hygiene.

Accu-Chek Safe-T-Pro Plus lancing devices fulfill these high requirements, and they allow intuitive ease of use.

The single-use lancets thus enhance safety and hygiene for both patient and healthcare provider.

YOUR BENEFIT

Reliable Prevention of Cross-infections and Needlestick Injuries

- Lancet protected from contamination by removable sterile cap
- Sterilized lancet, safely contained in the housing—No direct needle contact possible
- Lancet locked by a dedicated safety mechanism after use – Multiple use excluded
- Disposal of complete lancing device after usage—As a result there is no risk of injury
- The Accu-Chek Safe-T-Pro Plus can be used by healthcare professionals to obtain a capillary blood sample from a patient for measurement on Accu-Chek® Inform II, CoaguChek®, Reflotron® and Accutrend® instruments.

PRODUCT CHARACTERISTICS

- Ergonomic T-shaped design for easy handling
- Trigger button gives resistance thus preventing unintended activation
- Special cut and diameter of the needle to minimize pain
- 3 adjustable depth settings (1.3/1.8/2.3 mm) for greater flexibility and patient comfort
- Adaptable to different skin types.

COBAS H 232 POC SYSTEM

Expedite Your Cardiac Decisions with Rapid Results

Thanks to its compact, portable design, the **cobas h** 232 POC system can be easily deployed near the point of patient care where space is tight, be it at the bedside, in triage bays or in a designated lab area. The instrument is intended to be used in emergency care settings or CCU* for patients presenting with acute chest pain, dyspnea and other symptoms suggestive of acute cardiovascular disease. Studies have proven the effectiveness of cardiac marker testing with the **cobas h** 232 POC system in physician office settings, in particular where the use of NT-proBNP aids the diagnosis and assessment of heart failure. The system can also be used in pre-hospital settings such as ambulances or helicopters.

YOUR BENEFIT

Highly Versatile

- Suitable for use in different clinical settings, e.g. emergency room or GP office.

Allows Fast Patient Stratification

- Via a broad menu of individual tests
- Results available in a maximum of 15 minutes.

Easy Handling and Portability

- No sample preparation
- Automatic calibration
- No complicated setup procedures: intuitive, icon-based interface
- Maintenance-free
- Allows near-patient use at various locations.

Reliable Quantitative Measurements

- Roche CARDIAC® assays are validated by clinical studies and are comparable to Roche laboratory methods.

Safety

- Patient and operator ID entry and lockout
- Quality control lockout.

Control and Traceability

- Connection to the cobas POC IT solution allows extension of the testing network and ensures control of operators and quality assurance from the central laboratory
- Automatic recertification of operators through cobas academy to ensure use by trained operators only.

Optional integrated barcode scanner and external printer for greater safety and record keeping

* Cardiac care unit.

Product Characteristics

- Offers a wide range of parameters to help in the rapid diagnosis of acute coronary syndrome, heart failure, and venous thromboembolism (DVT and PE).

Parameter	*Time to result*
Myoglobin	8 minutes
D-dimer	
Troponin T	12 minutes
NT-proBNP	
CK-MB	

ROCHE CARDIAC® TROP T SENSITIVE TEST

Visual Test for the Rapid Diagnosis of Myocardial Infarction

Many patients seek medical attention only hours or even days after the onset of chest pain, especially on weekends. With the Roche CARDIAC Trop T Sensitive test you can make a diagnosis even several days (up to 10–14 days) after myocardial damage occurs.

The Trop T Sensitive is a visual troponin T test. Since it requires no system it can be easily deployed in rural areas near the point of patient care, at the bedside, in triage bays, emergency service areas, ambulances or a designated lab area. The Trop T Sensitive test is designed for qualitative determination of cardiac troponin T in the blood and elevated levels indicate acute mycardial infarction.

Results from a large prospective clinical trial* in Denmark indicate that implementation of qualitative pre-hospital troponin T testing in the ambulance vehicle by paramedics is feasible in most patients, including non-ST segment elevation myocardial infarction (NSTEMI) patients whose condition is not detected by the classical electrocardiogram.

YOUR BENEFIT

Highly Versatile

- Suitable for use in different clinical settings, e.g. emergency room, GP office or ambulance.

Fast Results

- Reliable yes/no result in 15–20 minutes.

Easy Handling and Portability

- Simple application that can be used anywhere
- No sample preparation
- Device independent.

Reliable Qualitative Measurements

- Proven test strip technology.

Cost-effective

- Requires no external measurement system
- Requires no special training

On the Spot Rule-in Acute Myocardial Infarction

- Specific cardiac marker—A positive result indicates myocardial damage
- Even if characteristic ECG changes are missing, a positive Roche CARDIAC Trop T Sensitive test with a non-ST-elevation myocardial infarction (NSTEMI) can aid the treatment decision.

PRODUCT CHARACTERISTICS

- Qualitative detection of troponin in anticoagulated (EDTA or heparin) venous whole blood

* Sørensen JT, Terkelsen CJ, Steengaard C. Prehospital troponin T testing in the diagnosis and triage of patients with suspected acute myocardial infarction. Am J Cardiol. 2011;107(10):1436-40.

- Reaction time: 15 minutes
- Positive result from a threshold (cut-off) of 100 ng/L
- Storage at 2–8°C (refrigerator)
- Test can be used immediately after removal from the refrigerator
- Storage for 1 week at room temperature (15–25°C)
- Roche CARDIAC Trop T Sensitive is available in 5 and 10 pack size.

COAGUCHEK® XS SYSTEM

Coagulation Self-testing Made Easy

The CoaguChek® XS system is a convenient, portable and user-friendly instrument for monitoring oral anticoagulation therapy. It determines the INR value (International Normalized Ratio) from a drop of capillary whole blood—simple, precise and reliable.

The CoaguChek XS system is ready for use anywhere at any time. Patients can use it for self-monitoring at home or on vacation.[1]

YOUR BENEFIT

Fast, Reliable Results

- Accurate PT/INR results in one minute
- Built-in quality control checks every strip automatically
- Lab-equivalent accuracy and better than laboratory precision1.

Simple Fingerstick Test

- Most patients prefer having a small drop of blood (just 8 µL) taken from a fingerstick to having blood drawn from a vein.

Independence and Reassurance

- Self-testing with a single instrument has the benefit of being accurate and reproducible.

Improved Patient Outcomes

- Frequent testing allows side effects to be minimized and increases the time spent within the therapeutic range range.[2]

[1] Kitchen DP, Munroe S, Kitchen S, Jennings I, Woods TAL, Walker ID. Results from the first year of an external quality assessment programme for the users of CoaguChek XS and CoaguChek XS Plus for monitoring INRs. Br J of Haematology. 2008;141 (suppl 1):188..

[2] Heneghan, et. al. Lancet 2006;367:404-11.

PRODUCT CHARACTERISTICS

- Test principle: Electrochemical determination of the PT time after activation of coagulation with human recombinant thromboplastin
- User interface: Icon-based LCD display; on/off, mem and set buttons
- Memory capacity: 300 test results with date and time
- Sample types: Fresh capillary or anticoagulant-free venous whole blood
- Easy blood application: top- or side dosing
- Measuring range: %Quick 5–120; Seconds: 9.6–96; INR: 0.8–8.0
- Data transfer: Infrared interface.

For more information please visit www.CoaguCheck.com

COAGUCHEK® XS PLUS SYSTEM COAGUCHEK XS PRO SYSTEM

Coagulation Monitoring for Healthcare Professionals

The CoaguChek XS Plus and the CoaguChek XS Pro systems are convenient, portable and user-friendly systems for monitoring oral anticoagulation therapy. They determine the INR value (International Normalized Ratio) from a drop of capillary whole blood—simple, precise and reliable. CoaguChek XS Plus and Pro systems have been developed exclusively for professional use.

They produce results equivalent to or better than1 those obtained with reference laboratory methods; results are also comparable to those obtained with the patient's device, the CoaguChek XS system, as they use the same technology and the same strips.

CoaguChek XS Plus system

CoaguChek XS Pro system

YOUR BENEFIT

Safety and Confidence

- Onboard control on every strip plus optional liquid controls
- Optional operator and QC lockouts
- Integrated barcode scanner with the CoaguChek XS Pro, for safe, easy patient identification
- Over 20 years' experience from Roche in INR monitoring.

Improved Workflow and Convenience

- Approximately 1 minute to get an accurate INR result from 8 µL whole blood
- Easy blood application: top- or side dosing.

Product Characteristics

- Test principle and measuring range is the same as on the CoaguChek XS system

- User interface: large touchscreen (TFT display—clear screen like a laptop); screen icons allow intuitive operation
- Memory capacity: 2,000 test results with date and time
- Liquid control available for dedicated QC requirements
- Extended data management capabilities.
  - Industry standard POCT1-A or Roche internal protocol for IT connectivity (to the cobas IT 1000 application)
  - Complete documentation of results including patient and operator identification.
- Automatic code chip identification to match lot-specific information with test strips in use
- Liquid control available for dedicated QC requirements.

For more information please visit www.CoaguChek.com

ACCUTREND® PLUS SYSTEM

Screening for Cardiovascular Risk Factors

The Accutrend Plus system is a flexible, hand-held point-of-care device for the key parameters used to detect cardiovascular disease:

- Total cholesterol
- Triglycerides
- Glucose and lactate

This cost-effective, all-in-one device provides rapid, yet accurate results.

YOUR BENEFIT

On the Spot Results

- Point-of-care lipid testing can substantially improve identification and management of dyslipidemic patients in primary care
- Make immediate recommendations regarding lifestyle or treatment, leading to improved patient compliance and loyalty.

Safety and Reassurance

- Built-in automatic performance testing and meter self-testing for reliable results.

Ease of Use

- Simplicity makes device ideal for testing in the physician office or in hospital settings.

Test	Measuring ranges		Measuring time	Sample material	Sample volumes	Operating conditions
	mg/dL	mmol/L				
Glucose	20–600	1.1–33.3	12 sec	• Fresh capillary blood	15–50 µL	18°–35°C
Cholesterol	150–300	3.88–7.76	180 sec	• Fresh capillary blood • Use of heparin-coated pipettes possible	15–40 µL	18°–35°C
Triglycerides	70–600	0.80–6.86	max. 174 sec	• Fresh capillary blood • Use of heparin-coated pipettes possible	10–40 µL	18°–30°C
Lactate	0.8–22 mmol/L		60 sec	• Fresh capillary blood • Use of heparin-coated pipettes possible	15–50 µL	5°–35° or 15°–35°C depending on concentration of analyte

Product Characteristics

- Convenient determination of cholesterol, triglycerides, glucose and lactate using capillary blood
- Positive control strip and parameter recognition are used for calibration
- Test strips can be stored at room temperature
- Can store up to 100 different measurements with date, time and flags
- Great precision and accuracy across the measuring range

REFLOTRON® PLUS SYSTEM
REFLOTRON® SPRINT SYSTEM

Flexible Testing to Support Your Clinical Decisions

The Reflotron® Plus system is a single-test clinical chemistry system which allows the measurement of 17 parameters from whole blood, plasma or serum – including liver and pancreas enzymes, metabolites, blood lipids, hemoglobin and potassium.

Immediate and reliable test results ensure quick performance and verification of the diagnosis without delay.

The system is suitable for primary care settings, as a back-up system in hospitals and private labs, at screening sites and for health check-ups.

YOUR BENEFIT

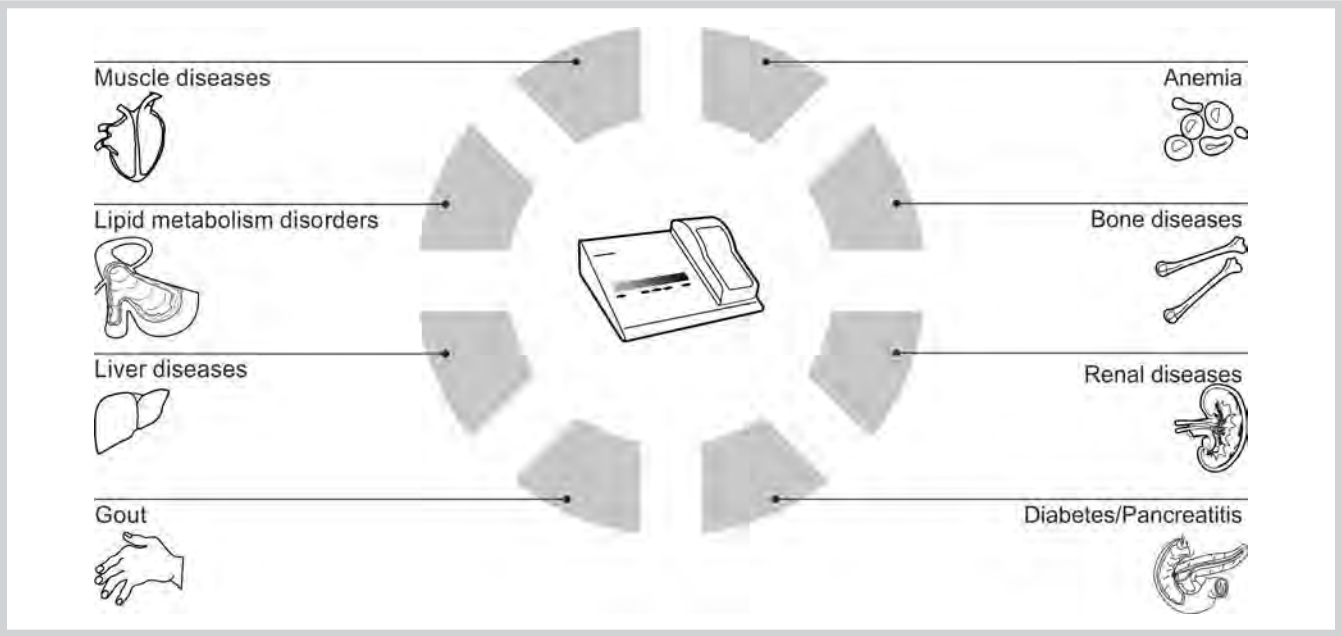
Reliability

- Test results, correlating well with standardized laboratory methods and validated in a number of clinical studies even from capillary samples
- No storage concerns due to excellent test strip stability
- Little waste and almost no maintenance.

Reflotron Plus system

Reflotron Sprint system

Covering a Wide Range of Daily Routine and Emergency Testing



Faster Clinical Decision Making

- Quick time to result
- No reagent preparation.

PRODUCT CHARACTERISTICS

- Throughput of Reflotron Sprint: Up to approx. 60 tests/hour
- Throughput of Reflotron Plus: Up to approx. 25 tests/hour
- Sample material: whole blood (capillary and venous) plasma or serum
- Sample volume: 30 µL
- Time-to-result: only 2–3 minutes (depends on parameter)
- Integrated printer: Immediate documentation of results
- Barcode reader and/or keyboard for patient and sample ID input.

COBAS B 101 SYSTEM

Managing Diabetes and Dyslipidemia at the Point of "Need"

The cobas b 101 system is an IVD test system offering HbA1c and a complete lipid profile (CHOL, HDL, LDL, TG) on one device at the point of care. Capillary blood, whole blood and plasma* can be used.

The system delivers fast and reliable results and is intended for professional use in a clinical laboratory setting or at point-of-care locations.

* Plasma for Lipid Panel only.

YOUR BENEFIT

Test Precision and Guideline Compliant

- Cobas b 101 system complies with all relevant standards and methods (IFCC/DCCT and CDC/NCEP).

Easy and Safe Operation

- Both tests can be performed from one finger prick
- No calibration needed, checking sample integrity, full process control, configurable display of results.

Fast Turnaround Time

- An intuitive 15 minutes workflow from patient preparation to result of both HbA1c and lipid panel.

PRODUCT CHARACTERISTICS

- User-friendly with a large touchscreen, full keyboard, and multiple language support
- Robust, maintenance- and calibration-free with a wide operating temperature and humidity range
- Connection to the cobas POC IT solution
- External printer or barcode scanner allow an improved workflow and documentation
- Data download to USB stick or direct to PC are possible.

Disc Features

- Sample volume easily from one finger stick, fast and easy with direct sample application (no capillaries, tubes or pipettes are needed)
  - HbA1c ≤2 µL in ≤340 sec
  - Lipids ≤19 µL in ≤385 sec
- Discs are color-coded and clearly labelled to support correct use. Flap for high operator safety
- Shelf life of more than 13 months
- Both capillary and venous whole blood can be used for lipids and HbA1c testing. Lipid testing can also be done with plasma.

Parameters and Measuring Range in the Therapeutically Important Range

- HbA1c disc:
  - IFCC: 20–130 mmol/mol
  - NGSP: 4–14 %
  - eAG*: 68–356 mg/dL (calculated from HbA1c)
- Lipid disc:
  - CHOL: 50–500 mg/dL
  - TG: 45–650 mg/dL
  - HDL: 15–100 mg/dL
  - LDL*: 1–476 mg/dL
  - Non-HDL and TC/HDL*

IFCC: International Federation of Clinical Chemistry
DCCT: Diabetes Control and Complications Trial
NCEP: National Cholesterol Education Program
CDC: Centers of Disease Control and Prevention
[1] Internal verification data with 4 native samples and 2 controls.
* calculated

MOLECULAR DIAGNOSTICS

Roche is a pioneer in molecular diagnostics. Since 1992 we have been providing innovative tests based on the Nobel Prize-winning polymerase chain reaction (PCR) technology.

Thanks to our wide range of products, services and solutions we are able to cover the needs of different types of hospitals and laboratories worldwide. Roche provides solutions for indication areas such as hepatitis, HIV, transplantation, women's health, oncology, genomics and microbiology. These solutions are designed to provide information that allows healthcare professionals to diagnose diseases and monitor patients' response to therapy. In addition we offer a range of products to identify the molecular characteristics of patients and diseases, thus enabling personalized healthcare.

Roche products also help to ensure the safety of blood and blood products by using Roche Molecular Diagnostics approved systems to screen donations.

Besides molecular diagnostic solutions, we also provide a range of innovative products for nucleic acid purification and PCR in the field of molecular biology.

For more information please visit www.molecular.roche.com

SOLUTIONS FROM ROCHE FOR MOLECULAR DIAGNOSTICS

Innovative, Reliable and Efficient

To meet the requirements for safe, high-quality PCR diagnostics, Roche has developed the concept of flexible, easy to combine system modules. Depending on test requirements and sample volumes, these modules can provide a customized, efficient solution for every laboratory.

YOUR BENEFIT

- **Efficient workflow**
- Innovative real-time PCR technology **meets international guidelines** for sensitivity and linear measurement range
- **Reliable results** due to AmpErase prevention of enzyme contamination, useof internal controls and automation.

Workflow Solutions for Molecular Diagnostics

Laboratory needs	*Sample purification*	*PCR system*
Women's health and genetic/oncology parameters		
• **Low to high throughput**	**cobas**® 4800 system	
	cobas x 480 instrument	**cobas z** 480 analyzer
		
• **High throughtput** • **Enhanced automation**	**cobas p** 480 instrument, **cobas** 4800 system	
		

Contd...

Contd...

Microbiology and special virology assays and customizable assay protocols		
• **Low and medium throughput** • **Customizable assay protocols**	High Pure or MagNA Pure LC 2.0 System	LightCycler® 2.0 System
		
Infectious diseases/virology		
• **Low throughput**	Manual	COBAS® TaqMan® 48 Analyzer
		
• **Medium throughput**	Manual or COBAS® AmpliPrep Instrument	COBAS® TaqMan® 48 Analyzer
		
• **High throughput** • **Back-up system** • **Flexibility**	COBAS® AmpliPrep Instrument	3 × COBAS® TaqMan® 48 Analyzer
		
• **High throughput** • **Full automation**	**cobas p** 630 instrument, COBAS® AmpliPrep/COBAS® TaqMan® system	
		
	cobas s 201 system (Blood screening)	
		
low <2,000 tests/year; medium >2,000 tests/year; high >6,000 tests/year.		

TEST OVERVIEW

Parameter	*Test kit*	*Detection*	***cobas s** 201 system*	*LightCycler® 2.0 Analyzer*	***cobas®** 4800/cobas z 480 instrument*	*COBAS® Amplicor/ Amplicor Analyzer*	*COBAS® TaqMan® Analyzer*
Viruses							
Cytomegalo	COBAS® AmpliPrep/COBAS® TaqMan® CMV test	quant	•				
	LightCycler® CMV quant test					•	
Epstein Barr	LightCycler® EBV quant test					•	
Hepatitis A	LightCycler® Hepatitis A Virus quantification kit					•	
Hepatitis B	COBAS® AmpliPrep/COBAS® TaqMan® HBV test, v2.0		•				
	COBAS® TaqMan® HBV test for use with High Pure system		•				
Hepatitis C	COBAS® AmpliPrep/COBAS® TaqMan® HCV qualitative test, v2.0	qual	•				
	COBAS® AmpliPrep/COBAS® TaqMan® HCV quantitative test, v2.0	quant	•				
	COBAS® TaqMan® HCV test for use with High Pure system, v2.0		•				
	LINEAR ARRAY HCV genotyping test	genot		•			
Herpes	LightCycler® HSV 1 and 2 qual test	qual and diff				•	
	cobas® HSV 1 and 2 test				•		
Human immunodeficiency	COBAS® AmpliPrep/COBAS® TaqMan® HIV test, v2.0	quant	•				
	COBAS® TaqMan® HIV test for use with High Pure system, v2.0		•				
	COBAS® AmpliPrep/COBAS® TaqMan® HLA-B*5701 screening test	qual	•				
	COBAS® AmpliPrep/COBAS® TaqMan® HIV qualitative (for research only)		•				
Human papilloma	**cobas** HPV test	qual/genot			•		
	LINEAR ARRAY HPV genotyping test	genot		•			
	AMPLICOR® Human Papillomavirus test	qual/genot		•			
Parvo B19	LightCycler® Parvo B19 quantification kit (for research only)	quant				•	
Varicella-zoster	LightCycler® VZV qual test	qual				•	
Other pathogens							
Chlamydia trachomatis/ Neisseria gonorrhoeae	**cobas®** 4800 CT/NG test	qual			•		
	COBAS® AMPLICOR CT/NG			•			
Chlamydia trachomatis	COBAS® TaqMan® CT test	qual	•				
Chlostridium difficile	**cobas®** 4800 Cdiff test				•		

Contd...

Contd...

Methyllicin resistant *Staphylococcus aureus*	LightCycler® MRSA advanced	qual and diff				•	
	cobas® MRSA/SA test				•		
Mycobacteria Tuberculosis	COBAS® TaqMan® MTB test	qual.	•				
Vancomycin resistant *Enterococcus*	LightCycler® VRE					•	
Sepsis pathogens							
Bacteria/Fungi	LightCycler® SeptiFast test MGRADE	qual and diff				•	
	LightCycler® SeptiFast mecA test MGRADE	qual and ident				•	
Blood screening							
HIV-1*, HIV-2, HCV, HBV	**cobas® TaqScreen** MPX Tests	qual/diff					•
B19V/HAV	**cobas® TaqScreen** DPX test						•
West Nile virus	**cobas® TaqScreen** WNV test	qual					•
Oncology							
BRAF	**cobas**® 4800 BRAF V600 mutation test	qual (mutation detection)			•		
KRAS	**cobas**® KRAS mutation test				•		
EGFR	**cobas**® EGFR mutation test				•		
PIK3CA	**cobas**® PIK3CA Mutation Test (research use only)	qual and ident			•		
BCR-ABL	LightCycler® t(9;22) quantification kit (for research only)	relative quant				•	
Genetics							
Factor V Leiden	Factor V Leiden kit	qual (mutation detection)				•	
Factor II	Factor II (Prothrombin) G20210A kit					•	
HLA-B*5701	COBAS® AmpliPrep/COBAS® TaqMan® HLA-B*5701 screening test	qual	•				

* Groups M and O
qual = qualitative; quant = quantitative, genot = genotyping; diff = differentiation; ident = identification

COBAS P 630 INSTRUMENT

The Pre-analytics Solution that Makes Life Easier

The **cobas p** 630 instrument offers in combination with the COBAS AmpliPrep/COBAS TaqMan system a fully automated pre-analytical solution for primary tube handling. The system automatically pipettes primary and secondary tubes and controls into sample input tubes for the COBAS AmpliPrep instrument.

The **cobas p** 630 instrument can be combined with up to 3 COBAS AmpliPrep Instruments and AmpliLink software to ensure full traceability of workflow.

YOUR BENEFIT

Efficiency

- Automated handling of primary and secondary tubes.

Flexiblility

- Compatible with a variety of sample tubes
- Modular design.

Full Traceability

- Barcode tracking from patient tube to result.

Process Surveillance

- Monitors liquid handling.

PRODUCT CHARACTERISTICS

- Uncapping and recapping of the sample tube
- Pipetting Roche controls from control tubes to sample tubes
- Pipetting samples from primary and secondary tubes to sample tubes
- Multiple tests can be ordered on a single primary tube
- Only one LIS interface required.

Unit Dimensions

- 112 cm wide, 101 cm deep, 90 cm high.

Sample Processing Throughput

- 320 samples on board
- 154 tubes per hour for 650 µL samples
- 149 tubes per hour for 1.0 mL samples
- 157 tubes per hour for 500 uL samples.

COBAS® AMPLIPREP INSTRUMENT

Nucleic Acid Purification Made Simple

The COBAS AmpliPrep Instrument automates purification of DNA and RNA using magnetic bead technology. Elimination of time-consuming and fault-prone manual sample preparation increases efficiency and safety in the laboratory. The COBAS AmpliPrep Instrument can be combined with the COBAS TaqMan or COBAS TaqMan 48 analyzer and thereby offer a custom solution for each PCR laboratory.

YOUR BENEFIT

Safety and Reliability

- Closed tubes for samples and purified nucleic acids minimize contamination

- Sample tracking with barcoded tubes prevents sample mix-ups.

Efficiency

- Handles up to 4 tests simultaneously; continuous reloading during the run
- Ready to use reagents—no aliquotting or mixing required
- Overnight runs
- Additional generic sample preparation for other PCR systems increases the versatility of the instrument.

PRODUCT CHARACTERISTICS

- Ready-to-use reagents in barcoded cassettes
- Detection of liquid level and clots
- Controllable via data station with AmpliLink software, for laboratory integration with LIS
- Barcoded data input.

Unit Dimensions

- 165 cm wide, 75 cm deep, 95 cm high.

Capacity

- 72 samples; up to 144 purifications per day.

Throughput

- approximately 15–24 samples/hr.

COBAS® TAQMAN® ANALYZER AND COBAS® TAQMAN® 48 ANALYZER

Innovation for Routine PCR

The COBAS TaqMan 48 analyzer is a compact benchtop instrument that minimizes manual steps and shortens analysis times with innovative real-time PCR technology. Two independent thermocyclers allow two parameters to be processed in parallel.

For higher throughput needs, a higher-capacity COBAS TaqMan 96 analyzer provides automated real-time amplification and detection of DNA or RNA for up to 96 samples and four assays at the same time. Samples can be prepared automatically on the COBAS AmpliPrep Instrument. The combination of innovation and flexibility ensures efficient workflow in routine PCR laboratories with low to medium throughputs. The COBAS TaqMan Analyzer combined with the COBAS AmpliPrep Instrument and docking station is the solution for higher throughput PCR.

COBAS® TaqMan® 48 Instrument

COBAS® TaqMan® Analyzer

YOUR BENEFIT

Efficiency and Reliability for Routine PCR

- Reliable results within 2–3 hours
- Sensitive, highly linear tests can handle both low titer and high titer samples in the same run
- Greater safety due to AmpErase enzyme contamination prevention and internal controls for detecting possible PCR inhibitors.

PRODUCT CHARACTERISTICS

COBAS TaqMan 48 Analyzer

- Compact desktop model
- 2 independent thermocyclers, each with 24 positions
- Real-time PCR assays using hydrolysis probes
- 48 samples in 2,5 to 3,5 hours (depending on parameters).

COBAS TaqMan Analyzer

- A docking station can combine COBAS AmpliPrep Instrument and COBAS TaqMan analyzer into a single, fully automated system that can perform sample preparation, PCR set-up and amplification/detection
- 4 independent thermocyclers, each with 24 positions
- Run time: 2.5–3.5 hours
- 192 samples in 24 hours.

TEST MENU

With Manual Sample Preperation

- HCV quantitative
- HBV quantitative
- HIV-1 quantitative
- *Chlamydia trachomatis* qualitative
- *Mycobacterium tuberculosis* qualitative

With Automated Sample Preparation

- HCV qualitative and quantitative
- HBV quantitative
- CMV quantitative
- HIV-1 quantitative
- HLA – B*5701
- HIV-1 qualitative*

COBAS® AMPLIPREP/COBAS® TAQMAN® HCV QUALITATIVE AND QUANTITATIVE TESTS, V2.0

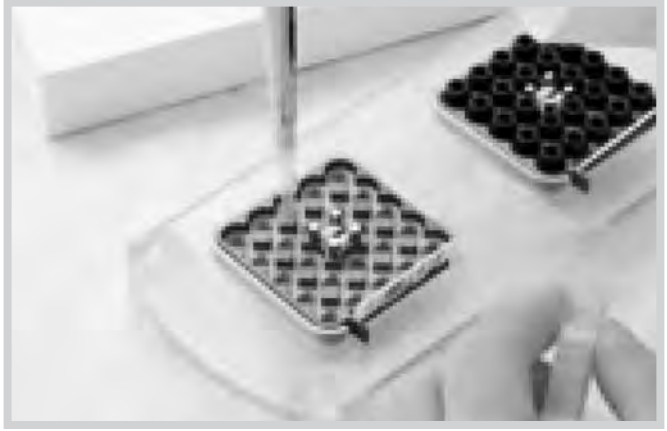
Empowering Change in HCV

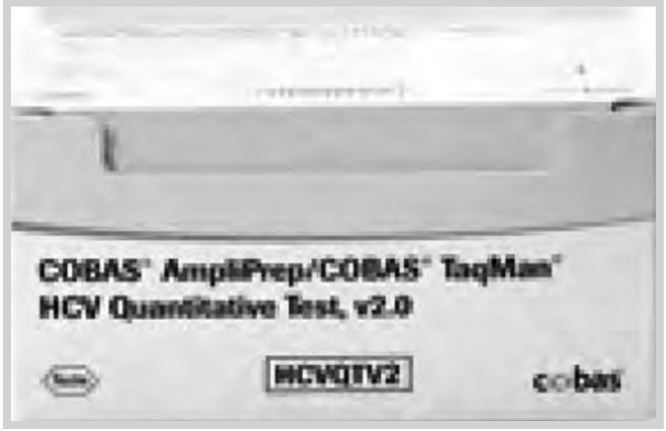
COBAS AmpliPrep/COBAS TaqMan HCV Qualitative Test, v2.0 and Quantitative Test, v2.0

The version 2.0 tests are developed with a lower input volume, and innovative dual-probe design provides improved sensitivity and precise detection across all genotypes for the new era of direct acting antiviral agents (DAAs) to distinguish true signal from background noise.

The COBAS AmpliPrep/COBAS TaqMan HCV Qualitative Test, v2.0

The test completes the molecular diagnostic tools in HCV diagnosis. It is indicated for patients who have clinical and/or biochemical evidence of liver disease and antibody evidence of HCV infection, and who are suspected to be actively infected with HCV. Detection of HCV RNA indicates that the virus is replicating and therefore is evidence of active infection.





YOUR BENEFIT

- Reliable results by enhanced mismatch tolerance and coverage of all genotypes
- Economic sample usage
- Excellent sensitivity to meet guidelines.

PRODUCT CHARACTERISTICS

- Kit configuration 72 tests/kit
- Sample types EDTA plasma and serum
- Sample input volume 650 µL
- Limit of detection 15 IU/mL
- Genotype inclusivity genotypes 1 through 6
- Diagnostic sensitivity 100%
- Specificity 99.9%.

Workflow

- Confirm active infection and monitor HCV viral load on the same system
- Flexible batch size with continuous loading
- Interleave with other COBAS TaqMan tests (HIV-1, HBV).

COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, v2.0

- The test can be used to assess the probability of a sustained viral response early in a course of antiviral therapy and to assess viral response to antiviral treatment as measured by changes in serum or plasma HCV RNA levels.

YOUR BENEFIT

- Precisely distinguish true signals from background noise for more accurate viral load results
- **Reliable results** by enhanced mismatch tolerance and coverage of all genotypes
- **Perfect tool to aid in response-guided therapy** with excellent sensitivity and specificity delivering accurate results
- **Economic sample usage** required which provides laboratory with enough left over sample for other laboratory testing.

PRODUCT CHARACTERISTICS

- Kit configuration 72 tests/kit
- Sample types EDTA plasma and serum
- Sample input volume 650 µL
- Limit of detection 15 IU/mL
- Linear range 15 IU/mL – 1E108 IU/mL
- Genotype inclusivity genotypes 1 through 6
- Diagnostic sensitivity 100%
- Specificity 100%.

Workflow

- Confirm active infection and monitor HCV viral load on the same system
- Flexible batch size with continuous loading
- Interleave with other COBAS TaqMan tests (HIV-1, HBV).

COBAS® TAQMAN® MTB TEST

Rapid MTB Detection

Tuberculosis is the world's most common infectious disease, with two million deaths annually. Due to the risk and severity of the disease, rapid diagnosis of the *M. tuberculosis*-complex is extremely important. Routine cultures are time-consuming and can take up to eight weeks. Microscopic examination of acid-fast smears is

Roche Offers a Complete Continuum of Care to Run the Key Tests for the Diagnosis and Management of HCV

HCV antibody test HCV RNA qualitative test: Confirmation of antibody-positive specimens		HCV RNA quantitative test: Viral load monitoring HCV		HCV RNA quantitative test: Viral load monitoring
Diagnosis	Treatment decision	On treatment	Evaluate treatment	End of treatment and follow-up (SVR)
	HCV RNA quantitative test: Viral load monitoring		HCV RNA quantitative test: Viral load monitoring	
Key steps in the diagnosis and management of HCV				

COBAS TaqMan 48 Analyzer and MTB kits

insensitive and nonspecific. The COBAS TaqMan MTB test has further improved the rapid diagnosis of tuberculosis by allowing direct detection of mycobacteria in clinical specimens.

YOUR BENEFIT

- **Fast results** in only 3.5 hours including sample preparation
- **Reliability of test results**
  - high sensitivity and specificity
  - clear differentiation of the pathogen from atypical mycobacteria (MOTT)
  - contamination protection through AmpErase System
- **Efficient workflow,** no manual steps required after sample preparation
- **Proven and safe sample preparation** with the AMPLICOR respiratory specimen preparation kit.

PRODUCT CHARACTERISTICS

- Detects pathogens of the *Mycobacterium tuberculosis complex (M. tuberculosis, M. bovis, M. africanum, M. microti)*
- Test is performed on the IVD CE-marked COBAS TaqMan 48 analyzer that allows variable batch sizes – between 1 and 48 tests per run
- Internal controls included in the same reaction batch
- Specificity: 99%
- Sensitivity: 0.46 CFU/PCR, corresponding to a calculated concentration of 18 CFU/mL sputum.

COBAS P 480 INSTRUMENT

Automating Your Primary Vial Preprocessing Steps

The **cobas p** 480 instrument reduces laboratory hands-on-time, and offers a fast, reliable way to uncap and recap PreservCyt® and SurePath liquid based cytology vials as well as **cobas**® PCR Media tubes. The instrument allows primary vials to be loaded directly onto the **cobas**® 4800 system, without a need to aliquot into a secondary vial. It provides significant workflow and sample integrity advantages improving lab workflow and eliminating repetitive motions.

YOUR BENEFIT

Improve Laboratory Efficiency

- Allows multiple vial types to be loaded in a single decapping operation
- Process 4 vials simultaneously

- High throughput operation allows a single instrument to support more than one analytic system.

Reduce Hands on Time and Eliminate Repetitive Motion

- Automated uncapping, recapping and vortexting
- Minimizes the risk of sample mix-up or user error
- Compatible with BD SurePath, Hologic PreserCyt and cobas PCR media vials
- Intuitive interface requires minimal training
- Barcode quality checks prevents costly delays in downstream processing.

Improve Sample Reproducibility and Process Reliability

- Automated vortexing processes specimens consistently from first to last, regardless of throughput
- Precision movements reduce opportunity for cross contamination
- Helps ensure reliability of the test results
- NO LIS or data connection required.

Replacement Caps Ensure a Quality Seal

- Quality seal ensures that the sample are well protected for transport, storage or other testing needs
- New replacement caps packaged for easy loading and automated recapping
- Replacement caps available for all compatible vial types
- Offers better seal integrity compared to parafilm or cellophane over pierced or open vial containers.

COBAS® 4800 SYSTEM V2.0

Keeping Pace with Changing Needs

The **cobas** 4800 system offers state-of-the-art, fully automated sample preparation, real-time PCR amplification/detection and easy-to-use software for multiple sample types (the detection of *C. trachomatis (CT), N. gonorrhoeae (NG)*, HPV (human papillomavirus) and an expanding menu of assays.

It consists of the **cobas x** 480 instrument for the nucleic acid extraction sample preparation and PCR pipetting and the **cobas z** 480 real-time PCR analyzer.

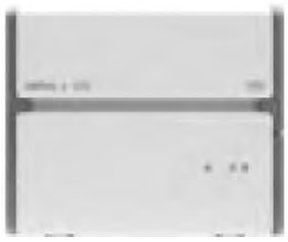
The **cobas z** 480 analyzer is also available as single system and can be used for parameters in the oncology field like BRAF, KRAS and EGFR.

YOUR BENEFIT

Reliable Results

- Proprietary kinetic algorithm software provides clear and precise answers reducing the need for retesting or interpretation.

Cobas x 480 instrument



Cobas z 480 analyzer

Efficiency

- By fully automated sample preparation and PCR set-up (for HPV and CT/NG)
- By bidirectional connectivity with your LIS for automated results reporting.

Flexibility

- Possibility to use multiple primary vial types
- User defined workflow software for free programmable PCR applications.

Load-and-go Reagents

- Save time and labor
- Low daily maintenance requirements.

TEST MENU

Cobas® 4800 HPV Test

- Only FDA approved hr HPV assay which simultaneously detects 14 high-risk HPV genotypes, including individual identification of HPV genotypes 16 and 18.

Cobas® 4800 CT/NG Test

- Test is designed to run as CT only.

NG Only or as CT/NG Combination

- Highest specificity for NG and detection of Swedish CT mutant and other variants due to dual target detection

Oncology Tests

- **Cobas**® 4800 BRAF V600 mutation test
- **Cobas**® KRAS mutation test
- **Cobas**® EGFR mutation test
- **Cobas**® PIK3CA mutation test (for research use only).

Hospital Aquired Infections

- **Cobas**® MRSA/SA test
- **Cobas**® Cdiff test

Viral Infections

- **Cobas** HSV 1 and 2 test.

PRODUCT CHARACTERISTICS

- Processes up to 376 samples in 10 h
- Bidirectional connectivity to LIS
- Easy to use software
- Automated result interpretation for HPV and CT/NG.

Components:

Cobas x 480 Instrument

- Fully automated nucleic acid purification
- Automated PCR set up
- Dimensions: 166 cm width, 90 cm depth, 101 cm high.

Cobas z 480 Analyzer

- Based on LightCycler® 480 technology
- 6 detection channels
- 96 well plate format
- Dimensions: 57 cm width, 59 cm depth, 50 cm high.

THE COBAS® HPV TEST

Know the Risk

Almost all cervical cancer is attributable to HPV, so knowing a woman's HPV status is important to ascertain her risk of cervical cancer and to determine clinical management.

The cobas® 4800 HPV test is the only clinically validated CE-marked, and FDA-approved assay, that simultaneously provides results on "high-risk" genotypes, including individual results on the highest-risk genotypes, HPV 16 and HPV 18, giving three results in just one test. HPV genotypes 16 and 18 are known to be responsible for more than 70 percent of all cervical cancer cases.

This test enables physicians to focus on the few patients who need more aggressive treatment or careful management, and reassures the vast majority of women they are at very low-risk, protecting them from potentially unnecessary interventions.

YOUR BENEFIT

Evidence Based

- Clinically validated in Roche's landmark ATHENA trial, the largest US based registration study for cervical cancer screening, including more than 47,000 women
- One in 10 women in the landmark ATHENA study who tested positive for either HPV genotype 16 or 18 had evidence of cervical pre-cancer, even though their pap was normal.

Clinically Relevant Results

- Knowing the patients HPV 16/18 status may impact patient management and allow better risk stratification of the patients at the highest risk.

Reort with Confidence

- Internal control for assurance of sample integrity
- No cross reactivity with low risk HPV genotypes
- Efficiency
- Suited for high volume screening programs
- By fully automated sample preparation workflow process, and unique efficiency feature.

PRODUCT CHARACTERISTICS

Coverage

- Identifies (types) HPV 16 and HPV 18 while concurrently detecting the rest of the high risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) at clinically relevant infection levels.

Sample Material

- Cervical cells collected in cobas® PCR cell collection media (Roche Molecular Systems, Inc.), PreservCyt® solution (Cytyc Corp.) and SurePath® preservative fluid (not approved in the US) (BD Diagnostics-TriPath)
- Sample volume of 1 mL is sufficient.

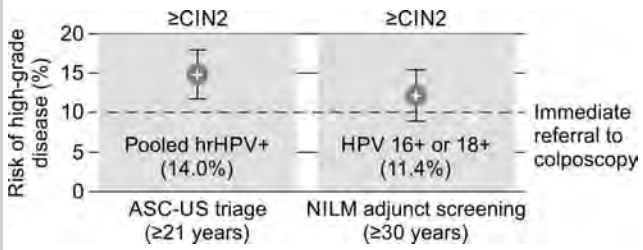
Test Principle

- Multiplex assay to detect 12 pooled high risk genotypes, with simultaneous individual genotyping for highest risk HPV 16 and 18
- Beta-globin acts as control for extraction and amplification.

Throughput

- up to 282 tests in less than 12 hours.

Absolute Risk of ≥CIN2 by Screening Strategies Assessed in ATHENA at Baseline



1 in 10 women ≥30 years of age with negative cytology who tested positive for HPV 16/18 using the cobas HPV test had underlying precancerous lesions. Women with negative pap cytology who are HPV 16+ and/or HPV 18+ and women with ASC-US who are pooled hrHPV+ share a similar absolute risk of precancer and should be managed similarly with immediate referral to colposcopy.

THE COBAS® ONCOLOGY TESTS

7–10 days is a Long Time to Wait when Everyday Counts

The cobas oncology portfolio exemplifies Roche's commitment to personalized healthcare. The tests detect mutations in key biomarkers which helps identify patients who are most likely to respond to certain drug treatments.

These clinically validated companion diagnostics help physicians make therapy decisions for patients suffering from metastatic melanoma, colorectal cancer, and non-small cell lung cancer. Due to the short testing time physicians can make decisions in hours instead of days when using alternative methods. The cobas oncology menu will be expanded during the next years.

YOUR BENEFIT

Reliable Results

- Complete and controlled IVD system consisting of cobas DNA sample Preparation Kit, cobas BRAF, KRAS, EGFR, and PIC3CA (RUO) mutation tests, and the cobas® 4800 system, v2.0.

Consistent, Objective and Reproducible Results

- Automated result interpretation and test reporting provide from laboratory to laboratory.

Fast Result Reporting

- Delivering patient results in <8 hours.

TEST MENU

cobas 4800 BRAF V600 Mutation Test

- Identifies which metastatic melanoma patients can be considered for BRAF inhibitor therapy, e.g. Zelboraf®
- Detects V600E mutations of the BRAF gene (<5% mutant copies in formalin-fixed, paraffin-embedded tissue [FFPET]); also sensitive to V600K and V600D
- 24 reportable results from a single test kit
- Only requires one 5 µm tissue section with >50 % tumor area for the PCR reaction.

cobas KRAS Mutation Test (CE-IVD)

- Offers broad mutation coverage of KRAS codons 12, 13 and 61 to identify colorectal cancer patients not likely to respond to anti-EGFR monoclonal antibody therapies, e.g. erbitux, vectibix
- Detects all of the reported mutations in codons 12, 13 and 61 of the EGFR gene (<5 % mutant copies in FFPET)

* In US, coverage is Exon 19 and 21 only

- 24 reportable results from a single test kit
- Only requires one 5 µm tissue sections with ≥10% tumor area for the PCR reaction.

Cobas EGFR Mutation Test

- Identifies patients with non-small cell lung cancer who benefit from anti-EGFR TKI therapy, e.g. Tarceva®
- Specific detection of 41 mutations (insertions and deletions) in exons 18, 19, 20 and 21* of the EGFR gene (≤5% mutant copies in FFPET)
- 24 reportable results from a single test kit
- Only one 5 µm tissue section with ≥10% tumor area for the PCR reaction.

Cobas DNA Sample Preparation Kit

- Clearly defined workflow
- Validated with FFPET samples
- Isolation time: 3–4 hours only.

Assay Specific Analysis Packages

- Software package containing cycling conditions, algorithms and calculations for automated interpretation and report of results.

COBAS® MRSA/SA TEST

Faster than a Spreading Infection

Staphylococcus aureus (SA) and methicillin-resistant *Staphylococcus aureus* (MRSA) infections represent a critical threat to public health. The cobas MRSA/SA test, performed on the cobas® 4800 system, provides innovative solutions for detecting both organism variances from a single nasal swab specimen, providing timesaving efficiencies and lifesaving answers.

YOUR BENEFIT

Exceptional Performance

- Quickly identify colonized patients and take decisive action
- Get the sensitivity and specificity that only PCR technology can deliver.

Greater Workflow Efficiencies

- Save time with first-of-its-kind primary sample vial loading
- Run MRSA/SA, Cdiff, and HSV 1 and 2 samples at the same time, on the same system
- Simplify data interpretation with patented, state-of-the-art software algorithms.

Automated Efficiency

- Run 6 to 94 specimens using the fastest, most advanced real-time PCR amplification and detection available today.

COBAS® CDIFF TEST

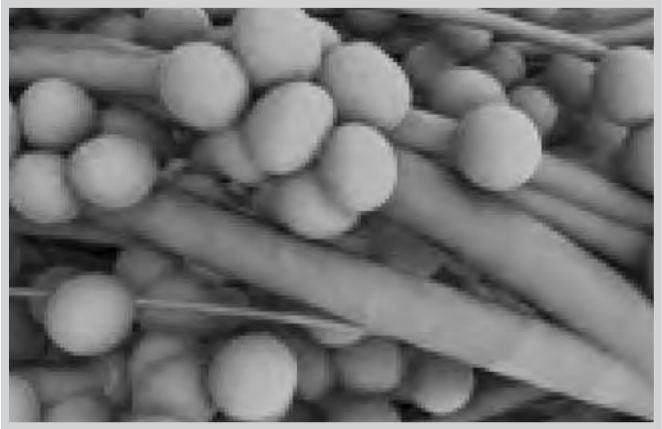
The Right Result the First Time

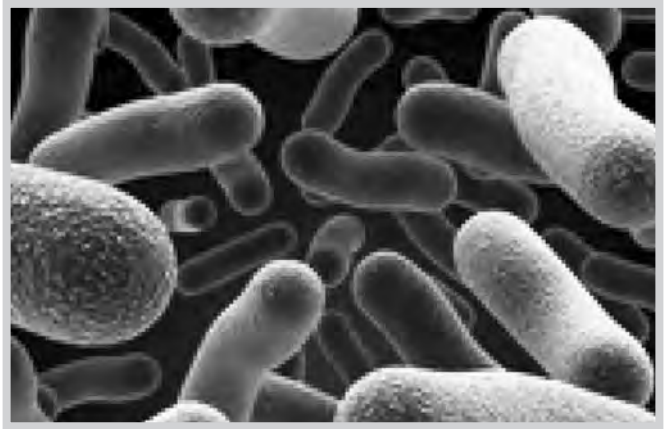
Clostridium difficile (C. difficile) infection is a major cause of diarrhea in healthcare facilities. By rapidly detecting Cdiff in patient stool samples, the **cobas** C diff test, which is performed on the **cobas**® 4800 system, provides accurate information for timely treatment and prevention.

YOUR BENEFIT

Exceptional Performance

- Selectively detects a specific C diff toxin gene directly from unformed stool samples using real-time PCR





- Generates robust results automatically, using patented, state-of-the art algorithms
- Detects the presence of 31 Cdiff toxinotypes and 20 ribotypes.

Confidence in Results

- Minimizes invalids and need for repeat testing resulting in cost efficiency
- Reduces possibilities for errors.

COBAS® HSV 1 AND 2 TEST

Bring More to Your Sexually Transmitted Infections Menu

Due to extremely different outcomes regarding recurrence, it is essential to determine whether a patient has type 1 or type 2 herpes simplex virus. The **cobas** HSV 1 and 2 test, which runs on the **cobas** 4800 system, offers exceptional sensitivity while delivering reliable answers that result in optimal patient treatment and management decisions.

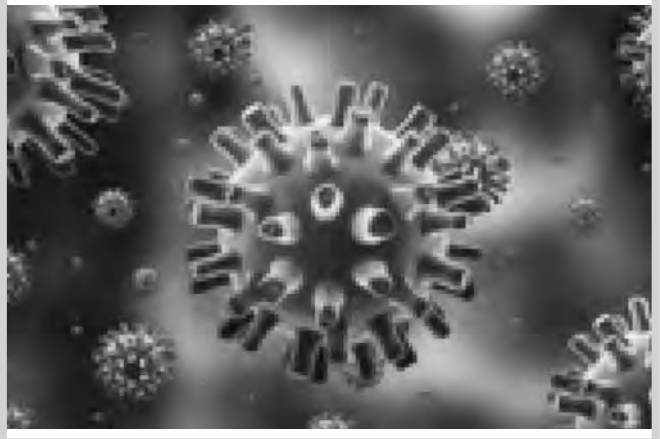
YOUR BENEFIT

Amplified Reliability

- Robust, dual-target detection amplifies two separate regions on each of the HSV-1 and HSV-2 genomes
- Optimizes sensitivity and specificity
- Ensures reliable results as new HSV strains emerge.

Reduced Hands-on Time

- Just load your primary sample vials on the **cobas** 4800 system and you're ready to go.



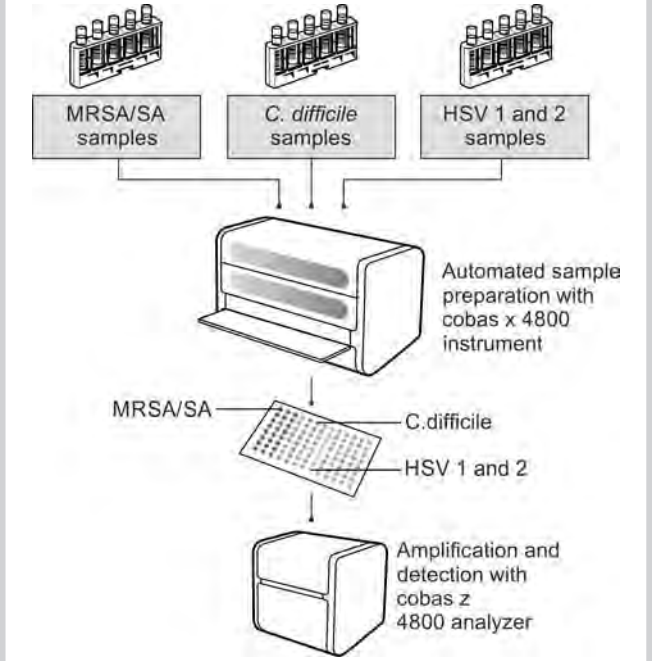
Multiple Assays, One System

- Efficiency at its best – 3 assays in one run: HSV 1 and 2, MRSA/SA and C diff.

Time-saving Flexibility—Use Different Primary Vials and/or Sample Types

- Confident patient tracking—from primary vial to final result.

Mixed Batch Testing on cobas® 4800 System



Parallel Sample Processing Offers the Flexibility to Run Different Tests and Sample Types, Including:

- Stool (**cobas** C diff test)
- Nasal (**cobas** MRSA/SA test)
- Anogenital lesions (**cobas** HSV 1 and 2 test).

COBAS S 201 SYSTEM

The First Multi-dye Nucleic Acid Testing (NAT) Screening System

The cobas s 201 system is a complete NAT solution able to meet both current and future needs of blood screening laboratories.

This system provides the efficiency and reliability of real-time polymerase chain reaction (RT-PCR) technology, modular automation, convenient ready-to-use reagents and a robust menu selection. New assays utilize multi-channel capabilities to provide real-time discrimination of major viruses.

The system is backed by world-class service and strong local support in over 140 countries.

YOUR BENEFIT

- **Full automation** including optional pooling and archiving with minimal hands-on time for the entire testing process
- **Confidence** in the test results through full process control
- Most comprehensive assays on the market with **ready-to-use reagents**
- **Built-in viral target resolution** through multi-dye technology makes confirmation testing obsolete

PRODUCT CHARACTERISTICS

Scalable, Modular System

- Flexible, mix-and-match scalability helps NAT labs work more efficiently
- Supports simultaneous multiple assay processing
- Accommodates integrated backup to maximize laboratory productivity.

Pooling and Data Management Server

- Single server, accommodating multiple instrument configurations and providing the added security of built-in redundancy.

TEST MENU

- Reagents are ready-to-use with built-in contamination control
- No freezers required, reagents are stored at 2–8°C
- Stabilized reagents obsoletes calibrations.

Cobas TaqScreen MPX Tests

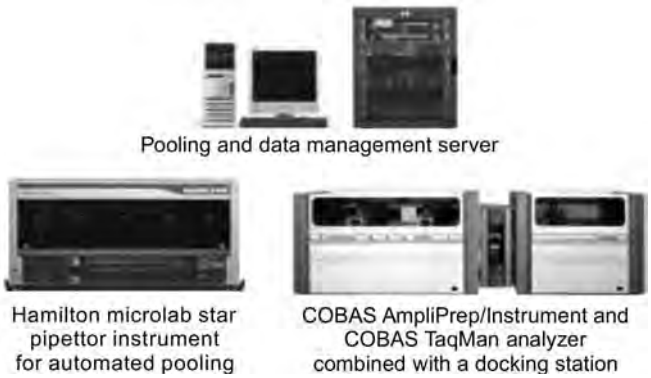
- Covers 5 critical viral targets (HIV-1 Group M, HIV-1 group O, HIV-2, HCV and HBV) in one easy-to-use assay
- Immediate virus discrimination in a single assay, no need for virus discriminatory testing.

Cobas® TaqScreen DPX Test

- Simultaneous quantitative detection
- of parvovirus B19V DNA and qualitative detection of HAV
- B19V target values are traceable to the WHO B19V International standard.

Cobas TaqScreen WNV Test

- Qualitative in vitro test for the direct detection of West Nile virus (WNV) RNA in human plasma



Pooling and data management server

Hamilton microlab star pipettor instrument for automated pooling

COBAS AmpliPrep/Instrument and COBAS TaqMan analyzer combined with a docking station

- Screening test for donations of whole blood and blood components
- Capable of detecting other members of flavivirus that have been implicated in fusion transmitted infectious disease.

LIGHTCYCLER® SYSTEMS

Excellence in Real-time PCR

Whether your interest is in gene expression profiling or in detecting genetic variations, there is a member of the LightCycler system family offering the analytical performance and throughput you need for your research. Supported by a broad range of software tools, real-time PCR based analysis can be performed in 32 capillaries or plastic tubes, interchangeable 96-/384-well plates, or using the unique 1536-well format or tube based formats.

For additional information see www.roche-applied-science.com

YOUR BENEFIT

High Precision

- Reproducible results independent of the sample position.

High Flexibility

- Suitable for all common assay formats and dyes.

High Sensitivity

- Even single copies can be detected.

High Operator Convenience

- Data analysis according to your needs.

Versatility

- Absolute or relative quantification, melting curve analysis or genotyping—the software offers all options.

Available Reagents

- Generic kits for PCR and RT-PCR
- Parameter-specific kits research use only
- Parameter-specific kits IVD
- Ready to use custom assays and panels for all available LightCycler systems (e.g. Universal ProbeLibrary and RealTime ready).

PRODUCT CHARACTERISTICS

	LightCycler® 2.0 Instrument	*LightCycler® 480 System (96/384)*	*LightCycler® 96 System*
Throughput	1– 32 reactions	1–96 or 1–384 reactions	1–96 reactions
Hardware	6 detection channels	5 excitation and 6 detection filters	
Disposable	Capillaries	96 or 384 multiwell plates	96 multiwell plates or tube strips
System features	• Excellent temperature homogeneity in all wells/vessels • No need for passive reference dyes • 40 cycles are possible in 40 minutes • Freely programmable protocols, data import and export, creation of macros and templates.		
Assay formats	SYBR Green I, hydrolysis and hybridization probes	SYBR Green I, hydrolysis and hybridization probes	SYBR Green I, hydrolysis probes
Reagents	• Generic kits for PCR and RT-PCR • Ready-to-use custom assays • Parameter-specific kits	• Generic kits for PCR and RT-PCR Ready-to-use custom assays and panels • Parameter-specific kits • Generic kits for PCR and RT-PCR • Ready-to-use custom assays and panels • Parameter-specific kits	

LightCycler® 2.0 Instrument is available as IVD in many countries.
Information about the low throughput LightCycler® Nano System and the high-throughput LightCycler® 1536 System is available on request.

LIGHTCYCLER® 2.0 INSTRUMENT

High Performance that Meets the Needs of IVD

The LightCycler 2.0 System is an innovative real-time PCR platform that uses a fluorescence detection system and high-quality reagents for a wide range of applications in in vitro diagnostics and in medical research.

It offers a multitude of innovative features, ranging from optimized validated software to six different detection channels.

YOUR BENEFIT

- **Safety and ease of use** in the IVD mode, including test-specific reagent kits, and PCR macros that can automate instrument programming, test analysis and result reporting
- The research mode offers **flexible programming, editing and user evaluation** - Versatility in application options, e.g. qualitative and quantitative detection, mutation detection by melting curve analysis and SNP genotyping
- Broad choice of detection formats

PRODUCT CHARACTERISTICS

- Compact desktop model
- 35 cycles in about fast 40 minutes
- Reaction batch of 1–32 samples 20 µL100 µL capillaries

- 6 detection channels for 530, 560, 610, 640, 670, and 710 nm
- Versatile detection formats: SYBR Green, hybridization probes, hydrolysis probes, SimpleProbe probes, Scorpion primers, and other FRET-based detection formats
- Online display of the PCR kinetics.

TEST KITS, VALIDATED FOR IVD

- CMV quantification
- EBV quantification
- HSV 1/2 detection and differentiation
- VZV detection
- MRSA advanced detection
- SeptiFast identification of bacteria and fungi
- SeptiFast mec A resistance screening
- Factor V mutation detection
- Factor II mutation detection.

For Medical Research

- HAV quantification
- Parvo B19 quantification
- VRE resistance screening
- Translocation (9;22) quantification

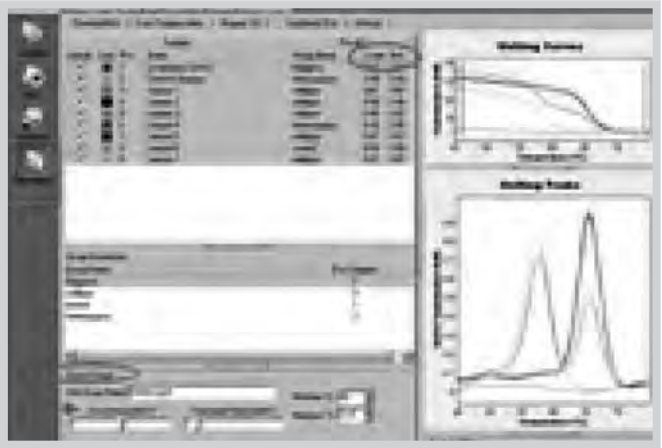
LIGHTCYCLER® SEPTIFAST TEST

Rapid Identification of Sepsis Pathogens

Sepsis is a leading, infectious complication for critically ill patients. It represents about 15% of all nosocomial infections. Despite improvements in medical care, sepsis is still a challenge for internal medicine. Any delay in the management of infection is deleterious, especially in patients whose illness is severe. Shortening this delay is of paramount importance. In the LightCycler Septi*Fast* test, Roche offers a molecular test that detects the presence of microorganisms responsible for approximately 90% of all sepsis cases seen on intensive care units.

				Samples	Cals		
Include	Color	Pos	Name	Combined	Target	Control
☑		1	no template control	Success	Negative	Success
☑		2	Negative Control	Success	Negative	Success
☑		3	Positive Control	Success	Positive	Success
☑		4	Sample 1	Positive	Positive	Success
☑		5	Sample 2	Positive	Positive	Success
☑		6	Sample 3	Negative	Negative	Success
☑		7	Sample 4	Positive	Positive	Success
☑		8	Sample 5	Positive	Positive	Success

Data display for a qualitative detection analysis



Genotyping analysis

YOUR BENEFIT

Broad Coverage of Sepsis Pathogens

- Approximately 90% of all potential sepsis pathogens are detected in a single PCR.

Fast Results with Minimal Sample Volume

- Detection within 6 hours starting with just 1.5 mL of whole blood.

Broad Application

- DNA detection also possible during antibiotic therapy
- Resistance screening possible with the LightCycler® SeptiFast mecA test.

25 Different Pathogens can be Identified with Dem LightCycler® SeptiFast Test

Gram (–) bacteria	*Gram (+) bacteria*	*Fungi*
• *Escherichia coli*	• *Staphylococcus aureus**	• *Candida albicans*
• *Klebsiella* (pneumoniae/oxytoca)	• CoNS (Coagulase negative Staphylococci)	• *Candida tropicalis*
• *Serratia marcescens*	• *Streptococcus pneumoniae*	• *Candida krusei*
• *Enterobacter* (cloacae/aerogenes)	• *Streptococcus species*	• *Candida glabrata*
• *Proteus mirabilis*	• *Enterococcus faecium*	• *Candida parapsilosis*
• *Pseudomonas aeruginosa*	• *Enterococcus faecalis*	• *Aspergillus fumigatus*
• *Acinetobacter baumannii*	• *Aspergillus fumigatus*	
• *Stenotrophomonas maltophilia*		

* If positive, resistance can be tested with LC SeptiFast mecA test.

LIGHTCYCLER® MRSA ADVANCED TEST

Enabling Improved Infection Control

The incidence of hospital-associated methicillin-resistant *Staphylococcus aureus* (MRSA) is on the rise around the globe. Studies in Europe and the United States suggest that 28–34% of patients infected with MRSA will even die from their infection. These findings have serious implications for patients, physicians, and hospitals. The increased rates of MRSA also have significant economic implications.

The LightCycler MRSA Advanced test offers a simple, flexible and reliable way to incorporate MRSA surveillance into your hospital's infection control program.

YOUR BENEFIT

- **Fast results:** Results available within 100 minutes
- **Simple:** Sample preparation procedure involves no pipetting steps
- **Flexible:** Validated for use with 3 different swabs and provided in a convenient, ready-to-use format
- **Reliable results:** The only rapid MRSA test containing the Roche AmpErase® enzyme, able to prevent carry-over amplicon contamination that lead to false positive results.

MagNA PURE SYSTEMS

Accelerate Your Lab Workflow

For 10 years, MagNA Pure systems represent safe, contamination-free, and reproducible isolation of highly pure nucleic acids. Hence MagNA Pure systems are the optimal solution for sample preparation in each molecular biology laboratory.

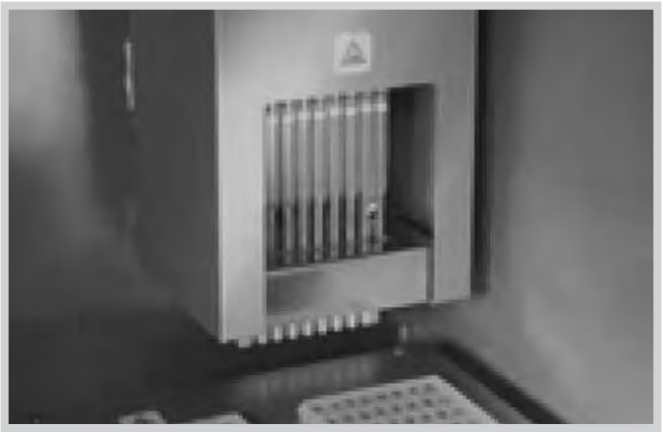
With the MagNa Pure 96 system, this technology is now also available for high throughput laboratories.

YOUR BENEFIT

Efficiency

- Walk-away systems with simple handling and standardized purification protocols.

Ensure Fast and Simple Operation



Reliability

- Proven isolation method based on magnetic bead technology.

Safety

- Cross-contamination-minimized sample preparation; closed housing, use of UV light, and convenient liquid waste discard.

Flexibility

- Isolation of highly pure DNA and RNA from pro- and eukaryotic organisms and different sample materials.

PRODUCT CHARACTERISTICS

	MagNA Pure Compact system	*MagNA Pure LC system*	*MagNA Pure 96 system*
Throughput	1–8 samples in about 30 minutes	1– 32 samples in about 60 minutes	8–96 samples in about 50 minutes
Hardware setting instrument	Benchtop with integrated PC	Benchtop with integrated PC Automated PCR setup integrated	Options for benchtop or continuous mode and sensors for load check
Run setup	Easy and convenient with single packaged, barcoded reagents	High flexibility multipack concept	Convenience and error prevention with prepacked, barcoded reagents
Run tracking	Barcoded tracking of individual samples and reagents	Barcoded tracking of sample plate	Barcoded tracking of sample plate and reagent trays
Flexible sample and elution volumes	100–1000 µL / 50–200 µL elution into single tubes	20–100 µL / 25–200 µL elution into plate	100–1000 µL / 50–200 µL elution into plate or single tubes

MagNA Pure 96 system is available as IVD in many countries.

TISSUE DIAGNOSTICS

Ventana Medical Systems, Inc, a member of the Roche Group, is one of the world's leading cancer diagnostic companies and is an innovator of tissue-based tests that enable the delivery of personalized healthcare to cancer patients.

The founder of Ventana, Thomas Grogan, MD, Professor of Pathology, University of Arizona, established the concept of a single, complete report covering all aspects of a patient's case, which helps to improve survivability.

Ventana is passionate about its mission to improve the lives of all patients afflicted with cancer by developing and delivering medical diagnostic systems and tissuebased cancer tests that are shaping the future of healthcare. VENTANA products provide healthcare professionals with a comprehensive solution for the critical steps involved in the analysis of tissue samples.

In addition, Ventana offers premier workflow solutions specially designed to improve laboratory efficiency and protect patient safety.

Recognizing the world's increasing medical needs, Ventana focuses on accelerating the discovery and development of new prognostic and predictive cancer tests that help enable personalized healthcare. These tests allow pathologists to analyze patient samples at the molecular, cellular and tissue level to help determine the best course of therapy for individual patients.

For more information please visit www.ventana.com

TISSUE DIAGNOSTICS

Leading Future Innovation

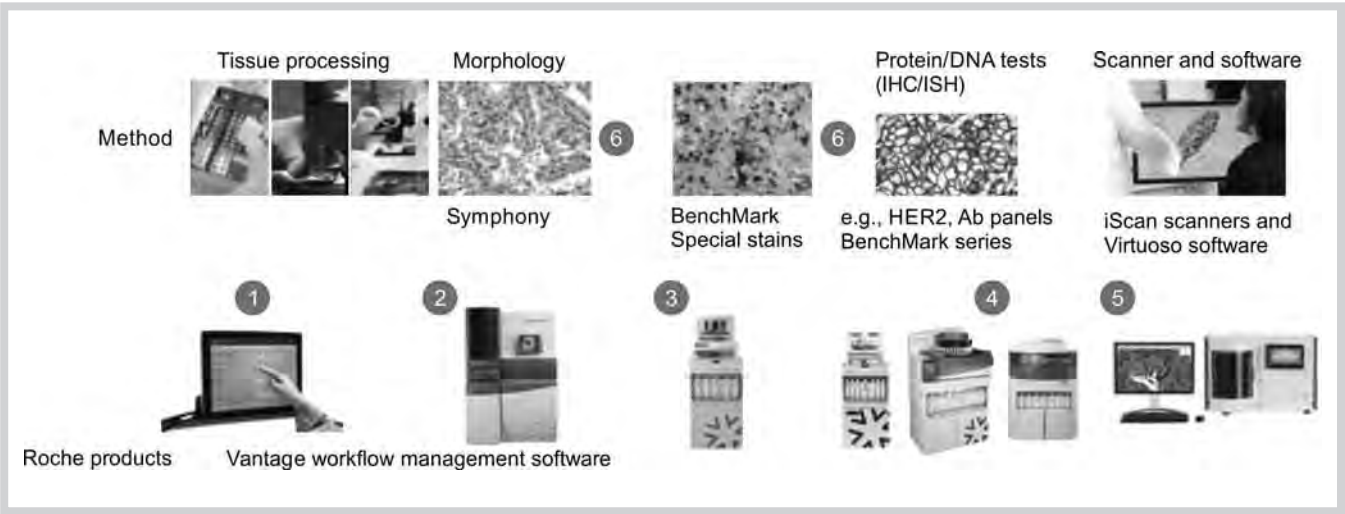
1. VANTAGE software
- Workflow solution from sample preparation to statistics monitoring
- Tracking of both samples and monitoring of the laboratory activity to help ensure quality
- Workflow consulting to optimise processes.

2. SYMPHONY platform
- Fully automated H and E staining
- Capacity up to 500 slides
- Integrated coverslipper.

3. BenchMark Special Stains instrument
- Fully automated special stains from
- baking to staining
- Capacity up to 20 slides per run
- Individual heater pads
- Pre-packed complete detection kits.

4. BenchMark IHC/ISH automated staining series
- Fully automated IHC* and ISH* systems, driven by easy-to-use barcoded slides and reagents
- Systems with different capacity available to fit small to large laboratories
- Open systems for antibodies.

5. Digital pathology
- Comprehensive digital pathology solution—from scanning and image viewing to customized reporting



- VENTANA iScan HT and iScan Coreo scanners—combine unprecedented flexibility, throughput and reliability
- VIRTUOSO image and workflow management software—designed for clinical laboratory use
- Industry-leading Companion Algorithm image analysis solution delivers consistent and objective results, time after time.

6. Reagents

- H&E, IHC*, ISH*, SpSt*
- More than 250 antibodies
- Ready-to-use and barcoded reagents.

SYMPHONY SYSTEM

Rethink H and E

Histology laboratories face a critical challenge—even in today's high-tech world, H and E slide preparation continues to be a labor intensive process. Each step requires significant time and effort, and can result in variable stain quality. The process also presents dangers that may compromise safety for patients through possible tissue cross contamination and laboratory technicians by exposing them to harmful chemicals.

The SYMPHONY system enables the only fully automated, one-touch H and E process that can minimize these issues and equip your lab with new levels of productivity, safety and quality.

Thanks to the SYMPHONY system, a technician can load slides on the system and walk away. When the automated process is complete, finished slides are coverslipped and ready for immediate presentation to the pathologist.

YOUR BENEFIT

Reduction of Errors and Mitigation of Risk

- Helps ensure positive patient identification and chain of custody by integrating the SYMPHONY system with the VANTAGE workflow solution
- Helps protect against cross-contamination with individual slide staining for every patient slide
- Reduce technician exposure to toxins with xylene-free SYMPHONY Clear.

Accurate and Reproducible Results

- Individual slide staining means no reagent carryover and stain degradation
- Application of fresh reagent on each slide produces exceptional clarity and enhanced visibility of microanatomic detail
- Excellent visualisation of microanatomic detail through the exceptional quality of high-definition H and E.

Faster Turnaround Times and Greater Efficiency

- Free your technicians to focus on additional value-added work with fully automated one-touch H and E slide processing
- Optimise lean workflow opportunities by integrating the SYMPHONY staining platform with the VANTAGE workflow solution.

PRODUCT CHARACTERISTICS

- Throughput: 160–200 slides per hour with continuous loading of up to 500 slides at a time
- Slide tray: universal tray holds up to 20 individual slides and can be stacked or "nested" for pathologist review
- "Slide Detect" ID: slide tracking supporting multiple barcode formats

* H and E = Hematoxylin and Eosin, ISH = in situ Hybridisation, IHC= Immunohistochemistry, SpSt = Special stains.

- Workflow: simultaneous processing of multiple slide trays including drying, deparaffinisation, staining and coverslipping
- Reagents: solutions are sealed, pre-packaged, ready-to-use and monitored with RFID for inventory management
- LIS connectivity through the VANTAGE workflow solution
- CareGiver remote support is an automated remote monitoring and diagnostics solution that enables continuous monitoring and remote service for SYMPHONY instruments.

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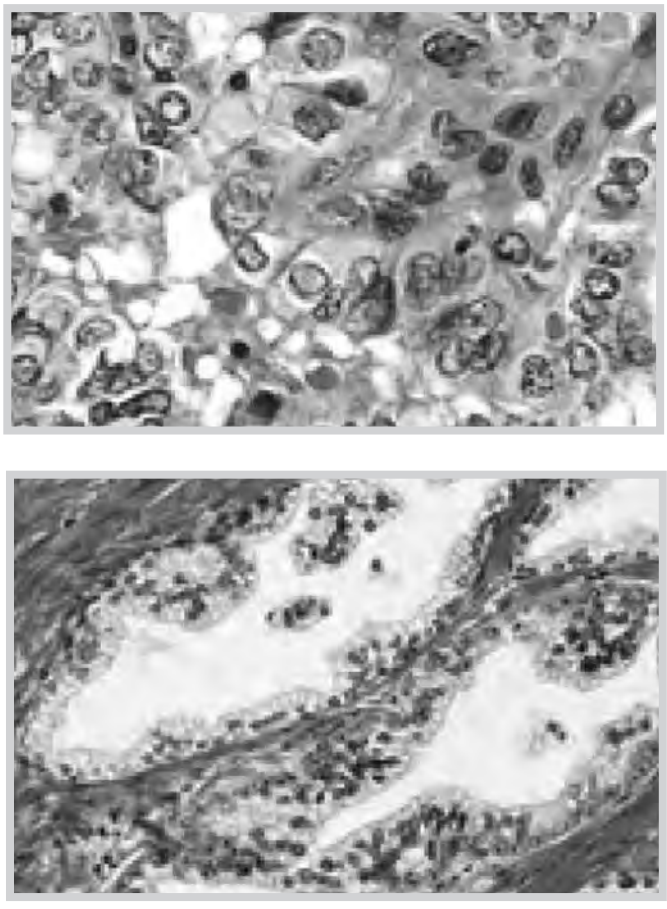

Gastric biopsy (above), Prostate biopsy (below).

BENCHMARK SPECIAL STAINS

Automated Slide Stainer

The new VENTANA BenchMark Special Stains automated slide stainer brings complete baking through staining to the histology laboratory for special stains, so your lab can consistently deliver exceptional quality. Productivity features such as random batch access, as well as bulk and waste fluid sensing, improve turnaround time and optimize workflow.

The BenchMark Special Stains instrument is operated by the Ventana System Software with an intuitive user interface. Reduce manual processes and improve your laboratory capabilities by delivering consistent, high-quality stains that help enable timely, accurate and efficient diagnosis.

YOUR BENEFIT

Superior Special Stains Workflow Efficiency

- Eliminate manual processes and temperature dependencies with automated deparaffinisation and independent slide heating.

Consistent Quality

- Full automation and standardised protocols reduces the variability inherent with manual staining techniques.

Reduced Risk

- Decreased technician risk with automated slide staining and reduced exposure to harmful chemicals.

PRODUCT CHARACTERISTICS

- Workflow: fully automated baking, deparaffinization and staining of special stains
- Slide carousel: 1–20 slides with independent temperature control for each position
- Reagent carousel: 25 reagent positions
- Slides 25 × 75 mm, 1 × 3" or 26 × 76 mm positively charged
- Bulk fluids: up to four bulk fluids in 3 to 6 liter on-board containers
- Modularity: 1–8 BenchMark Special Stains and BenchMark ULTRA systems may be controlled from one PC.

VENTANA Special Stains Reagents

- The new BenchMark Special Stains system brings reproducible, high-quality staining capabilities to every histopathology laboratory through ready-to-use and quality-controlled reagents. In addition, the system's new features of automated deparaffinization and independent slide heating enable consistent results to help ensure fast and accurate diagnosis.

Special Stains Menu

- AFB III
- Alcian Blue
- Alcian Blue for PAS
- Alcian Yellow
- Congo Red
- Diastase
- Elastic
- Giemsa
- GMS II
- Gram Stain
- Iron
- Jones Light Green
- Jones Hematoxylin
- Light Green for PAS
- Mucicarmine
- PAS
- Reticulum
- Steiner II
- Trichrome
- Green for Trichrome

PRIMARY ANTIBODIES

Over 250 Ready-to-use Clinical Reagents, Optimized for use on VENTANA Staining Platforms

Ready-to-use Antibodies

VENTANA antibodies, including a world-class breast panel, cover the pathology world's diagnostic requests. VENTANA antibodies include IVD/CE-IVD antibodies, as well as novel antibodies still in the research phase. Staining analysis is facilitated by advanced antibody performance and multiple detection technologies.

Breast	CEA (TF3H8-1)	CD63 (NKI/C3)
Calponin-1 (EP798Y)	CEA (CEA31)	Cytokeratin (34bE12), CONFIRM
Cytokeratin 14 (SP53)	CDX-2 (EPR2764Y)	Cytokeratin (AE1), CONFIRM
Cytokeratin 5/6 (D5/16B4)	COX-2 (SP21)	Cytokeratin 8 and 18 (B22.1 and B23.1), CONFIRM
E-cadherin (36), CONFIRM	Cytokeratin 7 (SP52), CONFIRM	Desmin (DE-R-11), CONFIRM
E-cadherin (EP700Y)	Cytokeratin 19 (A53-B/A2.26)	EMA (Epithelial Membrane Antigen) (E29), CONFIRM
Estrogen Receptor (ER) (SP1), CONFIRM	Cytokeratin 20 (SP33), CONFIRM	Ep-CAM (Epithelial Specific Antigen) (Ber-EP4)
GATA3 (L50-823)	DOG1 (SP31)	Factor VIII Related Antigen
GCDFP-15 (EP1582Y)	Glutamine Synthetase (GS-6)	Factor XIIIa (AC-1A1)
HER2 Dual ISH DNA Probe Cocktail assay, INFORM	Helicobacter pylori (SP48), VENTANA	Factor XIIIa (EP3372)
HER-2/neu (4B5), PATHWAY	MLH-1 (M1)	C1q, FITC
HER-2/neu (4B5), VENTANA	MSH2 (G219-1129)	C3, FITC
Ki-67 (30-9), CONFIRM	MSH6 (44), CONFIRM	C4, FITC
p120 (98)	MUC1 (H23)	Fibrinogen, FITC
p53 (DO-7), CONFIRM	MUC2 (MRQ-18)	Kappa, FITC
Progesterone Receptor (PR) (1E2), CONFIRM	PMS2 (EPR3947)	Lambda, FITC
Topoisomerase IIa (JS5B4), CONFIRM	*Dermatopathology*	HHV-8 (Human Herpes Virus Type 8) (13B10)
Cervical	Albumin, FITC	IgA (Immunoglobulin A)
CINtec® PLUS p16/Ki-67 dual stain	a-1-Antichymotrypsin (ACT)	IgA (Immunoglobulin A), FITC
(Cytology) (E6H4™ and 274-11 AC3)	a-1-Antitrypsin (AAT)	IgG (Immunoglobulin G)
CINtec® p16 Histology (E6H4)	CEA (CEA31)	IgG (Immunoglobulin G), FITC
Colorectal and Gastrointestinal	Carcinoembryonic Antigen (CEA) (TF3H8-1)	IgM (Immunoglobulin M)
Beta-catenin (14)	CD2 (MRQ-11)	IgM (Immunoglobulin M), FITC
BRAF-V600E (VE1)	CD3 (2GV6), CONFIRM	Macrophage (HAM-56)
c-KIT (9.7), PATHWAY	CD31 (JC70)	MART-1/melan A (A103), CONFIRM
Cadherin 17 (SP183)	CD34 (QBEnd/10), CONFIRM	Melanoma Associated Antigen (KBA.62)

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Melanoma Associated Antigen (PNL2)	CD15 (MMA), CONFIRM	IgM (Immunoglobulin M)
Melanoma Triple Cocktail (A103, HMB45, T311)	CD16 (SP175)	Kappa, CONFIRM
Melanosome (HMB45), CONFIRM	CD20 (L26), CONFIRM	Lambda, CONFIRM
MITF (C5/D5), CONFIRM	CD22 (SP104)	LMO2 (1A9-1), CONFIRM
Neurofilament (2F11)	CD23 (SP23), CONFIRM	LMO2 (SP51)
p53 (DO-7), CONFIRM	CD25 (4C9)	Lysozyme
p53 (Bp53-11)	CD30 (Ber-H2)	MUM1 (MRQ-43)
Podoplanin (D2-40)	CD31 (JC70)	Myeloperoxidase
S100 (4C4.9), CONFIRM	CD34 (QBEnd/10), CONFIRM	Oct-2 (MRQ-2)
S100 (Polyclonal), CONFIRM	CD38 (SP149)	PAX5 (SP34), CONFIRM
Synaptophysin (MRQ-40)	CD43 (L60)	PD-1 (NAT-105)
Synaptophysin (SP11), CONFIRM	CD45 (LCA) (2B11 and PD7/26)	SOX-11 (MRQ-58)
Tryptase (G3)	CD45 (LCA) (RP2/18), CONFIRM	Spectrin (RBC2/3D5)
Tyrosinase (T311), CONFIRM	CD45R (MB1)	T-bet (MRQ-46)
Vimentin (V9), CONFIRM	CD45RO (UCHL-1), CONFIRM	TdT
Vimentin (Vim 3B4), CONFIRM	CD56 (123C3), CONFIRM	TRAcP (9C5)
Hematopathology	CD56 (MRQ-42)	ZAP-70 (2F3.2)
ALK1 (ALK01), CONFIRM	CD57 (NK-1)	*Lung*
Annexin A1 (MRQ-3)	CD61 (2f2)	ALK (D5F3), VENTANA
bcl-2 (SP66)	CD68 (KP-1), CONFIRM	c-MET Total (SP44), CONFIRM
bcl-2 (124), CONFIRM	CD71 (MRQ-48)	Calretinin (SP65), CONFIRM
bcl-6 (GI191E/A8)	CD79a (SP18), CONFIRM	Carcinoembryonic Antigen (CEA) (TF3H8-1)
BOB.1 (SP92)	CD99 (O13), CONFIRM	CD56 (123C3), CONFIRM
c-Myc (Y69)	CD138 (Syndecan-1) (B-A38)	CEA (CEA31)
CD1a (EP3622)	Cyclin D1 (SP4-R)	CD56 (MRQ-42)
CD2 (MRQ-11)	Fascin (55k-2)	Chromogranin A (LK2H10)
CD3 (2GV6), CONFIRM	FoxP1 (SP133)	Cytokeratin (CAM 5.2)
CD4 (SP35), CONFIRM	Galectin-3 (9C4)	Cytokeratin 5/6 (D5/16B4)
CD5 (SP19), CONFIRM	Glycophorin A (GA-R2)	Cytokeratin 7 (SP52), CONFIRM
CD7 (SP94)	Granzyme B	Cytokeratin 17 (SP95)
CD8 (SP57)	HGAL (MRQ-49)	Cytokeratin 20 (SP33), CONFIRM
CD10 (SP67), VENTANA	IgA (Immunoglobulin A)	E-cadherin (36), VENTANA
CD13 (SP187)	IgD (Immunoglobulin D)	E-cadherin (EP700Y)
CD14 (EPR3653)	IgG (Immunoglobulin G)	EGFR E746-A750 del (SP111)

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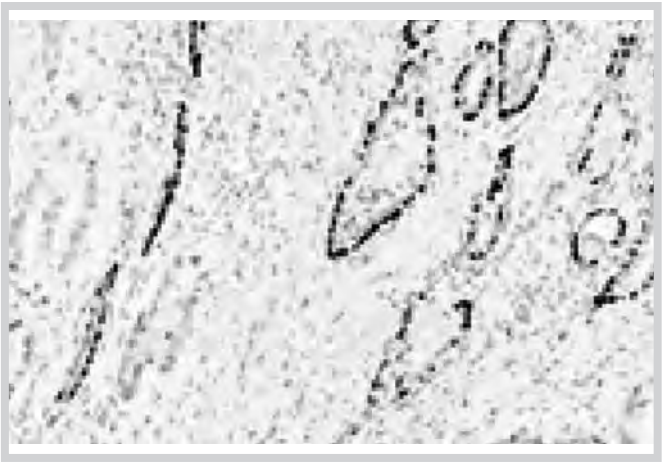
EGFR (Epidermal Growth Factor Receptor) (5B7), CONFIRM	Cytokeratin 5/6 (D5/16B4)
EGFR (Epidermal Growth Factor Receptor) (3C6), CONFIRM	Cytokeratin 7 (SP52), CONFIRM
EGFR L858R (SP125)	Cytokeratin 20 (SP33), CONFIRM
EMA (Epithelial Membrane Antigen) (E29), CONFIRM	ERG (EPR3864)
Epithelial-Related Antigen (MOC-31)	EZH2 (SP129)
Epithelial-Specific Antigen/Ep-CAM (Ber-EP4)	p63 (4A4), VENTANA
IGF-1R(G11), CONFIRM	PSA, CONFIRM
Mesothelial Cell HBME-1 (HBME-1)	PSA (ER-PR8)
MUC1 (H23)	PSAP (PASE/4LJ)
Napsin A (MRQ-60)	*Prostate*
NSE (MRQ-55)	Androgen Receptor (SP107)
p63 (4A4), VENTANA	Basal Cell Cocktail (34ßE12+p63), VENTANA
SOX-2 (SP76)	Cytokeratin (34ßE12)
Synaptophysin (MRQ-40)	Cytokeratin 5/6 (D5/16B4)
Synaptophysin (SP11), CONFIRM	Cytokeratin 7 (SP52), CONFIRM
TAG-72 (B72.3)	Cytokeratin 20 (SP33), CONFIRM
Thyroid Transcription Factor-1 (SP141)	ERG (EPR3864)
WT1 (6F-H2)	EZH2 (SP129)
Prostate	p63 (4A4), VENTANA
Androgen Receptor (SP107)	PSA, CONFIRM
Basal Cell Cocktail (34ßE12+p63), VENTANA	PSA (ER-PR8)
Cytokeratin (34ßE12)	PSAP (PASE/4LJ)

IHC DETECTION

Meet Your Needs and Everything Beyond IHC/ISH Detection

Ventana offers a comprehensive menu of optimized detection systems for use with our VENTANA BenchMark IHC/ISH automated slide stainers, allowing for the identification of targets by IHC and ISH.



IHC Detection Offerings

Choose from a comprehensive menu of detection chemistries (biotin and biotin-free based systems) and stains (DAB and Red) for high-quality IHC results:

- iVIEW DAB Detection Kit (biotin streptavidin)
- *ultra*View Universal DAB Detection Kit
- *ultra*View Universal Alkaline Phosphatase Red Detection
- OptiView DAB IHC Detection Kit.

ISH Detection Offerings

Our comprehensive menu of indirect, biotin-free detection systems and stains (Blue, Silver and Red) provides the options you need for high-quality ISH results:

- ISH iVIEW Blue Plus Detection Kit
- *ultra*View SISH Detection Kit
- *ultra*View SISH DNP Detection Kit
- *ultra*View Red ISH Detection Kit.

The OptiView DAB IHC Detection Kit offers advancements in detection technology, using a proprietary non-endogenous, biotin-free hapten technology that allows for exceptional range in sensitivity with extremely low background. Using VENTANA OptiView detection software with the BenchMark IHC/ISH platform provides the ability to optimize testing to achieve a desired level of sensitivity and improved turnaround times, with flexible protocols and workflow enhancements.

YOUR BENEFIT

OptiView DAB IHC Detection

Increase sensitivity

By increasing the numbers of HRP enzymes at each primary antibody site, OptiView provides unparalleled signal intensity, empowering you to achieve the level of intensity you desire for even the low-expressing antigens.

Enhance Stain Quality

Our synthetic, non-endogenous hapten system virtually eliminates background, even as signal intensity increases, to create the perfect view.

Customize Intensity

Unique chemistry and flexible software enable greater control to meet preferred staining intensity.

Improve Turnaround Time

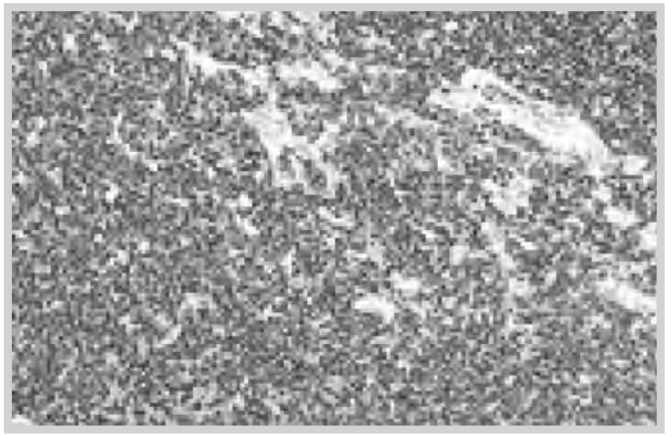
Amazing sensitivity and software flexibility allows you to reduce turnaround time by 30 minutes or more for most assays.

BREAST CANCER DIAGNOSTICS

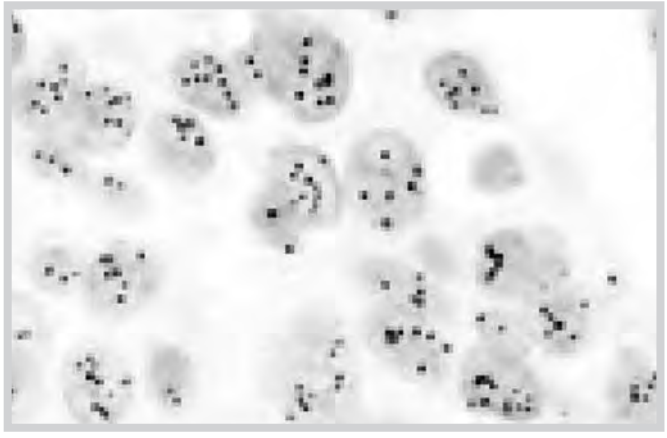
Empowering Clinical Confidence

Roche diagnostics delivers a comprehensive suite of validated immunohistochemistry and in situ hybridization diagnostic solutions for breast cancer—so you can deliver the right test, with clinical confidence.

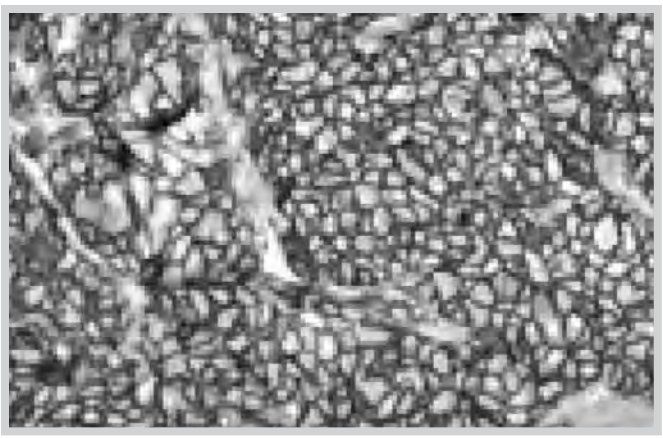
Our breast cancer predictive diagnostic offerings (HER2 IHC and ISH, ER, PR) in combination with our supporting diagnostic assays (Ki-67, p120 and E-cadherin) are fully automated on BenchMark IHC/ISH staining platforms that reduce the time to result and resources required compared to manual or semiautomated solutions.



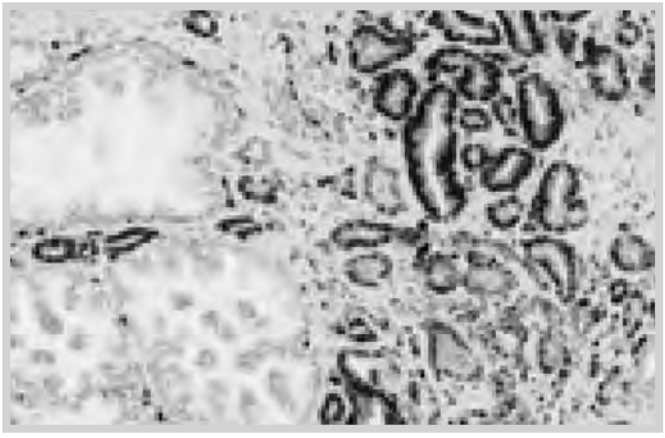
Cyclin D1 (SP4) on mantle cell lymphoma with OptiView DAB IHC Detection Kit.



Breast carcinoma INFORM HER2 Dual ISH DNA Probe Cocktail non-amplified; magnification: **40X.**



Breast carcinoma HER2 (4B5) positive Score: 3+; magnification: 40X.



Prostate carcinoma stained with ERG (EPR3864) Rabbit Monoclonal Primary Antibody

YOUR BENEFIT

Clinical Superiority

- High accuracy and clinical confidence in a short turn-around time to identify patients other assays can miss.

Analytical Superiority

- Specific and sensitive rabbit monoclonal antibodies, best-in-class probes and powerful detection systems.

Testing eFficiency

- Comprehensive breast cancer solution
- Fully automated assays, with digital pathology and workflow solutions.

PRODUCT CHARACTERISTICS

INFORM HER2 Dual ISH DNA Probe Cocktail Assay

- Brightfield detection allows evaluation of HER2 gene status with morphological context.

HER2 (4B5) Rabbit Monoclonal Antibody

- Clinical confidence with a world-class HER2 rabbit monoclonal antibody.

PROSTATE CANCER DIAGNOSTICS

Diagnostic Solutions and Innovative Tools for Emerging Utility

Our prostate cancer diagnostic portfolio can give you the confidence you need to improve patient care.

Empower your laboratory with our portfolio of biomarkers that deliver increased value for men's health. Our antibodies are pre-diluted and optimized for use on the BenchMark IHC/ISH series of automated platforms for efficient, reproducible staining quality. We continue to develop novel biomarkers with promising utility—such as the EZH2 (SP129) Rabbit Monoclonal Antibody and the Androgen Receptor (SP107) Rabbit Monoclonal Antibody.

ERG (EPR3864) Rabbit Monoclonal Primary Antibody

Developed for high sensitivity and specificity, the ERG (EPR3864) Rabbit Monoclonal Primary Antibody delivers:

- Specificity for prostate cancer which may aid in detection and diagnosis
- Ability to identify a molecular prostate cancer subtype
- High concordance to ERG FISH.

VENTANA p63 (4A4) Mouse Monoclonal Primary Antibody

The p63 (4A4) antibody empowers you to make informed, confident decisions.

- Consistently strong nuclear staining allows for easier interpretation
- Like high molecular weight cytokeratin 34βE12, p63 is specific and sensitive for basal cells in the prostate gland.

VENTANA Basal Cell Cocktail 34βE12+p63

Our Basal Cell Cocktail combines p63 (4A4) with 34βE12 to aid in the differentiation of benign and malignant prostatic lesions.

- Increases the sensitivity of basal cell detection
- Decreases staining variability
- Offers more consistent basal cell immunostaining.

HEMATOPATHOLOGY

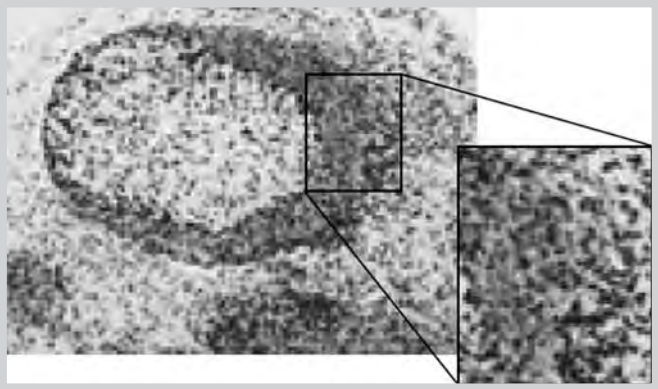
A Comprehensive Solution Helping You Detect and Subtype

We offer you over 65 cornerstone and novel hematopathology assays (IHC antibodies and molecular probes) that aid in the detection of lymphomas, leukemias and other hematopoietic diseases which provide:

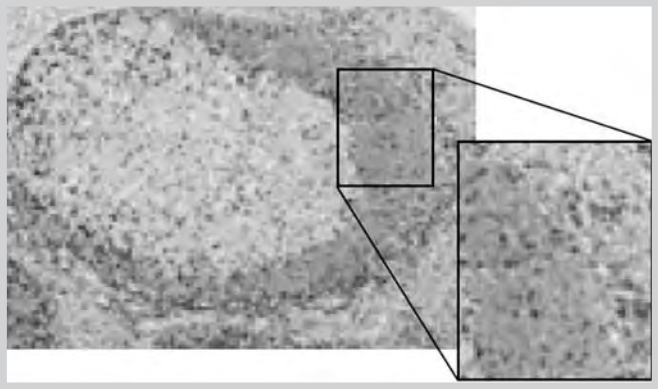
- Unparalleled sensitivity and specificity with OptiView DAB IHC detection
- Innovative automation (IHC/ISH) with VENTANA BenchMark IHC/ISH advanced staining platforms
- High-quality, ready-to-use reagents.

As you pursue more precise and personalized diagnosis and treatment options for your patients, no target should be beyond your detection.

CONFIRM CD5 (SP19) with OptiView DAB IHC Detection*



Competitor CD5 with DAB Detection



The dynamic power of the OptiView DAB IHC detection delivers unparalleled sensitivity and specificity so that you can:

- Detect even low-level expressing antigens
- Virtually eliminate background as signal intensity increases.

Mantle zones in normal tonsil are known to have very low levels of CD5 expression, as identified by techniques such as flow cytometry. This extremely low-level CD5 expression has been beyond detection with previously available IHC technology.[1] With OptiView DAB IHC detection, CD5 expression was detected in the mantle zone of the normal tonsil (internal study).

COLORECTAL DIAGNOSTICS

Assist in Diagnosis, Risk Stratification and Subtyping of Colorectal Cancer

Our colorectal cancer portfolio of primary antibodies assist in diagnosis, risk stratification, and subtyping while helping inform clinical decisions, and includes assays for BRAF V600E (VE1) and the most relevant DNA mismatch repair (MMR) proteins: MLH1 (M1), MSH2 (G219-1129), MSH6 (44), PMS2 (EPR947). MMR immunohistochemistry (IHC) testing in conjunction with BRAF V600E IHC testing allows for cost-effective colorectal cancer (CRC) subtyping within the anatomic pathology laboratory.[1]

Subtyping with MMR and BRAF V600E IHC tests

Defects/loss of expression in DNA MMR proteins are due to either inherited (Lynch syndrome) or sporadic mutations in the MMR genes, and typically manifest as microsatellite instability (mutations in short repetitive sequences of DNA).[2,3] IHC testing for MMR proteins allows identification of colorectal cancers with defects in MMR proteins and associated microsatellite instability. Combining MMR and BRAF V600E IHC testing facilitates additional CRC subtyping. In an MLH1-negative colorectal cancer (most common defect/loss), absence of BRAF V600E protein expression is associated with a higher likelihood of Lynch-associated CRC and a lower likelihood of sporadic CRC.[1]

Our MMR, BRAF V600E and other ready-to-use rabbit and mouse colorectal cancer IHC assays are supported by the fully automated Benchmark IHC/ISH platforms, innovative detection and efficient workflow solutions.

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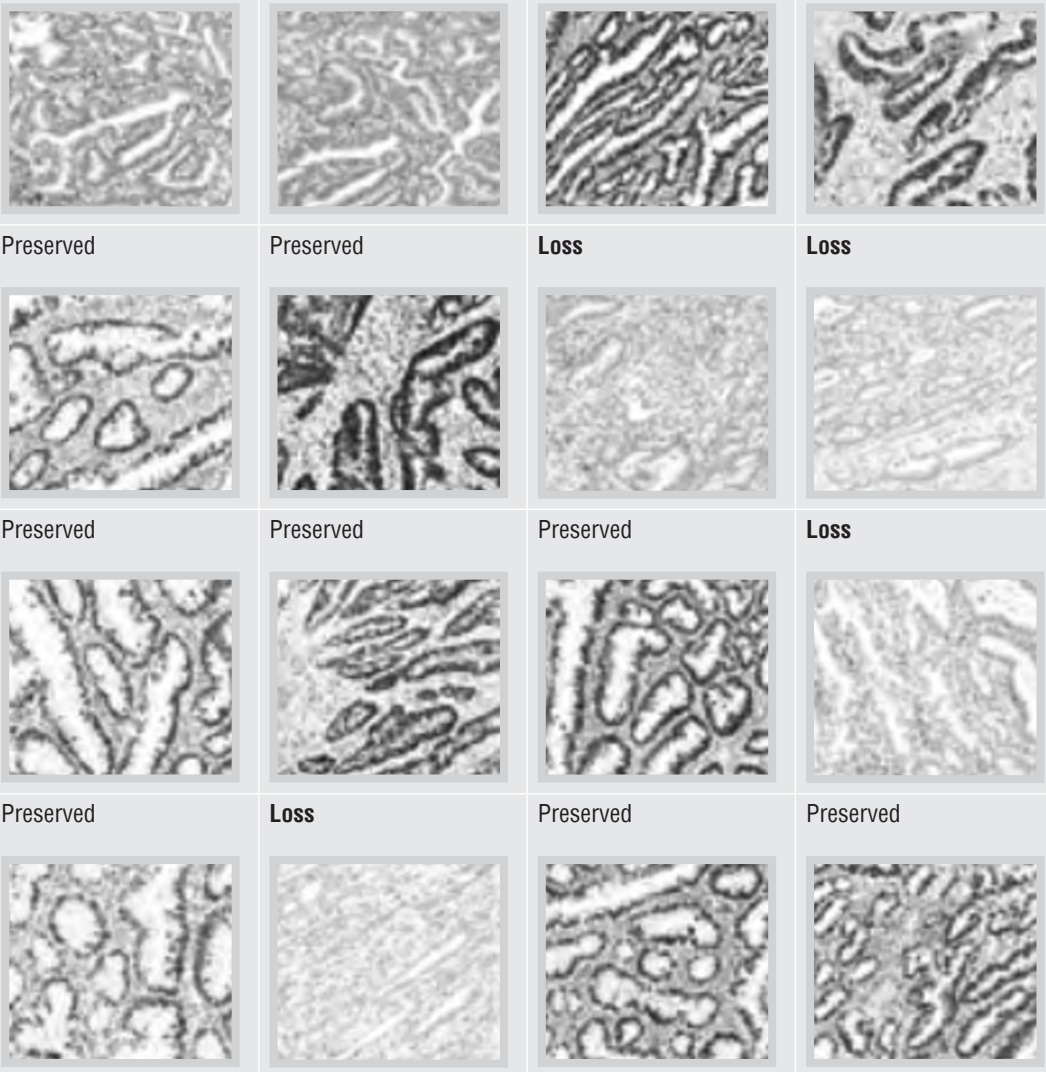
* Increased sensitivity powered by OptiView DAB IHC detection: positive staining for low-level CD5 protein expression in the mantle zone of the tonsil.

[1] Su W, et al. J Clin Pathol. 2000;53:395–7.
[1] Gausachs M, et al. Euro J of Hum Gen. 2012;20:762-8.
[2] Hatch S. et al. Clin Cancer Res. 2005; 11(6):2180-7.
[3] Sinicrope F. Nat Rev Clin Oncol. 2010;7:174-7.

Mismatch Repair IHC Staining Patterns in Colorectal Cancer

MMR mutations	*IHC result MLH1*	*IHC result PMS2*	*IHC result MSH2*	*IHC result MSH6*
MLH1 mutation	**Loss**	**Loss**	Preserved	Preserved
MSH2 mutation	Preserved	Preserved	**Loss**	**Loss**
MSH6 mutation	Preserved	Preserved	Preserved	**Loss**
PMS2 mutation	Preserved	**Loss**	Preserved	Preserved



Powered by the OptiView DAB IHC detection system.

LUNG CANCER DIAGNOSTIC SOLUTIONS

Driving Personalized Healthcare with Key Markers for Detection and Subtyping

The statistics associated with lung cancer clearly demonstrate the aggressive nature of this deadly disease, Roche Diagnostics offers a robust menu of tools to aid in the diagnosis of patients facing this challenge. "With the introduction of targeted therapies that can result in dramatically different outcomes based on subtype, the importance of accurate classification has been amplified."[1]

Our portfolio of products, which includes rabbit monoclonal antibodies, novel biomarkers and detection kits, delivers the high sensitivity and specificity needed from diagnostic assays.

Our antibodies are ready to use on the fully automated VENTANA BenchMark IHC/ISH staining platforms, reducing the time-to-result and resources required with manual or semi-automated solutions.

Differentiating Between Adenocarcinoma and Squamous Cell Carcinoma

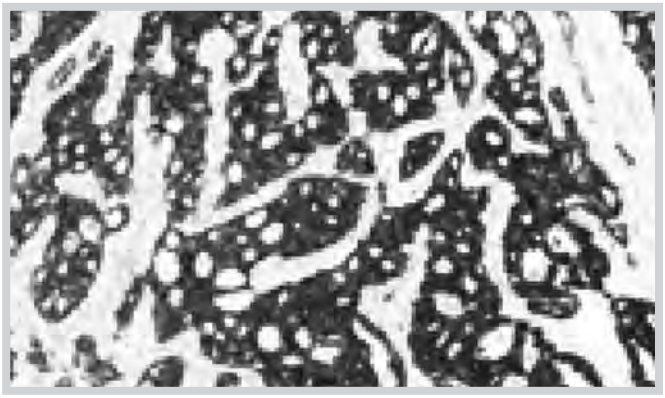
Confidently differentiate between lung adenocarcinoma (ADC) and squamous cell carcinoma (SCC) with four key markers, including the new thyroid transcription factor-1 (SP141) rabbit monoclonal primary antibody.

TTF-1 (SP141) detects lung carcinoids and was validated by third parties versus the SPT24 clone, demonstrating equal or better detection. "The TTF-1 (SP141) has a cleaner background and stronger staining intensity compared to clone [8G7G3/1]."*

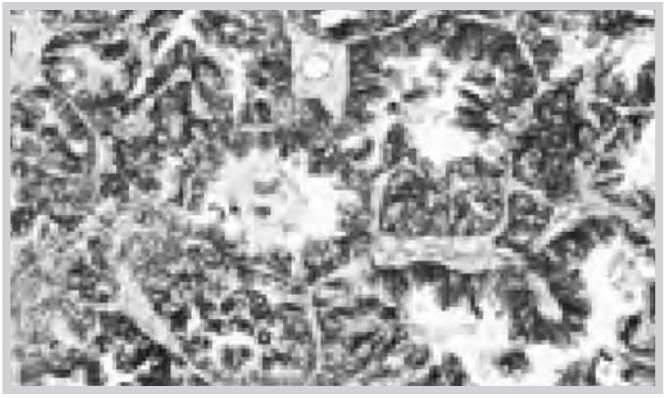
The combination of napsin-A, TTF-1, CK5/6 and p63 has been identified in some studies as the best IHC panel for differentiating ADC from SCC of the lung.[2]

Adenocarcinoma stained with TTF-1 (SP141) Magnification 20X

Gain a Clear View by Detecting ALK and c-MET Protein Expression



Adenocarcinoma stained with VENTANA ALK (D5F3), Magnification 20X



Adenocarcinoma stained with CONFIRM Total c-MET (SP44), Magnification 20X

VENTANA ALK (D5F3) Rabbit Monoclonal Primary Antibody

VENTANA ALK (D5F3) is indicated as an aid in identifying patients eligible for treatment with XALKORI (crizotinib). It is, therefore, critical that ALK positive patients are accurately identified. Shaw et al. highlights this importance and demonstrates that ALK testing via IHC represents a reliable and cost effective alternative to FISH.[3]

Clone D5F3 has been identified as "one of the most promising antibodies for the detection of ALK rearrangement in NSCLC." In a study of 296 patients with advanced NSCLC clinically referred for ALK testing, the "ultrasensitive" VENTANA ALK (D5F3) assay showed high correlation with FISH and 100% sensitivity and specificity.[4]

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* Dr Shalini Singh, Ventana Medical Systems, Inc.

[1] Tacha D, Yu C, Bremer R, Qi W, Haas T. Appl Immunohistochem Mol Morphol. 2012;20:201-7.

[2] Kim MJ, Shin HC, Shin KC, Ro JY. Ann Diagn Pathol. 2013;17:85-90.

[3] Shaw et al. J Natl Compr Canc. Netw. 2011;9:1335-41.

[4] Minca et al. J Mol Diagn. 2013;15(3).

CONFIRM Total c-MET (SP44) Rabbit Monoclonal Primary Antibody

CONFIRM Total c-MET (SP44) is directed against a membranous and/or cytoplasmic epitope present in human normal epithelial or tumour cells. This antibody may be used to aid in the identification of normal and neoplastic c-MET expressing cells.

"The pre-clinical evaluation demonstrated excellent specificity and sensitivity of the SP44 antibody and its suitability for determining Met protein expression on FFPE tissue."[5]

BENCHMARK IHC/ISH PLATFORM

Automated Slide Staining Systems

Minimize diagnostic lead time, maintain consistent high quality and streamline workflow in the histology laboratory with the BenchMark IHC/ISH instruments.

The BenchMark GX, BenchMark XT and BenchMark ULTRA instruments automate all slide preparation steps of immunohistochemistry (IHC), fluorescent IHC, in situ hybridisation (ISH) and Dual Color Silver tests. They have the flexibility you need to expand your test menu, process more slides and improve your turnaround time.

YOUR BENEFIT

Fully Automated

- Standardised IHC and ISH staining
- Dual and triple stains.

Flexibility

- Select from over 250 available VENTANA antibodies, or use your own antibodies
- Independent and simultaneous processing.

Optimal Quality

- Independent protocols for each slide
- Barcoded slides and reagents for case identification and traceability.

Workflow

- Higher throughput and faster turnaround times
- Increased laboratory productivity and reduced rework.

BENCHMARK SYSTEM FEATURES

Unique and innovative technology for best patient care by kinetically optimized reaction

- Individual heater pads
- Liquid coverslip controls evaporation and integrity
- Full slide coverage with 100 µL
- Air vortex mixing.

BenchMark GX System

- 20 slide positions
- 25 reagent positions
- Low to medium throughput
- Complete batching IHC and ISH diagnosis system.

BenchMark XT System

- 30 slide positions

[5] Koeppen H, Januario T, Filvaroff E. Mod Pathol. 2012;25;480A.

- 35 reagent positions
- Medium to high throughput
- Independent or simultaneous processing of IHC and ISH steps.

BenchMark ULTRA System

- 30 slide positions
- 35 reagent positions
- Flexibility to add/remove slides without impacting workflow
- Ability to add or remove reagents without interrupting cases in process
- Immediately process STAT and late-arriving samples
- Simultaneous IHC/ISH testing on a single platform.

LIS or VANTAGE Software Connection

- Connect multiple systems with a single computer or add a new system to existing ones
- Share reagents and protocols across instruments through Central Management software
- Download patient accession and test information from LIS to slide staining system to mitigate data entry errors.

DIGITAL PATHOLOGY

Eliminate the Boundaries of Time and Distance

Digital pathology enables anytime, anywhere access to slide images. By digitising the glass slides that pathologists typically view under the microscope care teams can collaborate in real time. Ventana is transforming the practice of pathology by developing innovative tools that help speed turnaround time and improve the evaluation of the most difficult IHC cases.

VENTANA digital pathology products consist of high performance scanners, image management software and power image analysis tools. When used together, our integrated staining platforms, assays, scanners, algorithms and workflow management solution optimise your process from staining to reporting. The result is increased workflow efficiency and enhanced medical value.

YOUR BENEFIT

Speed, Consistency

- Real-time collaboration for consults and education
- Expand access to remote expertise and specialists
- Consistency in the evaluation of key IHC breast biomarkers.

Optimize Case Management

- Speed case assembly with digital case management
- Archive and retrieve cases instantly, enhance patient reports with images and remote digital sign out.

Clinical Confidence

- Quantify what you see with the broadest menu of FDA-cleared companion algorithms for key breast markers
- Inform decision making with AP-LIS interfaces-integrated case data when you need it.

Comprehensive

- Whether you are looking to optimise your digital pathology system or are just starting out, our comprehensive solution is designed to empower you at every stage of adoption.

PRODUCT CHARACTERISTICS

Virtuoso Image Management Software

- Designed by pathologists for pathologists, the Virtuoso software provides access to slide images—anytime, anywhere

- Uniquely designed to support telepathology and remote consultation workflow
- Features secure role-based access and remote site configuration management tools.

VENTANA iScan HT Slide Scanner

- The industry's most powerful high-throughput bright-field scanner
- Continuous random access and STAT processing, which means new slides can be added without workflow interruption
- Excellent image quality and superior scanning speed at both 20X and 40X
- 360 slide capacity.

iScan Coreo Slide Scanner

- The most versatile scanner for low- to mid-volume use
- Excellent image quality and user-friendly, walk-away automation
- Multiple scanning objectives with manual change 4X, 10X, 20X, 40X
- Live microscopy mode for real-time collaboration.

Companion Algorithm Image Analysis Software

- The industry's broadest portfolio of clinically validated image analysis algorithms
- Comprehensive breast panel solutions
- VENTANA algorithms are cleared* as a system, including VENTANA scanner reagents, antibody clones and stainers. CE-marked and IVD use for all five key biomarkers: HER2, ER,PR, Ki-67 and p53.

VANTAGE WORKFLOW SOLUTION

A Proven System for Quality to Increase Patient Safety

Today's histology lab managers are under increasing pressure to improve laboratory workflow, sample tracking, quality and patient safety.

VANTAGE solutions have been designed to enable histology laboratories to address these challenges:

Our comprehensive solution for histology labs—hardware, software and workflow consulting—offers a commanding view of your complex operation from a single strategic perspective. It is an end-to-end product that automates, streamlines and integrates lab work and information flow to help provide maximum productivity and improvements to patient safety. The VANTAGE workflow solution is designed using Lean Six Sigma principles and includes expert workflow consulting support to help you obtain immediate and ongoing workflow benefits.

YOUR BENEFIT

Eliminate Redundancies, Reduce Errors

- Reduce data re-entry, relabelling and labelling errors with "one label, one time" technology and barcode scanners at every workstation.

Lean Workflow

- Prevent bottlenecks before they happen. The VANTAGE workflow solution gives you a clear view of your lab, so you can maintain optimal performance
- Collaborate with lean histology experts to improve your workflow
- Simplify workflow steps
- See a comprehensive dashboard of lab performance at any time
- Identify opportunities to improve quality, staffing and efficiency.

Establish Your Chain of Custody

- The VANTAGE workflow management system brings all of our automated platforms together, creating a chain of custody that encompasses your entire laboratory.

Full and Fast Control

- Locate any specimen, block or slide immediately
- Ask the VANTAGE system to locate any patient's slide, on any instrument, at any point in your process—and count on immediate, accurate results.

Full Transparency

- Populate patient details accurately
- Retrieve patient details with a quick barcode scan.

Product Characteristics

- Includes all VANTAGE connect characteristics
- Cassette verification/identification
- Slide label generation and management
- Harmonised unique slide identification
- Centralised instrument slide/test status
- Specimen chain of custody
- Block/slide tracking and locating
- Workflow process report and workload statistics
- QA/QC management and reports
- Specimen archive.

CONSULTANCY SERVICES

Healthcare budgets are continually being squeezed, which means laboratories and other diagnostic service providers are faced not just with operational but also commercial challenges.

Budget cuts, lack of personnel, limited space, attracting new customers and promoting the value of diagnostic services—all of these factors have become important considerations.

Based on our experience in serving laboratories for IVD testing, and supported by global and local experts, Roche provides consultancy services for all areas of testing, including molecular and tissue diagnostics.

Roche's mission is not only to help implement an optimal, future-proof solution but also to work with service providers in developing a service strategy that is able to cope with the many demands of a constantly changing market.

Inspiring Continuous Improvement

In a climate of deep financial crisis and acute competition, laboratories need to evolve their business into a model that allows them the flexibility to react efficiently to a very fast healthcare market dynamic.

Roche consultancy team can help you build the right, fact based strategy to meet both current and future demand. They will support you in the implementation of the strategy by building LEAN efficient processes and selecting the right equipment to precisely match the clinical needs securing a direct transfer of the value of your services into outstanding patient outcome.

YOUR BENEFIT

- Empower your people to embrace continuous performance improvement

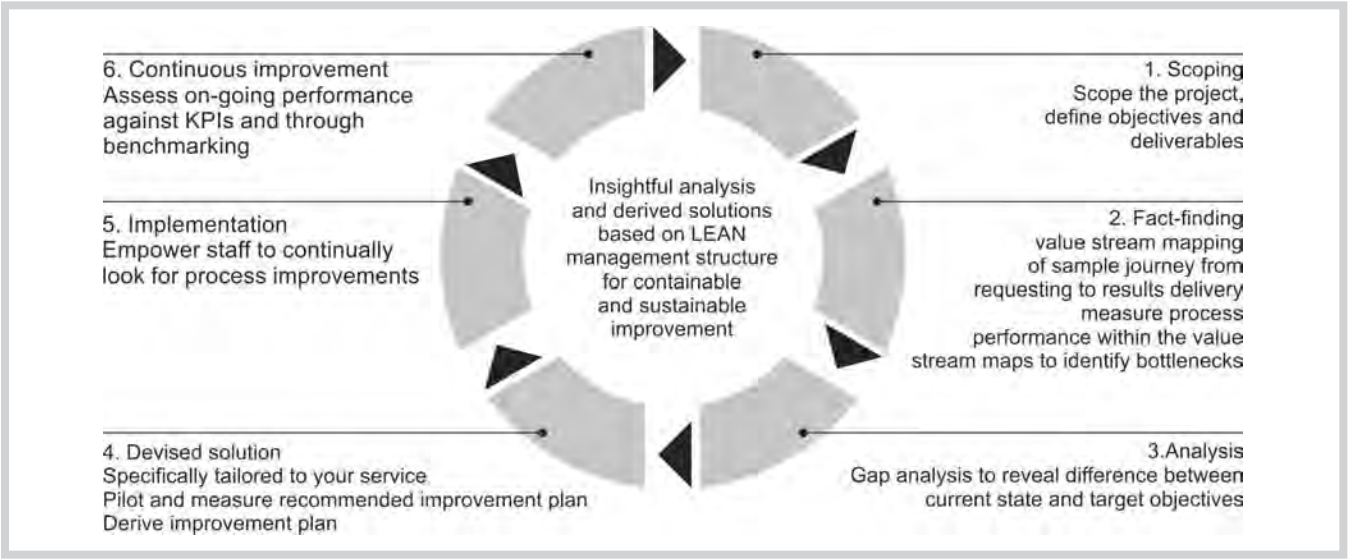
- Co-derived sustainable solutions with optimized workflow
- Rapid implementation according to fact based concept
- Increase operational efficiency and effectiveness
- A working environment with harmonised prosperity and performance
- Long term sustainable partnership.

Consultancy Process

Laboratory service performance improvement:

- Identification of strategic goals
- Analysis of main streams using LEAN management methodology to derive the optimum solution
- Implementation of proposed solution through a series of rapid improvement events which will validate the proposed solutions
- Monitoring of improvement through the benefit tracker which will indicate the status in concrete KPI's for each milestone.

A Structured Approach



SEQUENCING SOLUTIONS

Sequencing solutions provide researchers with innovative tools for the genome sequencing workflow, including instruments, reagents and target enrichment products. Roche's portfolio of next-generation sequencing products is driving advances in all areas of research including cancer, infectious diseases, inherited genetic diseases, immunogenetics, drug discovery, agriculture, environmental ecology and more.

Roche's 454 Sequencing systems spearheaded the post-Sanger era with the first next-generation sequencing system. Today, our expanded family of sequencing systems allows you to have this pioneering technology at your fingertips, regardless of the size of your laboratory. The GS FLX+ System and benchtop GS Junior System offer a unique combination of powerful next-generation sequencing throughput and long, accurate read lengths (up to 1,000 bp). The systems allow you to move quickly from sample to result with easy-to-interpret data and dedicated analysis software.

NimbleGen SeqCap EZ libraries and reagents enrich target DNA regions for a variety of next-generation sequencing platforms, allowing researchers to selectively sequence the human exome, human disease-associated genes, or genomic regions of interest in a wide range of non-human species. The broad portfolio of products with complete customization enables researchers to achieve best-in-class target enrichment efficiency and uniform coverage in variant detection.

Roche sequencing solutions offer researchers a clearer understanding of genomic structure and function in order to understand the impact of genes on biological processes. Through in-house research and collaborations with key opinion leaders, Roche is committed to delivering innovations in sequencing, now and in the future.

For more information please visit www.454.com

GENOME SEQUENCER FLX+ SYSTEM

Sanger-like Read Lengths—the Power of Next-Generation Throughput

Roche's portfolio of proven DNA sequencing and target enrichment solutions are advancing research in human health, agriculture, evolutionary biology, and more. The GS FLX+ System and benchtop GS Junior System offer the unique combination of powerful next-generation sequencing throughput and the familiarity of long Sanger-like read lengths (up to 1,000 bp).

NimbleGen SeqCap EZ Library products prepare DNA samples for a variety of next-generation sequencing platforms, allowing researchers to selectively sequence specific human exome and disease-associated regions. The broad portfolio of products with complete customization enables researchers to minimize sequencing costs in variant discovery studies.

YOUR BENEFIT

Fast Results

- Generate 700 million bases per 23 hours run.

More Comprehensive Data

- Take advantage of the Sanger-like read length up to 1 kb
- Includes powerful and easy-to-use data analysis SW.

Widest Application Range and Flexibility

- Cover all applications
- Gain project flexibility by utilizing different plate formats, gaskets and multiplex identifiers.

The Genome Sequencer FLX+ System—Sequence with Confidence

Up to 1,000 bp read length—get all the benefits of Sanger capillary sequencing with the power of next-gen throughput to take your research to the next level. Trusted results in over 1,300 publications:

- Identification of a novel arenavirus responsible for a series of fatal transplant-associated diseases in Australia

- Generation of the first complete genome and exome sequences from the huntergatherer people of southern Africa
- Sequencing of rearranged VDJ immune receptor loci tracks immune diversity and clonal lymphocyte population.

PRODUCT CHARACTERISTICS

Throughput	700 Mb per 23 hours run
Read length	Up to 1,000 bp
Consensus accuracy	99.997%
Data processing and bioinformatics	Perform data analysis without the need for enterprise scale IT solutions with preinstalled, easy-to-use software tools: • GS De Novo Assembler • GS Reference Mapper-GS Amplicon Variant Analyzer
Applications	• De novo sequencing • Re-sequencing • Sequence capture/targeted resequencing • Transcriptome analysis • Gene regulation studies • Epigenetic changes • Metagenomes and microbial diversity • Ancient DNA

GS JUNIOR SYSTEM

The Power of Next-generation Sequencing on Your Benchtop

The 454 GS Junior System brings the power of next-generation sequencing technology directly to your benchtop, opening the door to a new revolution in genomic research sequencing for every day and everyone.

Access to next-generation sequencing will no longer be limited to large facilities with the budget and infrastructure previously required to accommodate the high demands of the emerging technology.

YOUR BENEFIT

Integrated Next-generation Sequencing

- Established easy-to-use technology and Roche sequencing expertize.

Increased Lab Productivity

- Reproducible data, short run times and complete data analysis solutions.

Broad Application Versatility

- Due to read length, throughput, sensitivity and read accuracy.

PRODUCT CHARACTERISTICS

Research Application

- Unambiguously resolve highly complex genomic regions (e.g. HLA, IgH)
- Discover germline or somatic mutations in oncology (e.g. EGFR, KRAS, BRAF, PI3K, BRCA), hematology (e.g. TET2, CBL, RUNX1, RAS), and metabolic diseases (e.g. CFTR, MODY)
- Detect low-frequency variants such as rare drug-resistant viral mutations (e.g., HIV*)
- Throughput: >35 million high-quality, filtered bases per run
- Run time: 10 hours sequencing, 2 hours data processing
- Read length: ≈ 400 bp
- Accuracy: 99% accuracy at 400 bases
- Reads per run: 100,000 reads (on average)
- Sample input: gDNA, amplicons, cDNA, or BACs depending on the application
- Computing: HP desktop computer; all software is point-and-click.

GS Junior applications

- Zoom into critical genomic regions using amplicon sequencing of PCR products and sequence capture technologies
- Quickly perform haplotyping, genotyping, rare variant detection, structural variant detection, and heterozygote calling
- Analyze disease-associated regions in oncology and immunogenetics, or viral quasispecies present within infected populations in infectiology

NIMBLEGEN SEQUENCE CAPTURE

Confident and Efficient Genetic Variant Detection

Next-generation sequencing (NGS) target enrichment enables you to focus on your regions of interest in the human genome, hence greatly improving variant detection sensitivity, sample capacity and speed to results. Roche NimbleGen offers hybridization-based sequence capture enrichment tools. Compared to other hybridization-based enrichment technologies on the market, Roche NimbleGen products provide the highest capture efficiency and coverage uniformity available1,2, as a result of its superior design algorithms and proprietary probe synthesis technology.

Roche NimbleGen sequence capture products have enabled effective enrichment of a wide variety of genome regions from a broad range of sample types for high-fidelity detection of SNVs (single nucleotide variations), CNVs (copy number variations), indels (insertions and deletions), translocations and more.

YOUR BENEFIT

Most Relevant Content

- Uniform coverage of your target region, from the leader in custom designs, building highest confidence in variant detection and data reporting.

Proven Performance

- Best-in-class capture efficiency, proven by independent leading researchers year over year, leading to optimal sample throughput.

An Integrated Solution—from Sample Prep to Data Analysis

GS Junior Titanium Reagents and accessoires | Benchtop instrument and computer | Data processing and analysis software

Maximum Convenience

- Complete and cost-effective enrichment workflow coverage, from one source, greatly simplifying your validation process.

Product Characteristics

SeqCap EZ Library is a solution-based capture method that enables enrichment of the whole exome or customer regions of interest in a single test tube with up to 2.1 million overlapping probes.

- **SeqCap EZ Exome Libraries** enable enrichment of the whole exome. SeqCap EZ Exome Library v3.0 is based on the latest database builds and offering a 64 Mb sequence capture. SeqCap EZ Exome + UTR Library offers a 96 Mb design to capture the exome and untranslated regions (UTRs).
- **SeqCap EZ Choice Libraries** enable enrichment of customer regions of interest. SeqCap EZ Exome Plus provides a 64 Mb exome capture with the ability to add up to 50 Mb of your custom targets.
- **SeqCap EZ Designs** offer maximum performance for focused research areas, developed in collaboration with key opinion leaders. Designs are available for specific human genomic regions including a comprehensive cancer panel and a neurological disease panel.
- **NimbleDesign** is a free online tool that enables you to quickly and easily design SeqCap EZ Choice Libraries.

[1] Clark M, et al. Nat Biotech. 2011;♥doi:10.1038/nbt.1975.
[2] Bodi K, et al. J Biomol Tech. 2013;24(2):73-86. doi: 10.7171/jbt.13-2402-002.

ROCHE DIALOG

The consolidation and growth of medical laboratories is leading to ever-more complex processes and diagnostics systems are evolving constantly to keep pace.

This brings challenges for the people who use them. To make life easier, Roche has developed a one-stop solution that makes every aspect of laboratory management easier and more efficient.

The Changing World of Diagnostics

Introducing Roche DiaLog

A single platform designed to give you faster and more convenient online access to all the information and services you need.

YOUR BENEFIT

- Simplicity: one gateway to Roche

- Increased transparency of your processes
- Receive personalized support
- Stay up-to-date.

PRODUCT CHARACTERISTICS

Roche DiaLog: One point of entry to all Roche eServices. Access to Roche with just one login and password. Facilitates engaging interaction for a new form of direct two-way communication that's always open, personalized, simple and up-to-date.

eServices are applications to support your core business.

They include:

- Integration of multiple functions into one intuitive environment; providing one seamless path from stock management to tracking the delivery of your product
- A diverse range of eServices—or tools—that support you as you work, providing up-to-date content and boosting efficiency in your day-to-day tasks.
- Social platforms such as live chat, a forum, and a dedicated message service, which means that Roche specialists are always available to assist you.

Our aim is to create the world's leading diagnostics service for laboratory, office and mobile use. Always evolving in ways that will surprise you.

Appendix

Maximum permissible transport and storage times for analytes in blood (serum, plasma) and cerebrospinal fluid.

CLINICAL CHEMISTRY, SERUM, PLASMA AND BLOOD

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma 20-25°C -20°C to 4-8°C*	*Stabilizer*	*Comments*
Acid phosphatase, prostatic. immunol 4 m:8 d:8 d	1 h, unstable ↓ 1 d:8 h:2 h	Without stabilizer serum or 20 µL Stabilized to pH 4-5 serum	5 mg $NaHSO_4$/mL Add stabilizer 10% acetic acid/mL serum	Serum > plasma after-separation of
Adrenocorticotropic hormone (ACTH)	Unstable↓, stabilize in aprotinin/EDTA plus mercaptoethanol	6 w ? ?	Aprotinin 400 kU/mL mercaptoethanol 2 µL/mL	
Alanine aminotransferase (ALAT. ALT)	4 d ↓	2 d 7 d 3 d		
Albumin	6 d	3 m 3 m 3 m		
Aldosterone	1 d ↓	4 d 4 d 4 d	EDTA	Not applicable to outpatients
Alkaline phosphatase total bone	4 d ↓ 4 d	2 m 7 d 7 d 2 m 7 d 7 d		
Aluminum	Days	1 yr 2 w 1 w		
Ammonia	15 min in EDTA, Heparin ↑	3 w 2 h 15 min Stabilized by serine and borate	Serine 5 mmol/1 and borate 2 mmol/1	Easily contaminated by sweat ammonia
α-Amylase (AMYL). total pancreatic	 4 d ↓ 7 d ↓	 1 yr 7 d 7 d 1 yr 7 d 7 d		
Androstendione	1 d ↓	1 yr 4 d 1 d		
Antisreptolysin	?	? 2 d 2 d		

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma 20-25°C -20°C 4-8°C*	*Stabilizer*	*Comments*
α_1-Antitrypsin	?	3 m 3 m 7 d	Heparin plasma recommended	EDTA and citrate ↓
Apolipoprotein A-I	?	3 d 1 d		Do not freeze
Apolipoprotein B	?	3 d 1 d		Do not freeze
Aspartate Aminotransferase (ASAT, AST)	7 d ↓	2 w 7 d 4 d		
Bicarbonate	Unstable (+ 4°C, 30 min)	6 m 7 d 1 d	Keep closed	1 h after opening the tube
Bilirubin, conjugated total	 7 d in dark ↓ 7 d in dark ↓	 6 m 7 d 2 d 6 m 7 d 1 d	 Darkness required when stored > 8 h	
C-peptide	?	4 w d h		
C-reactive protein (CRP)	?	3 yr 8 d 3 d		
C3 complement	1 h	8 d 8 d 4 d		Dependent on antibody. During storage C_{3C} increases –C3 decreases
C4 complement	1 h	? 2 d 2 d		
CA 125	?	3 m 5 d 3 d		
CA 15-3	?	3 m 5 d ?		
CA 19-9	?	3 m 5 d ?		
CA 72-4	?	3 m 7 d ?		
Cadmium	1 d in trace element tube	? ? ?	Special tube	Released from red stopper
Calcitonin	1 h, stabilized with aprotinin (400 kU/mL)	? ? ?	Aprotinin 400 kU/mL	
Calcium, total	2 d ↓	8 m 3 w 7 d		
Calcium. ionized (actual)	15 min ↑	2 h	Use Ca-titrated heparin; keep light	pH-dependent Whole blood recommended only. Stable in gel tubes as primary tubes for 8 h after centrifugation
Carbohydrate deficient transferin (CDT)	? /	yrs 7 d ?		

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma -20°C 4-8°C 20-25°C*	*Stabilizer*	*Comments*
Carcinoembryonic antigen (CEA)	?	6 m 7 d 1 d		
Catecholamines Noradrenaline Adrenaline Dopamine	1 h if not stabilized	4 w 2 d 1 d 6 m if stabilized	Glutathione + EDTA, 1.2 mg/mL	Special tube necessary EDTA plasma separated within 15 min and frozen at –20°C
Chloride	1 d ↓	yrs 7 d 7 d		
Cholesterol, total HDL- LDL-	7 d ↑ 2 d ↑ 1 d ↑	3 m 7 d 7 d 3 m 7 d 2 d 3 m 7 d 1 d		
Cholinesterase (CHE)	7 d ↓	3 m 17 d 17 d		
Coeruloplasmin	?	3 m 2 w 8 d		
Copper	7 d	yrs 2 w 2 w	Special tube	Contamination
Corticotropin				See ACTH
Cortisol	7 d	3 m 7 d 7 d		
Creatine kinase (CK) total MB (CK-MB)	7 d ↓ 7 d ↓	4 w 7 d 2 d in dark 4 w 7 d 2 d in dark	Darkness SH reagent	CK-BB not stable without stabilizer Including immunoassays
Creatinine	3 d ↑	3 m 7 d 7 d		Also enzymatic procedure
CYFRA 21-1	?	6 m 4 w 2 d		
Dehydroepiandro-sterone sulfate (DHEAS)	2 d ↓	yrs 2 w 1 d		
Electrophoresis. Protein-	?	3 w 3 d 1 d		
Erythropoietin	?	? ? 2 w		Shipped frozen
Estradiol	1 d	1 yr 3 d 1 d		
Estriol	?	1 yr 2 d 1 d		
Ethanol	2 w	? 6 m 2 w	EDTA/Heparin	Evaporation, closed tubes
Fatty acids, free	30 min ↑	2 d 12 w 30 min		Freeze serum immediately

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma -20°C 4-8°C 20-25°C*	*Stabilizer*	*Comments*
Immunoglobulins				
IgA	?	6 m 3 m 3 m		
IgD	?	6 m 7 d 7 d		
IgE	?	6 m 7 d 7 d		
IgG	?	6 m 3 m 3 m		
IgM	?	6 m 3 m 7 d		
Insulin	63 min	6 m 1 d 4 h	Plasma preferable	
Iron	2 h↑	yrs 3 w 7 d		EDTA, oxalate, citrate interfere
Lactate	< 5 min, unstable ↑↑ deproteinization recommended, glycolysis inhibitors	? 3 d 3 d ? 6 d 3 d	Mannose-fluoride, oxalate iodoacetate, deproteinization	
Lactate dehydrogenase (LDH)	1 h ↑	4 w 4 d		All isoenzymes serum > plasma
Lead	?	? ? 7 d		Special tubes
Lipase	? h	1 yr 7 d 7 d		
Lipoprotein (a) (Lpa)	?	3 m 2 w ?		
Lutropine (LH)	7 d	1 yr 3 d 1 d		
Magnesium	1 d ↑	1 yr 7 d 7 d		
Myoglobin	1 h ↓	3 m 1 d 2 h		
Neuron-specific enolase	2 h ↑ (heparin)	3 m 3 d ?	Heparin plasma	Serum > plasma (platelets, hemolysis)
Osmolality	?	3 m 1 d 3 h		
Osteocalcin	15 min	Stabilized 14 d ? Unstable	EDTA (5 mmol/1) and aprotinin (2500 kU/mL)	
Parathyrin (PTH) intact	6 h (24 h in EDTA blood)	? 1 d 6 h	EDTA	Method-dependent
Phosphate, inorganic	1 h ↑↑	1 yr 4 d 1 d		
Potassium	1 h ↑↑	1 yr 1 w 1 w		Serum > plasma
Progesterone	7 d	1 yr 3 d 1 d		
Prolactin	2 d	1 yr 3 d 1 d		No EDTA. citrate
Prostatic-specific antigen (PSA)	1 d	3 m 2 d 1 d		

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma -20°C 4-8°C 20-25°C*	*Stabilizer*	*Comments*
Protein electrophoresis	?	3 w 3 d 1 d		
Protein total	1 d	yrs 4 w 6 d		Plasma > serum (fibrinogen)
Rheumatoid factor (RF)	?	4 w 3 d 1d		
Selenium	2 d	1 yr 2 w 1 w		Contamination
Sodium-	4 d ↓	1 yr 2 w 2 w		
Testosterone	7 d 1 day in women ↑	1 yr 3 d 1 d		
Thyroglobulin	2 d	4 w 3 d 1 d		
Thyrotropine (TSH)	7 d	3 m 3 d 1 d		
Thyroxine (T_4)	7 d	4 w 7 d 2 d		
Thyroxine-binding globulin (TBG)	7 d	4 w 5 d 5 d		
Transferrin	?	6 m 8 d 8 d		
Triglycerides	7 d ↑	yrs 7 d 2 d		Decrease ol triglycerides, increase of free glycerol, but only minor increase of total glycerol
Triiodothyronine (T_3)	?	3 m 8 d 2 d		
Troponin T	?	3 m 1 d ?		
Troponin I	?	? ? ?		
Urea	1 d ↑	1 yr 7 d 7 d		
Uric acid	7 d ↑	6 m 7 d 3 d		
Vitamin A	?	2 yrs 4 w ?		Light ↓.
Vitamin B1 (thiamin)	?	1 yr ? ?		
Vitamin B2 (riboflavin)	?	4 w ? ?		Light ↓
Vitamin B6 (pyrid-oxal phosphate)	Unstable, use EDTA plasma	Days hs 30 min	EDTA plasma	Light ↓ darkness
Vitamin B12	Unstable, use EDTA	8 w 4 h 15 min	EDTA, darkness	Light ↓

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma -20°C 4-8°C 20-25°C*	*Stabilizer*	*Comments*
Vitamin C	3 h (4°C)	3 w 3 h ? only with stabilizers	Metaphosphate 63 mg/mL; deproteinized	
Vitamin D, 1.25-Dihydroxy-cholecalciferol	3 d	? ? 2 d		
25-Hydroxy-cholecalciferol	3 d	? ? 2 d		
Vitamin E (tocopherol)	8 h ↓	1 yr 4 w ?		
Vitamin K (transphyllochinone)	Unstable	3 m Un- ? stable		UV light ↓, use extraction
Zinc	30 min	1 yr 2 w 1 w	Special tubes	Contamination from stoppers

HEMATOLOGY, EDTA BLOOD

Analyte	*Stability in blood at 20–25°C* *M C B S O*	*Stability in blood at 4–8°C*	*Stabilizer*	*Comments*
Differential leukocyte count		Do not store at refrigerator temperature	Dry blood smear stable	Lower filling (higher EDTA concentration) decreases stability
Band neutrophils	3 h 8 h 12 h 2 h 4 h			
Segmented neutrophils	3 h 9 h 12 h 8 h			
Eosinophils	9 h 12 h 7 d 2 d			
Basophils	12 h 2 d 1 d ↑			
Monocytes	3 h 7 h 12 h 2 h ↑↑			
Lymphocytes	3 h 9 h 12 h 7 d 4 d			
Erythrocytes	7 d	7 d		Analyzer-dependent
Hematocrit (Hct, PCV)	1 d	7 d	K_2-EDTA superior to K_3-EDTA with centrifuge	
Hemoglobin (Hb)	7 d	7 d		
Leukocytes	7 d	7 d		
MCV	1 d	7 d		
Reticulocytes	1 d	1 d		
Thrombocytes	7 d	7 d		

M = Microscopic
C = Coulter (STKS®)

Contd...

Contd...

Analyte	*Stability in blood at 20-25°C* *M C B S O*	*Stability in blood at*	*Stabilizer*	*Comments 4-8°C*
B = Bayer/Technicon (H2®)) S = Sysmex (NE 8000) O = Others (COBAS® Argos)				

COAGULATION AND ESR, CITRATED BLOOD/PLASMA

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in plasma –20°C 4-8°C 20-25°C*	*Stabilizer*	*Comments*
Antithrombin 111	8 h	4 w 2 w 7 d		
D-Dimer	8 h	6 m 4 d 8 h		
Erythrocyte sedimentation rate (ESR)	2 h	- - -		Temperature-dependent
Factor II	?	4 w ? 6 h		
Factor V	?	4 w 4 h 4 h		Centrifuge at 4°C
Factor VII	?	? Unstable 8 h		
Factor VIII	?	2 w 4 h 4 h		Freeze to store > 4 h
Factor IX	4 w	? ? 4 h		
Factor IX: Ag	?	? ? ?		
Factor X		4 w ? 6 h		
Factor XI	?	? ? ?		
Factor XII	?	4 w ? 2 h		
Factor XIII	?	4 w ? 4 h		
Fibrin monomers	Unstable ↑↑	? ? 3 h	See FDP	
Fibrinogen	8 h	4 w 7 d 7 d		
Fibrinogen degradation products (FDP)	Unstable ↑↑	4 w 1 d ?	10 U thrombin and 150 IU Kallikrein/mL blood	Heparin inhibits thrombin effect
Fibrinopeptide A	?	? 2 h ?		
Capillary quick	?	4 w 2 d 6 h		
Hepatoquick				

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in plasma –20°C 4-8°C 20-25°C*	*Stabilizer*	*Comments*
Partial thromboplastin time (APTT)	4 h	4 w 8 h 4 h		
Protein C	?	4 w 7 d 8 h		
Protein S	?	4 m 4 h 4 h		
Quick, prothrombin time (PT)	8 h	4 w 1 d 1 d		Reagent-dependent
Reptilase time	?	4 w 4 h 8 h		
Thrombin coagulase	?	? ? 8 h		
Thrombin time	2 h	4 w 8 h 4 h		Reagent-dependent
von Willebrand factor (vWF)	?	6 m 7 d 2 d		

** Do not cool whole blood samples, global tests are accelerated (activation of factor VII, XI, XII).

BLOOD GASES AND WHOLE BLOOD ELECTROLYTES

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in blood at refrigerator temperature*	*Stabilizer*	*Comments*	
pH	15 min* ↓	2 h**		Decrease due to formation of lactate. Increase due to loss of CO_2	
pCO_2	15 min* ↑	2 h**	Close, cooling to 6°C	4-Decrease due to loss into surrounding air	Do not cool plastic containers, and do not store them for more than 15 min
Bicarbonate, base excess	15 min* ↓	4- 2h**		pH-dependent change	If the storage interval is longer, use glass containers and cool them
pO_2	15 min* ↓	2 h**		Increase, if not tightly closed	
Calcium ion, actual	15 min* ↑	2 h**	Heparinate, Ca-titrated and elecIrolyte-balanced		
Sodium	1 h*	1 h**			

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in blood at refrigerator temperature*	*Stabilizer*	*Comments*
Potassium	1 h* ↑↑	1 h**		
Chloride	1 h*	1 h**		

* Stored in closed plastic tubes
** Stored in ice-water cooled glass

THERAPEUTIC DRUG MONITORING, TOXICOLOGY (BLOOD)

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma*			*Stabilizer*	*Comments*
		–20°C	*4-8°C*	*20-25°C*		
Acetylsalicylic acid	?	?	?	?		
Carbamazepine	2 d	4 w	7 d	2 d		
Cocaine		4 d	4 w	<30 min	Fluoride, pH 5	
Cyclosporine	?	?	3 d	?	EDTA	Monoclonal
Diazepam			5 m	5 m		
Digitoxin	?	6 m	3 m	2 w		
Digoxin	?	6 m	3 m	2 w		
Disopyramide	?	5 m	2 w	?		
Ethosuximide	?	5 m	4 w	?		
Gentamicin	4 h	4 w	4 w	4 h		
Lidocaine	?	?	6 h	?		
Lithium	1 h ↓	6 m	7 d	24 h	Accumulates in red cells	
Methotrexate	?	6 m	3 d	?	Protect from light	
Nitrazepam		1 w	1 w		Light	
Phenobarbital	2 d	6 m	6 m	6 m		
Phenytoin	2 d	5 m	4 w	2 d		Not stable in SST-tubes

Contd...

Contd...

Analyte	*Stability in blood at room temperature and tendency of change thereafter*	*Stability in serum/plasma -20°C*	*4-8°C*	*20-25°C*	*Stabilizer*	*Comments*
Primidone	?		5 m	4 w	?	
Procainamide	?		6 m	2 w	?	
Quinidine	?		?	1 d	?	
Tetrahydrocannabinol (THC)			6 m	6 m	2 m	Sodium azide
Tetrahydrocannabinol)-9-carboxylic acid (THCA)			6 m			
Theophylline			3 m	12 w	12 w	
Tobramycin	?		4 w	3 d	Unstable	
Valporic acid	2 d		3 m	7 d	2 d	
Vancomycin	?		?	?	?	

URINE

Analyte	*Stability in urine at -20°C*	*4-8°C*	*20-25°C*	*Stabilizer*	*Comments*
Albumin	6 m	4 w	7 d		Do not freeze (nephelometry)
Aluminum	1 yr	7 d	3 d		
δ-Aminolaevulic acid	4 w	4 d	1 d	pH 6-7, stabilized with 0.3 % $NaHCO_3$	Drugs Light↓
α Amlylase	3 w	10 d	2 d		
Amphetamine	1 yr	?	?		
Bence Jones-Protein (light chains κ, γ)	6 m	4 w	7 d		
Benzoylecgonine	4 m	?	?	pH 5, ascorbic acid	Unsilanized glass containers
Calcium	3 w	4 d	2 d	Acidified, pH < 2	Crystallization at cool temperature
Catecholamines (noradrenaline, adrenaline, dopamine)	Unstabilized 20 d Stabilized 1 yr	4 d 1 yr	Acidified, pH < 2 4 d 3 w		

Contd...

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Analyte	*Stability in urine at -20°C*	*4-8°C*	*20-25°C*	*Stabilizer*	*Comments*
Citrate	4 w')	?	1 d**)	5 mL 1 % thymol	Storage acidified
Cocaine	4 m	3 w	?	pH 5, ascorbic acid	Unsilanized glass containers
Codeine	1 yr	?	?		
Copper	1 yr	7 d	3 d		
Cortisol	Stable	?	Unstable		
Creatinine	6 m	6 d	2 d		
Cystine	1 yr	3 m	7 d	HCl	
Glucose	2 d	2 d	2 h	Pregnancy, diet	Bacteria decrease stability
5-Hydroxyindole acetic acid	2 d	2 d	2 h	Acidified	

**Stored with HCl (pH< 1.7) **Thymol added*

Analyte	*Stability in urine at -20°C*	*4-8°C*	*20-25°C*	*Stabilizer*	*- Comments*
Hydroxyproline	5 d	5 d	5 d	Acidified	
Iron	yrs	7 d	3 d		
Immunoglobulin G (IgG)	Unstable	4 w	7 d		Do not freeze (nephelometry)
Lysergic acid diethylamide (LSD)	2 m	4 w	4 w		
α2-Macroglobulin	?	7 d	7 d		
Magnesium	1 yr	3 d	3 d	Acidified, pH < 2	
α1-Microglobulin	6 m	4 w	7 d		
Morphine	1 yr	?	?		
N-Acetyl-β-D-glucosaminidase (β-NAG)	4 w	7 d	1 d		
Osmolality	3 m	7 d	3 h		
Oxalate	4 m (at pH 1.5)	Unstable 1 h	pH < 2 (HCl)	Vitamin C ↑	

Contd...

Contd...

Analyte	*Stability in urine at* *-20°C*	*4-8°C*	*20-25°C*	*Stabilizer*	*- Comments*
pH		Unstable↑		Thymol	Increase by NH_4 formation
Phosphate, inorganic	?	?	2 d at pH <5.0	Add thymol	Precipitates at alkaline pH
Porphobilinogen	4 w	7d at pH 6-7	4 d at pH 6-7	pH 6 - by adding 0.3 % $NaHCO_3$	Acid pH↓
Porphyrins	4w	7d	4 d at	pH 6- by adding 0.3 %	Light↓
Total porphyrin Uroporphyrin Heptacarboxyporphyrin Hexacarboxyporphyrin Pentacarboxyporphyrin Coproporphyrin Tricarboxyporphyrin Dicarboxyporphyrin	Stabilized pH 6-7			$NaHCO_3$	
Potassium	1 yr	2 m	45 d		
Protein	4 w	7 d	1 d		
Pyridinium crosslinks (collagen crosslinks)	?	?	7 d	5 mmol/L Na-formate	UV-sensitive, stable at day light
Sediment Bacteria Casts (hyaline and others) Epithelial cells Erythrocytes Leukocytes	 24 h days hours 1-4 h 1-4 h	 24 h at osm >300 24 h at pH< 6.5 < 1 h at pH > 7.5		50% glycerol in 7% gelatine with 0.5 % thymol (storage in closed glass slides)	Do not freeze
Sodium	1 yr	45 d	45 d		
Test-strip fields Bacteria Erythrocytes Ketone bodies (acetoacetate) Leukocytes Protein Specific gravity		 24 h l-4 h 1-4 h	 hours ? 1 d hours at pH < 7.5 Unstable (pH effect)		

Contd...

Contd...

Analyte	*Stability in urine at* *-20°C*	*4-8°C*	*20-25°C*	*Stabilizer*	*- Comments*
Tetrahydrocannabinol-9 carboxylic acid (THCA)	1 yr	1 yr	10 w	Albumin	
Urea	4 w	7 d	2 d	pH < 7 ammonia is not	Stability less if included
Uric acid	Unstable, unless alkalized 4 d			pH > 8	precipitation at pH < 7
Vanilmandelic acid (VMA)	yrs	7 d at pH 3-5		7 d	pH 3-5 pH 3-5

CSF

Analyte	*-20°C*	*Stability in CSF* *4-8°C*	*20-25°C*	*Stabilizer*	*Comments*
Albumin	1 yr	2 m	1 d	EDTA tubes Up to 1 h: Do not cool.	Glucose, lactate: stability depends
Stability depends					
Glucose	Months	3 d	5 h ↓	Up to 3 h: Transport on ice	on cell content
IgG	Unstable	7 d	1 d	No additives No partial fixation	IgG: Freezing is not recommended
Lactate	Months 24 h	3 h ↑		Long-term storage:	Leukocytes, tumor cells:
Leukocytes smears. tumors cells	—	3.5 h	1-2 h	–70°C in glass or poly-propylene vessels that can be tightly closed	Store cells as dry
Protein, total	1 yr	6 d	1 d		

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