M.Sc. in MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

1st Semester

Prepared By

Paramedical & Allied Health Science Dept.

MMLT

MIDNAPORE CITY COLLEGE

Midnapore City College

PHYSIOLOGY AND HAEMATOLOGY LAB MANUAL

Code: MMLT 195

1. The Compound Microscope

Introduction

The microscope is one of the most commonly used instruments in the medical and life sciences colleges, and in clinical laboratories. Students of physiology use it in the study of morphology of blood cells and in counting their numbers. They will use it in histology, histopathology and microbiology and later in various clinical disciplines. Before using a microscope, the students must familiarize themselves with its different parts and how to use it and take its care. It will be discussed under the following heads:

1. Parts of the Microscope
A. The support system
B. The focusing system
C. The optical (magnifying) system
D. The illumination system.
i. Source of light
ii. Mirror
iii. Condenser.
2. Physical Basis of Microscopy
A. Visual acuity
B. Resolving power
C. Magnification
D. Numerical aperture
E. Image formation
F. Working distance
G. Calculation of total magnification.
3. Protocol (Procedure) for the Use of Microscope
A. Focusing under low power (100 x)
B. Focusing under high power (450 x)
C. Focusing under oil immersion (1000 x)
D. "Racking" the microscope.

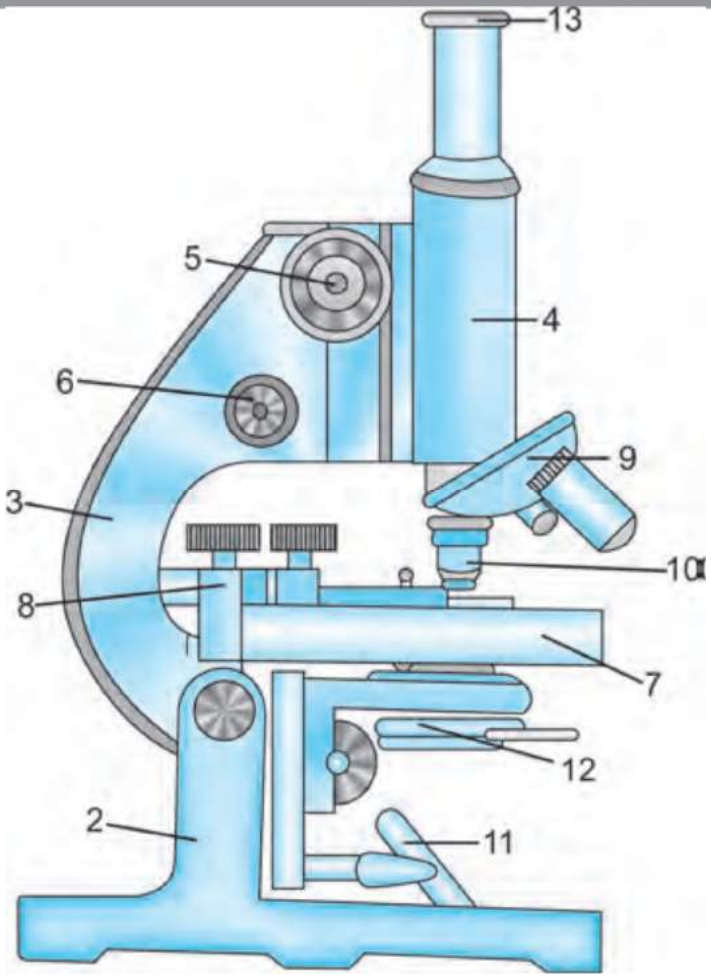


Figure 1: Compound microscope: (1) Base, (2) Pillars, (3) Handle, (4) Body tube, (5) Coarse adjustment screw, (6) Fine adjustment screw, (7) Fixed stage, (8) Mechanical stage, (9) Fixed and revolving nose pieces, (10) Objective lenses, (11) Mirror, (12) Condenser, and (13) Eye-piece

1. PARTS OF THE MICROSCOPE

A. The Support System

The support system functions as a framework to which various functional units are attached:

i. **Base.** It is a heavy metallic, U- or horseshoe-shaped base or foot, which supports the microscope on the worktable to provide maximum stability.

ii. **Pillars.** Two upright pillars project up from the base **(Figure 1)** and are attached to the C-shaped handle. The hinge joint allows the microscope to be tilted at a suitable angle for comfortable viewing.

iii. **Handle (the arm or limb).** The curved handle, which projects up from the hinge joint supports the focusing and magnifying systems.

iv. **Body Tube.** Fitted at the upper end of the handle, either vertically or at an angle, the body tube is the part through which light passes to the eyepiece, thus conducting the image to the eye of the observer. It is 16–17 cm in length, and can be raised or lowered by the focusing system.

v. **The Stage.** It has two components: the **fixed stage** and the **mechanical stage.**

a. Fixed stage. It is a square platform with an aperture in its center, and fitted to the limb below the objective lenses. The slide is placed on it and centered over the aperture for viewing. The converging cone of light emerging from the condenser passes through the slide and the objective into the body tube.

b. Mechanical stage. It is a calibrated metal frame fitted on the right edge of the fixed stage. There is a spring-mounted clip to hold the slide or counting chamber in position while two screw-heads move it from side to side and forwards and backwards. The vernier scale on the frame indicates the degree of movement. In some microscopes the screw-heads are mounted on a common spindle under the fixed stage.

B. The Focusing System

The focusing system consists of **coarse** and **fine** adjustment screw-heads. It is employed for raising or lowering the optical system with reference to the slide under study till it comes into focus. Thus, the adjustments place an objective lens at its optimal working distance, i.e. its focal length. There are two coarse and two fine adjustment screws working on a double-sided micrometer mechanism, one pair (one coarse and one fine) on either side. If one coarse (or fine) adjustment is turned, its partner on the other side also rotates at the same time. It is, therefore, not sensible to use both hands on the coarse or the fine screws simultaneously.

The **coarse adjustment** moves the optical system up or down rapidly through a large distance via a rack and pinion arrangement. The fine adjustment works in the same way but several rotations of the screw-head are required to move the tube through a small distance; e.g. one rotation moves the tube by 0.1 mm or less. The fine adjustment is usually graduated in l/50ths, where each division corresponds to a movement of 0.002 mm of the tube. It is employed for accurate focusing.

C. The Optical (Magnifying) System

The optical system consists of the body tube, the eyepiece, and the nosepiece that carries the objectives. It can be raised or lowered as desired.

i. The body tube. The distance between the upper ends of the objectives and the eyepiece is called the tube length, which is 16–17 cm. The distance between the upper focal point of the eyepiece and the lower focal point of the objective is called **the optical tube length,** which is about 25 cm (A × 10 lens will produce an image 10 times the diameter of the object as it naturally appears when held at 25 cm from the eye).

ii. The eyepiece. The eyepiece fits into the top of the body tube. Most microscopes are provided with 5 ×, 8 ×, and 10 × eyepieces, though 6 × and 15 × are also available. Each eyepiece has two

lenses—one mounted at the top, the **'eye lens',** and the other, the **'field lens'** is fitted at the bottom. The field lens collects the divergent rays of the primary image (see below) and passes these to the eye-lens, which further magnifies the image.

iii. The nosepiece. It is fitted at the lower end of the body tube and has two parts: the **fixed** nosepiece, and the **revolving** nosepiece. The latter carries interchangeable objective lenses. Any lens can be rotated into position when desired, its correct position being indicated by a 'click'.

iv. Objective lenses (also called objectives, or; simply 'lenses').

Three spring-loaded objectives of varying magnifying powers are usually provided with the student microscope. In some cases, there is a place provided for a 'scanning' lens as well. Each objective has a cover glass which forms its outer covering and protects it. Though each lens can be unscrewed for cleaning, the students are not supposed to remove them.

The magnifying power of each lens and its numerical aperture (NA) rather than its focal length, are etched on each.

The objective lenses are

a. Low-power (LP) Objective (10 ×; NA = 0.25; focal length = 16 mm).

The LP objective in common use magnifies 10 times. It is used for initial focusing and viewing a large area of the specimen slide. The numerical aperture (NA) of this lens is always less than that of the condenser in most microscopes. In order to achieve focus, therefore, the NA has got to be closely matched by reducing the light reaching the specimen under study. This is achieved by lowering the condenser and partially closing the iris diaphragm. (See below).

b. High-power (HP) Objective (45 ×; NA = 0.65; focal length = 4 mm).

This lens magnifies the image 45 times. Because of its higher magnification, it is used for more detailed study of the material before switching to oil immersion lens. The NA of HP lens is almost equal to, or slightly less than that of commonly used condenser. Therefore the latter has to be slightly raised and the iris diaphragm opened to get more light and maximum clarity in focusing.

c. Oil-immersion (OI) Objective (100 ×; NA = 1.30; focal length = 2mm).

The OI lens magnifies the image 100 times. Since the lens almost touches the slide it has to be immersed in a special medium (most commonly cedar wood oil), a drop of which is first placed on the slide. The oil is used to increase the NA and thus the resolving power of the objective. Since the NA of OI objective is always greater than that of the condenser, the latter has to be raised to its highest position and iris diaphragm fully opened. As this lens gives (with an eyepiece of 10 ×) a total magnification of 1000 times, it is employed for detailed study of the morphology of blood cells and tissues.

d. Scanning Objective (3 ×; NA = 0.10; focal length = 40 mm).

This objective, a very low power lens, magnifies the image 3 times. It is used for scanning (or viewing) a much larger area on the slide.

Parfocal system. The objectives these days are so constructed that when one lens (LP, for example) is in focus, the others are more or less in focus. Thus switching from one lens to another (e.g. from

LP to HP) requires only a little turn of fine adjustment to bring the image into sharp focus. This arrangement of lenses is called "parfocal system."

D. The Illumination System

No microscope can function optimally unless proper illumination (lighting) is provided. All the light that will reach the eye should come from the specimen under study. Light from any other part of the slide will tend to obscure the details. Such extra (extraneous) light is called glare. The illumination system must, therefore, provide uniform, soft, and bright illumination of the entire field of view. Two factors are involved in providing such uniform illumination:

i. The construction and position of the condenser.
ii. The size of the iris diaphragm.

Types of Illumination. The compound microscopes work on six types of illumination: **'Bright-field' or 'light' microscope. This is the usual student microscope** that uses white light, either external or internal, as the source of illumination.

Seen under this light, the objects look darkish or colored, contrasted against a lighted background. The **other types** of illumination systems include: **Dark-field microscope, Phase-contrast microscope. Fluorescent microscope, Polarizing microscope,** and **Interference-contrast microscope.** (See below for their brief descriptions).

The **illumination system** of the bright-field microscope consists of: a source of light, and a mechanism to condense the light and direct it into the specimen under study.

i. Source of light. The light source may be outside the microscope or within the microscope.

External light source. It may be the diffuse, natural daylight (sunlight) reflected and scattered by the atmosphere and its dust particles and reflected from the buildings. On bright, sunny days, the north daylight, which is a distant light source, is ideal for routine student work.

If daylight is not available, or is not sufficient, an artificial source of light—a fluorescent tube, or an electric lamp housed in a lamp box with a frosted glass window, fitted on the worktable can provide enough light.

Internal light source. In most microscopes, there is a provision to remove the mirror and fit an electric microscope lamp in its place. This unit has frosted tungsten lamp to provide uniform white light.

ii. The mirror. A double-sided mirror, in fact two mirrors, one flat or plane and the other concave, fitted back to back in a metal frame is located below the condenser; it can be rotated in all directions. The plane mirror is used with a distant source of light (natural, or daylight). The parallel rays of light are reflected parallel into the condenser. The concave mirror, on the other hand, is employed when the light source is near the microscope. The divergent rays of light are reflected as parallel rays and directed into the condenser.

iii. The condenser ('Substage' or 'substage condenser'). The condenser is a system of lenses fitted in a short cylinder that is mounted below the stage. It can be raised or lowered by a rack and pinion, and focuses the light rays into a

solid cone of light onto the material under study. It also helps in resolving the image.

a. The lens system. The commonly used substage is Abbe-type condenser. It is composed of two lenses which should be corrected for spherical and chromatic aberrations.

Since the condenser is a lens system, it has a fixed NA, which should be equal or less than that of the objective being used. Raising or lowering the condenser can vary its NA. And with the axes of the two being the same, all the light passing through the condenser is collected by the objective, thus allowing maximum clarity.

b. The iris diaphragm. It is fitted within the condenser. A small lever on the side can adjust the size of the aperture of the diaphragm, thus allowing more or less light falling on the material under study. Reducing the size of the field of view (i.e. by narrowing the aperture) decreases the NA of the condenser. Thus, proper illumination includes a combination of position of light source, regulation of light intensity, position of condenser, and regulation of the size of field of view.

c. Filter. A metal ring can accommodate a pale blue or green filter since monochromatic light is ideal for microscopy.

• Generally, when viewing clear preparations under low power, we need less light, but more illumination is required when studying stained preparations under oil-immersion lens.

2. Collection of Blood Samples

Since blood is confined within the cardiovascular system, the skin has to be punctured before blood can be obtained. There are two common sources of blood for routine laboratory tests: *blood **from a superficial vein*** by puncturing it with a needle and syringe, or ***from skin capillaries*** by skin-prick. Arterial blood and blood from cardiac chambers may be required for special tests. None of these samples can be called a representative sample because there are minor variations in their composition.

ASEPSIS

The term asepsis refers to the condition of being free from septic or infectious material—bacteria, viruses, etc. The skin is a formidable barrier to the entry of foreign invaders and the first line of defence against bacteria and other disease-causing microorganisms which are present in abundance on the skin and in the air. Therefore, puncturing the skin always poses the danger of infection.In order to achieve asepsis, the following aspects need to be kept in mind:

A. Sterilization of Equipment

All the instruments to be used for collecting blood— syringes, needles, lancets, and cotton and gauze swabs—should preferably be sterilized in an autoclave. The old practice of boiling glass syringes and needles in tap water is now obsolete. Irradiated and sealed, single-use syringes, needles, lancets and blades are now freely available and are in common use.

B. Cleaning/Sterilization of Skin

Though it is impossible to completely sterilize the selected site for skin puncture, every aseptic precaution must be exercised. The selected area need not be washed and scrubbed unless grossly dirty. If washed, the area should be allowed to dry before applying the antiseptics because these agents do not act well on wet skin. At least 2–3 sterile cotton/gauze swabs soaked in 70% alcohol, methylated spirit, or ether should be used to clean and scrub the area. Cotton swabs are likely to leave fibers sticking to the skin and provide an undesirable contact, or they may appear as artifacts in a blood film. But if they are used, the final cleaning should be done with gauze swab.

C. Prevention of Contamination

Any material used for skin puncture, or the operator's hands may cause contamination. Therefore, once the site has been cleaned and dried, it should not be touched again. Care must be taken to prevent contamination until the puncture wound has effectively closed/healed.

THE BLOOD SAMPLE

The term "blood sample" refers to the small amount of blood—a few drops or a few milliliters—obtained from a person for the purpose of testing or investigations. These tests are carried out for aiding in diagnosis and/ or prognosis of the disease or disorder.

A. Sources and Amount of Blood Sample

i. Capillary blood. The skin and other tissues are richly supplied with capillaries, so when a drop or a few drops of blood are required, as for estimation of Hb, cell counts, BT and CT, blood films, micro chemical tests, etc, blood from a skin puncture (skin-prick) with a lancet or needle is adequate.

ii. Venous blood. When larger amounts (say, a few ml that cannot be obtained from a skin puncture) are needed as for complete hematological and biochemical investigations, venous blood is obtained with a syringe and needle by puncturing a superficial vein. In infants, venous blood may
have to be taken from the femoral vein, or the frontal venous sinus.

iii. Arterial blood. When arterial blood is needed for special tests such as blood pH, gas levels, etc, an artery such as radial or femoral is punctured with a syringe and needle. This, however, is not a routine procedure.

iv. Cardiac catheterization. Blood from a heart chamber, taken through a cardiac catheter, may be required for special tests.

B. Containers for Blood Sample

A container is a receptacle into which blood is transferred from the syringe before sending it to the laboratory. Clean and dry 10 ml glass test tubes, collection bottles such as clean and dry 10 ml

discarded medicine vials, glass bulbs, etc are the usual ones in use. A container may or may not contain an anticoagulant depending on whether a sample of blood/plasma, or serum is required.

***For a sample of whole blood or plasma*.** The blood is transferred to a container containing a suitable anticoagulant. This is to prevent clotting of blood.

***For a sample of serum*.** No anticoagulant is used. The blood is allowed to clot in the container and serum is collected as described later.

Obviously, capillary blood does not require a container or anticoagulant.

C. Differences Between Venous and Capillary Blood

The differences between these two sources of blood are given in **Table 1-1**.

COMMONLY USED ANTICOAGULANTS

Anticoagulants are substances employed to delay, suppress, or prevent clotting of blood. They are classified into 2 groups: the *in vitro* (outside the body) anticoagulants, and the in vivo (in the body) anticoagulants. The commonly used *in vitro anticoagulants* include: EDTA, trisodium citrate, double oxalate, sodium fluoride, heparin, and ACD and CPD-A mixtures. The use of fluoride and heparin is limited to pH, blood glucose and gas analysis. The *in vivo* anticoagulants include: heparin and dicoumarol derivatives (warfarin, dicoumarin). Thus, heparin is both an *in vivo* and an *in vitro* anticoagulant.

COLLECTION OF VENOUS BLOOD

Puncturing a vein and withdrawing blood from it will be demonstrated to you because it requires some degree of skill and confidence. It needs assistance and complete aseptic precautions. (In due course of time, you will also learn to do venepuncture).

For a Sample of Whole Blood or Plasma. (Plasma = Blood minus all the blood cells). Draw blood from a vein as described below and transfer it from the syringe to a container containing a suitable anticoagulant. Mix the contents well without frothing. A sample of whole blood is now ready for tests. If plasma is desired, centrifuge the anticoagulated blood for 20–30 minutes at 2500 rpm, as described later. Collect the supernatant plasma with a pipette and transfer it to another container. (The packed RBCs will be left behind).

For a Sample of Serum. (Serum = Plasma minus fibrinogen and all the clotting factors). Transfer the blood from the syringe to a container *without any anticoagulant* in it, and keep it undisturbed. After the blood has clotted in an hour or two and the clot shrunk in size, the serum will be expressed. Remove the supernatant serum with a pipette and transfer it to a centrifuge tube. Centrifuge it to remove whatever red cells may be present. Clear serum can now be collected with another pipette.

APPARATUS AND MATERIALS

Keep the following equipment ready before venepuncture:

1. Disposable gloves. These should always be worn before venepuncture.
2. Sterile, disposable, one-time use, 10 ml syringe with side nozzle. Two 22-gauge needles with short bevels (the flattened puncturing points).
3. 10 ml test tubes, or vials, with or without anticoagulant.
4. Sterile gauze pieces moist with 70% alcohol/ methylated spirit.
5. Tourniquet. A 2–3 cm wide elastic bandage with Velcro strips to keep it securely in place. (It will be used to obstruct the venous return and make the veins prominent just before venepuncture). Alternately, a blood pressure cuff attached to its apparatus, or a 'twisted' handkerchief can serve the purpose.

PROCEDURES

1. Seat the subject comfortably on a chair with an arm rest, or near a table. The subject, if nervous, may lie down on a bed. Reassure the subject by your approach and conversation.
2. Examine both arms in front of the elbows to locate a suitable vein. Ask your assistant to compress the upper arm with his hands to make the veins prominent. The antecubital (medial basilic) vein is embedded in subcutaneous fat and is usually sufficiently large to take a wide-bore needle. It also runs straight for about 3 cm, and is usually palpable—even in obese subjects. If the vein is neither visible nor palpable, try the other arm. (You should avoid superficial veins because they are notoriously slippery. Veins above the ankle or on the back of the hand may have to be used).
3. Once a suitable vein has been selected, support the subject's arm over the edge of the table. Wash your hands with soap and water, dry them on a sterile towel, and put on the gloves. Ask your assistant to open the syringe pack. Take out the syringe and attach the needle (it is attached/ detached with a little twist), with its bevel facing you.
4. Ask your assistant to apply the tourniquet about 2-3 cm above the elbow to obstruct the venous return. The subject may open and close her fist to increase the venous return and make the veins engorged (filled) with blood. If the vein is still not sufficiently prominent, a few 'slaps' with your fingers over the region may do so. Clean the skin over the selected vein with gauze and alcohol and allow it to dry. With the fingers of your left hand supporting and steadying the elbow from behind, stretch the skin over the vein downward with your left thumb placed about 4 cm below the vein. This traction fixes the vein and prevents its slipping when it is punctured.
5. With the piston pushed in, the side nozzle towards the subject's arm, and the bevel of the needle facing you, hold the syringe between your fingers and thumb of the right hand.
6. With the first finger placed near the butt of the needle, puncture the skin and push in the needle under the skin with a firm and smooth thrust, at an angle of 15-20° to the skin.
7. Slightly pull the plunger back with your thumb and little finger to produce a little negative pressure in the syringe. Advance the needle gently along the vein and puncture it from the side, a few mm ahead of the skin puncture. This prevents counterpuncture of the far wall of the vein and formation of a hematoma (local leakage of blood).

8. As the vein is punctured, all resistance will suddenly cease and blood will start to enter the syringe. With the needle still in the vein, and supporting the syringe with your left hand, gently pull the plunger back with the thumb and fingers of your right hand. Do not withdraw blood faster than the punctured veins is filling as too much pressure applied to the plunger is likely to cause mechanical injury and hemolysis of red cells. The subject may open and close the fist to enhance venous return.
9. When enough blood has been collected, release the tourniquet and press a fresh swab over the skin puncture. Withdraw the needle gently but keep the swab in position. Ask the subject to flex the arm and keep it so to maintain pressure on the puncture site till the bleeding stops. The arm may be raised above the head for a minute or so if required.
10. Hold the syringe vertical with the plunger supported, and remove the needle with a slight twist. Expel the blood gently into the container; do not apply force as it may cause mechanical injury to red cells. Gently shake, or swirl the container between your palms so that the anticoagulant (if used) mixes well with the blood without frothing.
4. Ask the subject to keep the swab in position till the bleeding from the puncture site stops.

COLLECTION OF CAPILLARY BLOOD (SKIN-PRICK METHOD)

In most medical colleges and clinical laboratories, trained laboratory technicians give the skin prick because an anxious student may only make a superficial prick on her or her partner's finger. This will force her to squeeze the finger that will expel tissue fluid along with blood to come out of the puncture site. The dilution of blood will, thus, nullify the results. *Hence for clinical work, venous blood is always preferred.* Skin-prick may be used on the bedside of a patient, or in an emergency when it is not convenient to take a venous sample. With a little practice, and confidence, the students should be able to give skin pricks to their work-partners with confidence.
• Capillary blood is also called **"peripheral blood"** as it comes out of the peripheral vessels (capillaries) in contrast to venous blood.

Selection of Site for Skin Prick

In adults and older children, capillary blood is generally obtained from a skin puncture made on the tip of the middle or ring finger, or on the lobe of the ear. In infants and young children in whom the fingers are too small for a prick, the medial or lateral side of the pad of the big toe or heel is used. The site for skin-prick should be clean and free from edema, infection, skin disease, callus, or circulatory defects.

APPARATUS

1. **Blood Lancet/Pricking needle.** Disposable, sterile, one-time use, blood lancets (flat, thin metal pieces with 3–4 mm deep penetrating sharp points) are commercially available and should be preferred. Lancets with 3-sided cutting points and mounted in plastic are also suitable. However, lancets with thin and shallow points are not satisfactory.

Ordinary, narrow-bore **injection needles** are useless since they only make shallow cuts rather than deep punctures. However, wide-bore (22 gauge) needles may be used in an emergency or if blood lancets are not available.

A **cutting needle** with 3-sided cutting point (used by surgeons) can serve the purpose well.

Pricking gun. A spring-loaded pricking gun that has a disposable, 3-sided sharp point, and a loading and releasing mechanism, is ideal because the depth of the puncture can be preselected. After pulling back the release lever, and thus "loading" the gun, it is placed on the ball of the finger and the release lever pressed. The subject does not see the sharp point and the pain is thus minimized.

2. Sterile gauze/cotton, moist with 70% alcohol/ methylated spirit.

3. Glass slides, pipettes, etc. according to requirements.

PROCEDURES

All aseptic precautions must be taken. The person giving the prick should wash his/her hands with soap and water, and wear gloves if possible.

1. Clean and vigorously rub the ball of the finger with the spirit swab, followed by a final cleaning with dry gauze. (Scrubbing increases local blood flow).

Allow the alcohol to dry by evaporation for the following reasons:

i. Sterilization with alcohol/spirit is effective only after it has dried by evaporation.

ii. The thin film of alcohol can cause the blood drop to spread sideways along with alcohol so that it will not form a satisfactory round drop.

iii. The alcohol may cause hemolysis of blood.

2. Steadying the finger to be pricked in your left hand, apply a gentle pressure on the sides of the ball of the finger with your thumb and forefinger to raise a thick, broad ridge of skin. (Do not touch the pricking area).

3. Hold the lancet between the thumb and fingers of your right hand, and keeping it directed along the axis of the finger, but slightly "off" center so as to miss the tip of the phalanx (i.e. not too far down or too far near the top of the nail bed), prick the skin with a sharp and quick vertical stab to a depth of 3–4 mm and release the pressure. The blood should start to flow slowly, spontaneously and freely (without any squeezing)—if a good prick has been given.

4. Wipe away the first 2 drops of blood with dry, sterile gauze as it may be contaminated not only with tissue fluid, but also with epithelial and endothelial cells which will appear as artifacts in the blood film.

5. Allow a fresh drop of blood of sufficiently large size (about 3–4 mm diameter) to well up from the wound, and make a blood smear, or fill a pipette as the case may be.

6. Clean the area of the prick with a fresh swab and ask the subject to keep the swab pressed on the wound with his/her thumb till the bleeding stops, which occurs in a minute or so.

3. Measurement of Pulse rate

Principle:

Pulse/heart rate is the wave of blood in the artery created by contraction of the left ventricle during a cardiac cycle. The strength or amplitude of the pulse reflects the amount of blood ejected with myocardial contraction (stroke volume). Normal pulse rate range for an adult is between **60-100 beats per minute**. A well-trained athlete may have a resting heart rate of 40 to 60 beats per minute, according to the American Heart Association.

Procedure:

Ask whether the patient has walked, climbed stairs, or otherwise exerted themself in the last 20 minutes. If not, you can proceed. If the answer is yes, wait 20 minutes before taking the reading. This will help to prevent false readings.

Make sure the patient is relaxed and comfortable.

Place the tips of your first and second finger on the inside of the patient's wrist.

Press gently against the pulse. Take your time to note any irregularities in strength or rhythm.

If the pulse is regular and strong, measure the pulse for 30 seconds. Double the number to give the beats per minute (e.g.: 32 beats in 30 seconds means the pulse is 64 beats per minute). If you noticed changes in rhythm or strength, you must measure the pulse for a full minute.

Record the pulse rate (the number of beats per minute) in the patient's notes and describe its strength and rhythm. Compare the pulse rate with the values in the Table land record whether the pulse is normal, slow or fast. Any abnormalities should be recorded and reported to the senior nurse and doctor.

Strength of the pulse is a very subjective measurement, but an experienced nurse will compare it with what has been felt previously in other patients. Describe the puse as ‘weak’, ‘faint’, ‘strong' or' bounding’.

Think about the rhythm of the pulse. Is it regular? If irregular, in what way? Cardiac problems may present as a regular missed beat, for example, so is the irregularity regular (described as regularly irregular) or is there no pattern (described as irregularly irregular)?

Discuss with your patient the result of the pulse measurement and if any further investigations are required.

Wash and dry your hands.

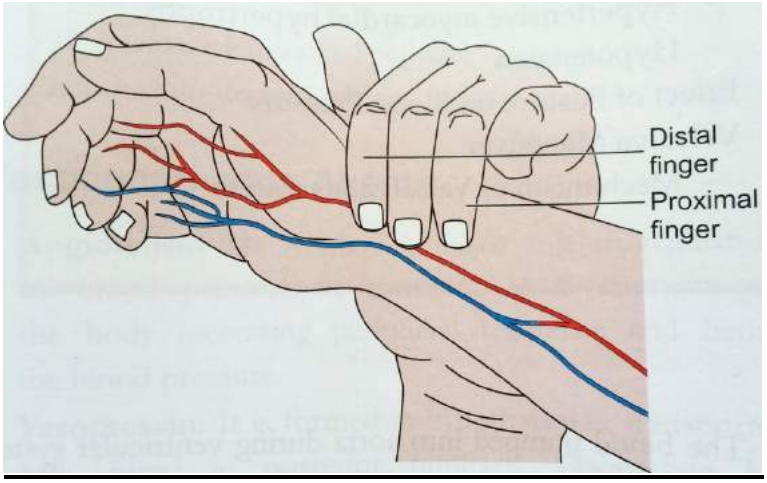
Pulse point	Area of palpation
Temporal pulse	Over the temple, in front of ear on superficial temporal artery
Carotid pulse	In the neck along anterior border of sternocleidomastoid muscle on common carotid artery
Brachial pulse	In cubital fossa along medial border of biceps muscle on brachial artery

Radial pulse	Over the thumbside of wrist between tendons of brachioradialis and flexor carpi radialis muscles on radial artery
Ulnar pulse	Over the little fingerside of wrist on ulnar artery
Femoral pulse	In the groin on femoral artery
Popliteal pulse	Behind knee, in the popliteal fossa on popliteal artery

Measurement of redial artery pulse –

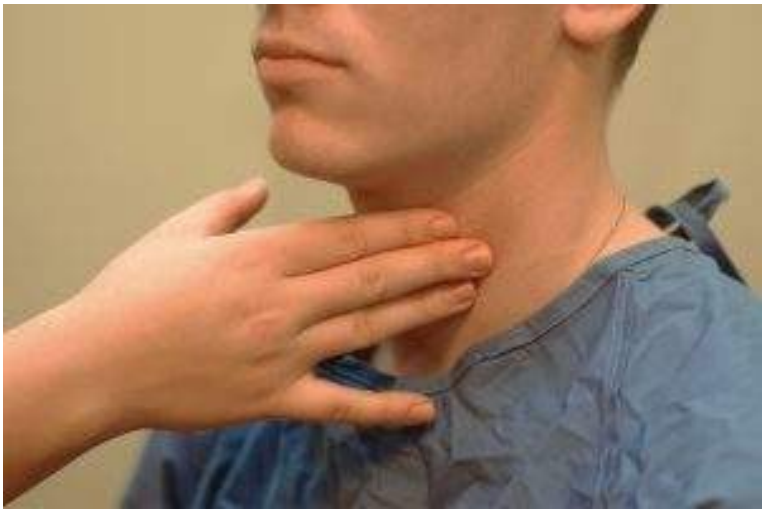
1. With the palm up, look at the area between wrist bone and the tendon on the thumb side of wrist. Radial pulse can be taken on either wrist.
2. Use the tip of the index and third fingers of other hand to feel the pulse in radial artery between wrist bone and the tendon on the thumb side of wrist.

3. Apply just enough pressure so can feel each beat. Do not push too hard, it will obstruct the blood flow.
4. Watch the second hand on watch or a clock to count how many times.
5. Record your pulse rate.



Measurement of carotid artery pulse –

Ask the client to sit upright. Locate the carotid artery medial to the sternomastoid muscle (between the muscle and the trachea at the level of the cricoid cartilage, which is in the middle third of the neck). With the pads of your three fingers, gently palpate the carotid artery, one at a time.



Evaluation Of Pulse

1. Rate :-
   Count the pulse for 1 min / at least 30 sec
   Normal : 60 – 100 /min
   Tachycardia : >100 /min
   Bradycardia : <60 /min

❖ Increased pulse rate due to- exercise, fever, anxiety, hyperthyroidiam and atrial and ventricular trachycardias.
Decreased pulse rate in bradycardia, hypothyroidism and incomplete heart blocks.

2. Rhythms –
   Assessed by palpating radial artery
   It is noted regular or irregular.
   Under normal sinus bradycardia or sinus trachycardia pulse appears at regular intervals.
   Irregular pulse rhythm is a feature of extra systole, Atrial Tachyarrhythmia, atrial fibrillation.
   Observation-
   Regularly irregular : Atrial Tachyarrhythmia with fixed AV block .
   Irregularly irregular : Atrial / ventricular ectopic, Atrial Fibrillation.

3. Volume

   Assessed by palpating – carotid artery

   Correlates with stroke volume.

   High volume – elderly person, anxiety, Aortic regurgitation, fever, thyrotoxicosis.

   Observation: Normal / High/ Low volume.

4. Character of blood vessel-

- Thickened and palpable / soft and not palpable.
- It is usually evaluated at right carotid artery.
- It becomes more prominent in some abnormal conditions such as anacrotic pulse, water hammer pulse, pulsus paradoxus.

5. Character of pulse :

   Catacrotic / Aanacrotic

Palpitation / measurement format for the arterial pulse rate (beats/min).

- Measurement No….
  - Name of subject-
  - Age –
  - Sex-

Pulse Rate	Carotid artery pulse (Beats/min)	Radial artery pulse (Beats/min)
Resting (Before Exercise) (15 sec x 4)		
Just After step test for 5 minutes		
After 10 minutes of resting		
After 20 minutes of resting		
After 30 minutes of resting		

- Evaluation of resting pulse (before exercise) - -

1. Rate-
2. Rhythm –
3. Volume-
4. Character of arterial wall-
5. Character of pulse-

Interpretation of resting results: -

……
……

4. Measurement of blood pressure

The term blood pressure refers to the force exerted by the blood as it presses against and attempts to stretch the walls of blood vessels. Although blood exerts this outward force throughout the CVS, the term blood pressure, used unqualified, refers to systemic arterial blood pressure (others are: venous, capillary pressure, etc). The blood pressure (BP) is not steady (unchanging) throughout the cardiac cycle but fluctuating, i.e. it is pulsatile. It rises and falls; reaching its maximum during systole of the heart, when it is called the systolic blood pressure (SBP); and falling to its minimum during diastole of the heart when it is called diastolic blood pressure (DBP) Measurement of blood pressure is an important clinical procedure as it provides valuable information about the cardiovascular system (CVS) under normal and disease conditions.

PRINCIPLE:

A sufficient length of a single artery is selected in the arm (brachial artery), or in the thigh (femoral artery). The artery is first compressed by inflating a rubber bag (connected to a manometer) placed around the arm (or thigh) to stop the blood flow through the occluded section of the artery. The pressure is then slowly released and the flow of blood through the obstructed segment of the artery is studied by:

i. Feeling the pulse—the palpatory method.
ii. Observing the oscillations of the mercury column—the oscillometric method, and
iii. Listening to the sounds produced in the part of the artery just below the obstructed segment—the auscultatory method.

PROCEDURES:

The subject may be lying down (supine) or sitting, but should be mentally and physically relaxed and free from excitation and anticipation.

Lay the arm bare up to the shoulder and record the blood pressure first with the palpatory method, followed by auscultatory method. The upper arm on which the BP cuff is to be tied must be at the level of the heart. (In the supine position, the arm resting on the bed will be nearly at the heart level. In the sitting position the arm resting on the table of a suitable height will be at the correct level).

In obese subjects, the cuff may be applied on the forearm with the stethoscope placed over the radial artery for auscultatory method. (If no sounds are heard a reasonably reliable determination can be obtained by palpation at the wrist).

Palpatory Method (Riva Rocci 1896)

1. Make the subject sit or lie supine and allow 5 minutes for mental and physical relaxation.
2. Open the lid of the apparatus until you hear the “click”. Release the lock on the mercury reservoir and check that the mercury is at the zero level. If it is above zero, subtract the difference from the final reading. If it is below zero, add the required amount of mercury to bring it to zero level.

3. Place the cuff around the upper arm, with the centre of the bag lying over the brachial artery, keeping its lower edge about 3 cm above the elbow. Wrap the cloth covering around the arm so as to cover the rubber bag completely, and to prevent it bulging out from under the wrapping on inflation. The cuff should neither be too tight nor very loose.
4. Palpate the radial artery at the wrist and feel its pulsations with the tips of your fingers. Keeping your fingers on the pulse, hold the air bulb in the palm of your other hand and tighten the leak valve screw with your thumb and fingers.
5. Inflate the cuff slowly until the pulsations disappear; note the reading then raise the pressure another 30–40 mm Hg.
6. Open the leak valve and control it so that the pressure gradually falls in steps of 2–3 mm. Note the reading when the pulse just reappears. **The pressure at which the pulse is first felt is the systolic pressure**. (It corresponds to the time when, at the peak of each systole, small amounts of blood start to flow through the compressed segment of the brachial artery). Deflate the bag quickly to bring the mercury to the zero level.

7. Record the pressure in the other arm. Take 3 readings in each arm, deflating the cuff for a few minutes between each determination.

Advantages of palpatory method.

This method avoids the pitfall of the auscultatory method in missing the auscultatory gap.

Disadvantages of palpatory method:
This method measures only the systolic pressure, the diastolic pressure cannot be measured.
This method lacks accuracy because the systolic pressure measured by it is lower than the actual by 4–6 mm Hg. It assumes that the first escape of blood under the cuff will cause pulsations in the peripheral artery (radial in this case). However, there is no evidence that the amount of blood that escapes when the artery first opens is enough to produce a pulse wave detectable by the fingers. Thus, definite pulsation may not occur until the cuff pressure has been reduced by 6–8 mm Hg.

Auscultatory Method (Korotkoff, 1905)

1. Place the cuff over the upper arm as described above, and record the BP by the palpatory method.
2. Locate the bifurcation of brachial artery (it divides into radial and ulnar branches) in the cubital space just medial to the tendon of the biceps which can be easily palpated in a semi-flexed elbow as a thick, hard, elongated structure. Mark the point of arterial pulsation with a sketch pen.
3. Place the chest-piece of the stethoscope on this point and keep it in position with your fingers and thumb of the left hand (if you are right-handed).
4. Inflate the cuff rapidly, by compressing and releasing the air pump alternately (sounds may be heard as the mercury column goes up). Raise the pressure to 40 to 50 mm Hg above the systolic level as determined by the palpatory method.
5. Lower the pressure gradually until a clear, sharp, tapping sound is heard. Continue to lower the pressure and try to note a change in the character of the sounds.

These sounds are called Korotkoff sounds and show the following phases:

Phase I This phase starts with a clear, sharp tap when a jet of blood is able to cross the previously obstructed artery. (Sometimes this phase may start with a faint tap, especially when the systolic pressure is very high). As the pressure is lowered, the sounds continue as sharp and clear taps. This phase lasts for 10–12 mm Hg fall in pressure.

Phase II The sounds become murmurish and remain so during the next 10–15 mm Hg fall in pressure when they again become clear and banging.

Phase III It starts with clear, knocking, or banging sounds that continue for the next 12 to 14 mm Hg pressure, when they suddenly become muffled.

Phase IV The transition from phase III to phase IV is usually very sudden. The sounds remain muffled, dull, faint and indistinct (as if coming from a distance) until they disappear. The muffling of sounds and their disappearance occurs nearly at the same time, there being a difference of 4–5 mm Hg (i.e. phase IV lasts for 4–5 mm Hg).

Phase V This phase begins when the Korotkoff sounds disappear completely. If you reduce the pressure slowly, you will note that total silence continues right up to the zero level.

6. Take 3 readings with the auscultatory method and repeat 3 readings on the other arm.

7. Effects of posture, gravity, and muscular exercise on blood pressure are discussed in the next experiment.

Effect of postural changes in Blood Pressure

The effect of changes in posture depends on whether these are recorded immediately after standing from supine position, or after prolonged standing. They also depend on whether a person stands against a support (e.g. a wall), or is standing 'free' and still.

Immediate Effect. As the person assumes erect position, blood tends to pool in the lower parts of the body (especially in the veins) due to gravity. This decreases the venous return, and hence CO and BP. A pooling of 250–300 ml of blood can decrease the systolic pressure by 10–15 mm Hg. However, within some 8–10 seconds the sino-aortic baroreceptor mechanism restores BP to normal level.

Effect of Prolonged Standing. If a person stands still, especially against a support, more than 500 ml of blood may pool in the lower body. Also, increased capillary hydrostatic pressure causes fluid to be filtered out into the tissues, which further reduces venous return. The CO and BP fall, resulting in cerebral ischemia that causes the person to fall down unconscious. The fainting is actually a homeostatic mechanism, as it restores venous return, CO and BP, thus relieving cerebral ischemia.

PROCEDURES

1. Allow the subject to rest and relax for a few minutes in the supine position. Record the heart rate (pulse rate) and BP by the palpatory method and auscultatory method (later on by auscultatory method alone). Disconnect the cuff from the BP apparatus.
2. Ask the subject to sit up and immediately record the BP and heart rate (HR). Repeat the determinations after 1 minute, 2 and 5 minutes.
3. Make the subject lie down again and rest for a few minutes. Then record the BP and HR. Now ask him to suddenly stand up, and record the BP and HR.
4. Record your observations in your workbook.

Interpretation of Results

Measurement of arterial blood pressure by palpatory method-

- Measurement No….
  - Name of subject-
  - Age –
  - Sex-

	Pulse Dis-appears (mmHg)	Pulse Re-appears / Systolic blood pressure (mmHg)
Resting (Before Exercise)		
Just After step test for five minutes		
After 10 minutes of resting.		
After 20 minutes of resting.		
After 30 minutes of resting.		

Interpretation of your results: -

……

Measurement of arterial blood pressure by auscultatory method-

- Measurement No….
  - Name of subject-
  - Age –
  - Sex-

	Systolic BP (mmHg)	Diastolic BP (mmHg)	Pulse pressure (mmHg)	Mean arterial pressure (mmHg)
Resting (Before Exercise)				
Just After step test for five minutes				
After 10 minutes of resting.				

After 20 minutes of resting.				
After 30 minutes of resting.				

Interpretation of your results: -

……

Postural changes and Blood Pressure

Measurement No….

- Name of subject-

Three posture	Systolic BP (mmHg)	Diastolic BP (mmHg)	Pulse pressure (mmHg)	Mean arterial pressure (mmHg)
In supine				
In sitting				
In standing				

Interpretation of your results: -

……

5. Measurement of respiratory rate

Principle:

Respiratory rate (RR) is an essential vital sign and a fundamental element of patient assessment. Generally, changes in RR occur automatically in response to a physiological demand but it is possible to consciously increase or decrease RR for short periods. Changes from as little as three to five breaths per minute (bpm) may indicate a change in the patient's condition and are often the first sign of deterioration. RR therefore provides a baseline for future comparisons and helps determine the patient's acuity.

Other key indications for measuring RR include:

- Post-operative monitoring;
- Detecting complications such as pulmonary oedema associated with blood transfusion and administration of intravenous fluids;
- Identifying patients who are deteriorating and are critically ill;
- Evaluating response to treatment – for example use of opiates, which can cause respiratory depression;
- Monitoring chronic lung disease such as chronic obstructive pulmonary disease, and

response to treatment;

- Monitoring patients receiving oxygen therapy

Procedure

1. Wash hands with soap and water to reduce infection risk.
2. Position the patient in a comfortable position. Maintain a constant temperature to prevent shivering, which can increase RR. If possible, remove bulky clothing or bed covers from the upper part of the patient's body to facilitate counting RR and observing depth, symmetry and pattern of breathing. If the patient is sitting, their feet must be flat on the floor; sitting with legs suspended can reduce venous return, which may increase heart rate and subsequently RR.
3. If a patient has been prescribed oxygen, ensure the oxygen mask or nasal cannula is correctly positioned and recorded on the observation chart before recording RR.
4. Allow the patient to rest, if possible, for 20 minutes before taking the measurement. RR may increase after activity, giving an abnormal baseline. Some medication can affect RR so this should also be taken into consideration.
5. While you are preparing the patient, observe their respiratory function, for example, whether they can talk in full sentences. Taking a breath mid-sentence or one-word answers may be a sign of respiratory distress.
6. Note whether the patient is alert and orientated to time and place. Changes in cognitive status, such as confusion, may be due to hypoxia, cerebral injury or side-effects of medication, such as opiates.
7. It may be useful to assess RR at the same time as pulse rate or oxygen saturation. This will give a more accurate rate and minimise any subconscious influence, as patients may alter their breathing if they know they are being observed.
8. Using a watch with a second hand, count breaths (number of times the chest moves up and down) for a full minute. This length of time is needed as changes can occur in the respiratory pattern and rate.
9. While observing the RR, note the rhythm, which may indicate signs of underlying illness. Respirations should be regular with equal pause between each breath.
10. Observe the patient's lips for signs of cyanosis (blue tinge), which may indicate hypoxia (low oxygen saturation [SpO2]). Pulse oximetry is a valuable tool to measure SpO2 but it has limitations when a patient's peripheral circulation or condition is compromised – for example, through tremor, shivering, hypovolaemia, hypothermia, heart failure or vaso-constriction.
11. Record the RR on the observation chart and report any abnormalities.

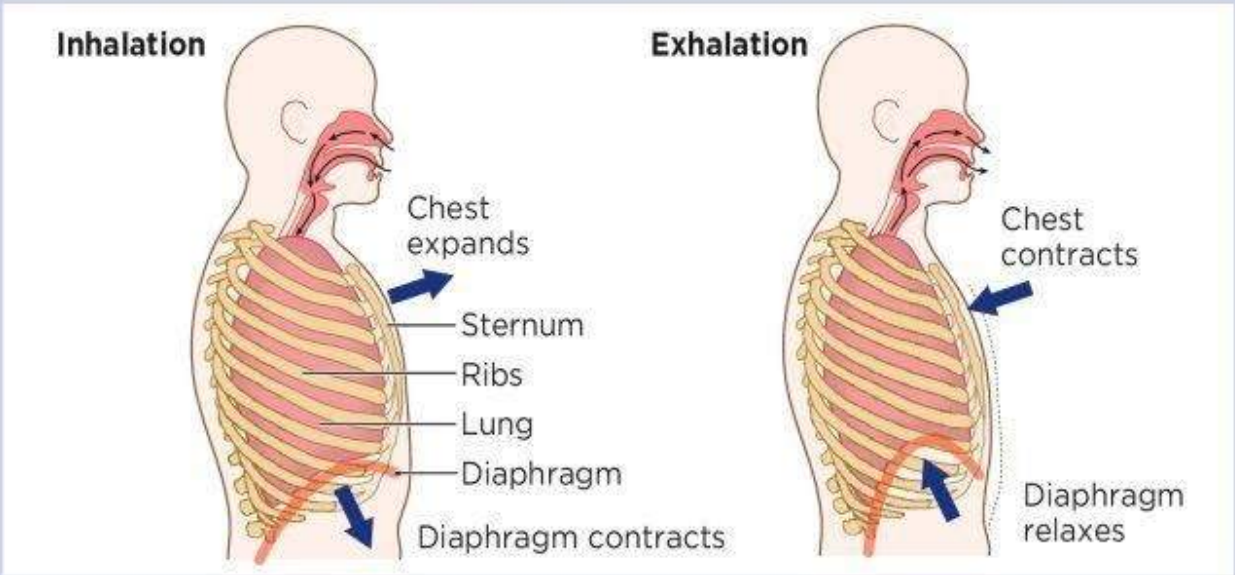
Normal and abnormal RR

Table 1. **Respiratory rate classification in adult patients**

RR	Range
Eupnoea (normal relaxed breathing)	12-20bpm (Royal College of Physicians, 2017)
Normal range >65 years	12-25bpm (Rodrigues-Molinero et al, 2013)
Normal range >80 years	10-30bpm (Rodrigues-Molinero et al, 2013)
Bradypnoea (slow RR)	<12bpm (RCP, 2017)
Tachypnoea (fast RR)	>20bpm (RCP, 2017)

bpm = breaths per minute; RR = respiratory rate

Fig 1. **Chest movements with inhalation and exhalation**



Measurement of respiratory rate (breaths/min).

- Measurement No….

- Name of subject-
- Age –
- Sex-

Pulse Rate	Breathing rate
Resting (Before Exercise)	
Just After step test for 5 minutes	
After 10 minutes of resting	

Interpretation of resting results: -

……

Clinical significances:

Tachypnoea

- Anxiety, Emotional distress, Pain, Fever, Exercise, Asthma, Pulmonary embolism
- Pneumonia
- Acute respiratory distress syndrome
- Anaphylaxis
- Heart failure
- Shock
- Diabetic ketoacidosis
- Neuromuscular disorders
- Chronic obstructive pulmonary disease

Bradypnoea

- Depression of the respiratory centre
- Opioid overdose
- Increased intracranial pressure
- Diabetic coma, Exhaustion caused by severe airway obstruction
- Sleep apnoea, Obesity hypoventilation syndrome

6. Hemocytometry (Cell Counting)

HEMOCYTOMETRY

Hemocytometry is the procedure of counting the number of cells in a sample of blood; the red cells, the white cells, and the platelets being counted separately. It is assumed that the cells are homogenously mixed (suspended) in the plasma in all regions of the body. However, even under physiological conditions, there are slight differences (e.g. higher red cell counts in venous and capillary blood than in arterial blood) which, though minor, are accentuated by muscular exercise, changes in posture, meals etc. Nevertheless, important clinical information can be obtained if cell counts are done carefully on a venous blood sample.

Principle

Since the number of blood cells is very high, it is difficult to count them even under the microscope. This difficulty is partly overcome by diluting the blood to a known degree with suitable diluting fluids and then counting them.
The sample of blood is diluted in a special pipette and is then placed in a capillary space of known capacity (volume) between a specially ruled glass slide (counting chamber) and a coverslip. The cells spread out in a single layer which makes their counting easy. Knowing the dilution employed, the number of cells in undiluted blood can then easily be calculated.

Units for Reporting

The result of cell counting is usually expressed as "so many cells per cubic millimeter (c mm; mm3; µl) of blood". For example, RBC count = 5.0 million/cmm. The SI unit, however, is ……cells per liter of blood.
1 mm3 = 1 µl = 10–6 liter
1 µl × 106 = 1 liter

Significance of Counting the Cells in a Chamber

How much important information can be obtained from this method? Does counting the cells in this traditional manner give useful clinical information as compared to counting them in electronic counters?

Automatic Electronic Cell Counters

The electronic cell counter uses volumetric impedence method. An electrolyte solution (diluent) containing suspended blood cells is aspirated through the aperture. Two electrodes are located close to the aperture and a constant current flows between them. When a blood cell passes through the aperture, the resistance between the electrodes momentarily increases and a very small voltage change occur corresponding to the resistance. The voltage signal is amplified and sent to the electronic circuit. The data is then corrected by the CPU and displayed on the screen.

RBC Counting

The counting of red cells in a chamber is a time-consuming and tedious procedure and difficult to perform accurately even in the hands of experts. Therefore, RBC counting, because of their great

number and high dilution employed, has not much of clinical value. (However, it is needed in the calculation of some blood standards). By the time changes occur in their number, the diagnosis is already clear to the clinician.

WBC Counting

The white cell counting is comparatively simpler to carry out and also more reliable.

Sources of Error in Cell Counting

Three important sources of error, pipette error, chamber error, and field error (see Expt 1-8 for details) can produce a variation of as much as 10–15%, or even more in the hands of a student. On the other hand, hemoglobin and packed cell volume are easy to determine and give enough information about the blood picture.

Steps in Hemocytometry

The whole process of cell counting involves the following steps:

1. Keeping all the equipment ready
2. Getting a sample of blood
3. **Pipetting,** i.e. filling the pipette with blood and diluting it
4. **Charging,** i.e. filling the counting chamber with diluted blood
5. Counting the cells and reporting the results.

HEMOCYTOMETER

The hemocytometer set consists of the following:

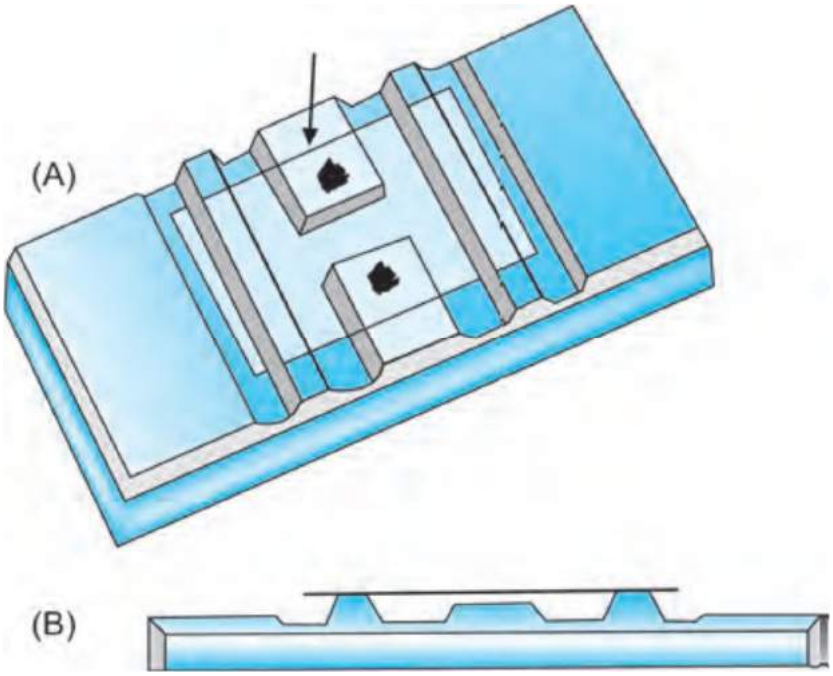
1. **The diluting pipettes** Two different glass capillary pipettes, each having a bulb, are provided for counting RBCs and WBCs. (These pipettes are sometimes called “cell pipettes” or blood pipettes. The third pipette that the students will be using is the hemoglobin pipette, which does not have a bulb).
2. **The counting chamber** It is a thick glass slide, appropriately ruled with a counting grid, i.e. squares of varying dimensions.
3. **Coverslips** Special coverslips having an optically plane and uniform surface should be preferred over ordinary coverslips.
4. **RBC and WBC** diluting fluids.
5. **Watch glasses** •Spirit swabs, •Blood lancet/ needle, etc.

Improved Neubauer Chamber (Counting Chamber)

The counting chamber **(Figure)** is a single, solid, heavy glass slide. Extending across its middle third are 3 parallel platforms (pillars, or flanges) separated from each other by shallow trenches (moats, gutters, or troughs). The central platform or “floorpiece” (sometimes also called the plateau) is wider, and exactly 0.1 mm (one-tenth of a mm) lower than the two lateral pillars. The floorpiece is divided into two equal parts by a short transverse trench in its middle

as shown in **Figure**. Thus, there is an H-shaped trench or trough enclosing the two floorpieces. The two lateral platforms can support a coverslip which, when in position, will span the trenches and provide a capillary space 0.1 mm deep between the under surface of the coverslip and the upper surface of the floorpieces.

Identically ruled areas, called "counting grids", consisting of squares of different sizes, are etched on each floorpiece. The two counting grids allow RBC and WBC counts to be made simultaneously if needed, or duplicate samples can be run.



The Counting Grid

The ruled area on each floor piece, the counting grid, has the following dimensions:

- Each counting grid **(Figure)** measures 9 mm2 (3 mm × 3 mm). It is divided into 9 large squares, each 1 mm2 (1 mm ×1 mm).
- Of these 9 squares, the 4 large corner squares are lightly etched, and each is divided by single lines into 16 medium-sized squares each of which has a side of 1/4 mm, and an area of 1/16 mm2 (1/4 mm × 1/4 mm). These 4 large corner squares are employed for counting leukocytes and are, therefore, called WBC squares **(Figure)**.
- The central densely etched large square (1 mm × 1 mm), called the RBC square, is divided into 25 medium-sized squares, each of which has a side of 1/5 mm.
- Each of these medium squares is set off (separated) from its neighbors by very closely placed double lines (tram lines) or triple lines. These double or triple lines extend in all directions beyond the boundaries of the 9 mm2 ruling, i.e. in between all the WBC squares around the central RBC square.

• Each of the 25 medium squares (side = 1/5 mm), bounded by double lines (which are 0.01 mm apart) or triple lines, is further divided into 16 smallest squares by single lines. Thus, each smallest square has a side of 1/5 × 1/4 = 1/20 mm, and an area of 1/400 mm^2.

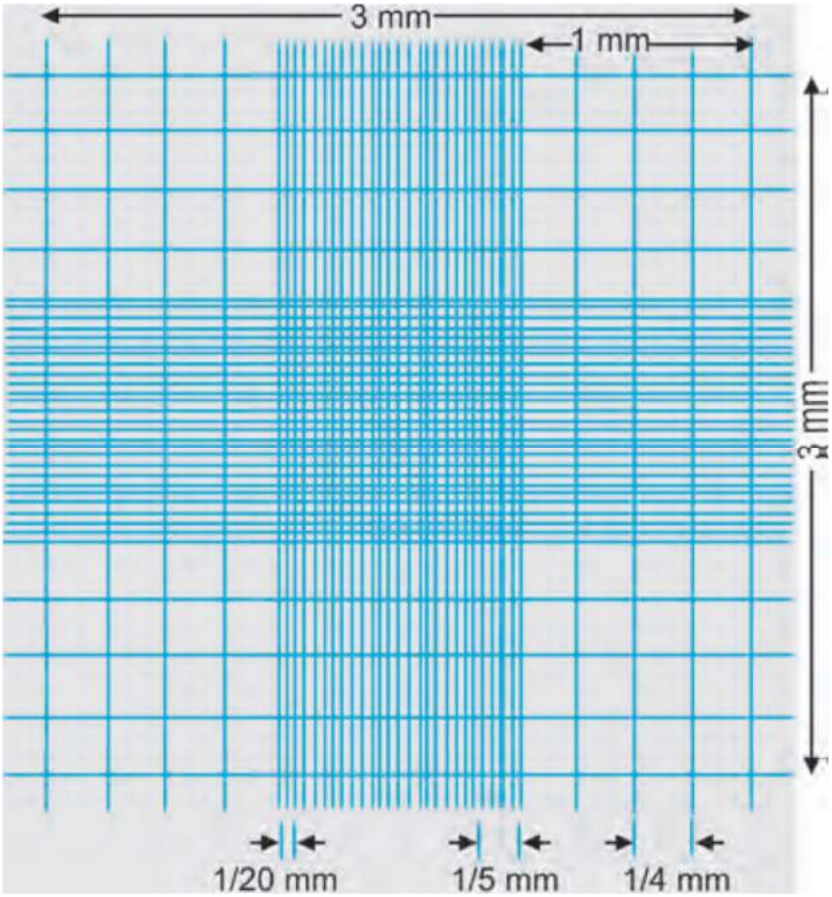


Figure: The counting grid of improved Neubauer's ruling. The four corner groups of 16 squares (side = 0.25 mm) are used for counting WBCs. The RBCs are counted in 5 groups of 16 smallest squares each (side = 0.05 mm). The grid also provides a convenient scale for measuring the size of small objects like parasite eggs.

For RBC Counting

The red cells are counted in 4 corner groups and one central group of medium squares, each of which has 16 smallest squares, i.e. in a total of 80 smallest squares.
Area of smallest square = 1/20 mm × 1/20 mm
= 1/400 mm^2
Since the depth of the chamber is 1/10 mm, the
Volume of the smallest square = 1/400 ×1/10 = 1/4000 mm^3.

For WBC (Total Leukocyte) Counting

This count is done in the 4 corner groups of large squares, each of which has 16 medium squares.
Area of one medium = 1/4 mm × 1/4 mm square = 1/16 mm^2.
Volume of this square = 1/16 mm2 × 1/10 mm = 1/160 mm^3.

7. Measurement of total count of RBC.

PRINCIPLE:

The blood is diluted 200 times in a red cell pipette and the cells are counted in the counting chamber. Knowing the dilution employed, their number in undiluted blood can easily be calculated.

APPARATUS AND MATERIALS

1. RBC pipette: It should be clean and dry and the bead should roll freely.

2. Improved Neubauer chamber with coverslip. These should be clean and dust free.

3. Microscope with LP and HP objectives and 10 x eyepiece.

4. Disposable blood lancet/pricking needle.

• Sterile cotton/gauze swabs

• 70% alcohol/methylated spirit.

5. Hayem's fluid (RBC diluting fluid): The ideal fluid for diluting the blood should be isotonic and neither cause hemolysis nor crenation of red cells. It should have a fixative to preserve the shape of RBCs and also prevent their autolysis so that they could be counted even several hours after diluting the blood if necessary. It should prevent agglutination and not get spoiled on keeping. All these properties are found in Hayem's fluid.

Composition of Hayem's fluid.

Sodium chloride (NaCl) 0.50 g

Sodium sulfate (Na_2SO_4) 2.50 g

Mercuric chloride ($HgCl_2$) 0.25 g

Distilled water 100 ml

Dissolve all these chemical in distilled water and filter several times through the same filter paper. Discard the solution if a precipitate forms.

• Sodium chloride and sodium sulfate provide isotonicity so that the red cells remain suspended in diluted blood without changing their shape and size. Sodium sulphate also acts as an anticoagulant, and as a fixative to preserve their shape and to prevent rouleaux formation (piling together of red cells)

• Mercuric chloride acts as an antifungal and antimicrobial agent and prevents contamination and growth of microorganisms.

PROCEDURES

1. Place about 2 ml of Hayem's fluid in a watch glass.

2. Examine the chamber, with the coverslip 'centred' on it, under low magnification. Adjust the illumination and focus the central 1 mm square (RBC square on the counting grid) containing 25 groups of 16 smallest squares each. All these squares will be visible in one field. Do not change the focus or the field. • Admitting too much light is a common cause of the inability to see the grid lines and squares clearly.

3. Move the chamber to your work-table for charging it with diluted blood. (It can be charged while on the stage, but it is more convenient to charge it on the table).

4. **Filling the pipette with blood and diluting it:** Get a finger-prick. Wipe the first 2 drops of blood and fill the pipette from a fresh drop of blood up to the mark 0.5. Suck Hayem's fluid to the mark 101 and mix the contents of the bulb for 3–4 minutes as described earlier.

5. Charging the chamber: Observing all the precautions, fill the chamber with diluted blood.

• Since the RBC pipette is a slow-speed pipette, it will need to be kept at an angle of 70–80° while charging the chamber.

6. Move the chamber to the microscope and focus the grid once again to see the central 1 mm square with the red cells distributed all over.

• Wait for 3–4 minutes for the cells to settle down because they cannot be counted when they are moving and changing their positions due to currents in the fluid. During this time draw a diagram once again showing the RBC square. Then draw 5 groups of 16 squares each, showing their relative positions—the 4 corner groups and one central group for entering your counts.

7. Counting the cells: Switch over to high magnification (HP lens) and check the distribution of cells. If they are unevenly distributed, i.e., bunched at some places and scanty at others, the chamber has to be washed, dried, and recharged.

8. Move the chamber carefully and bring the left upper corner block of 16 smallest squares in the field of view. (There are no smallest squares above and to its left).

Rules for Counting

Note that the immediate boundary of each smallest square is formed by the 4 lines forming the square (side: 1/20 mm; area: 1/400 mm2) the other lines of the tram or triple lines do not form part of the boundary of that square.

i. Cells lying within a square are to be counted with that square.

ii. Cells lying on or touching its upper horizontal and left vertical lines are to be counted with that particular square.

iii. Cells lying on or touching its lower horizontal and right vertical lines are to be omitted from that square because they will be counted with the adjacent squares. In this way you will avoid counting a cell twice. (You may omit cells lying on the upper horizontal and left vertical lines and count those lying on its lower and right lines. But whichever method is chosen, it is best to follow it for all cell counts).

• While counting the cells, continuously "rack", the fine adjustment up and down so that cells sticking to the underside of the coverslip are not missed

• An occasional WBC (may be 1 in 600-700 RBCs) may be seen—appearing greyish and granular but it is not to be counted with the red cells.

9. We have already focused the upper left block of 16 smallest squares in the high power field. First count the cells in the upper 4 horizontal squares from left to right, then come down to the next row and count the cells in each square from right to left. Then count the cells in the 3rd row from left to right, and in the 4th row, from right to left. As the counts are made, enter your results in the appropriate squares drawn in your workbook, showing the count in each square.

• Count once more in these 16 squares and note the result in your work-book. The difference between the two counts should not be more than 10.

10. Move the chamber carefully till you reach the right upper corner block of 16 smallest squares (there are no smallest squares above and to the right of this group), and count the cells as before. Then move on to the right lower corner and then left lower corner groups, and finally count the cells in the central block of 16 smallest squares.

Thus, the counting will have been done in 80 smallest squares, i.e., in 5 blocks of 16 squares each.

OBSERVATIONS AND RESULTS

Add up the number of cells in each of the 5 blocks of 16 smallest squares. A difference of more than 20 between any 2 blocks indicates uneven distribution.

A. Calculation of dilution obtained (dilution factor).

Recall that the dilution with this pipette can be 1 in 100 or 1 in 200 depending on whether blood is taken to mark 1.0 or 0.5.

Thus, the dilution factor is = Final volume attained (100 parts) Volume of blood taken (0.5 part)

B. Calculation of volume of fluid examined. We know the count in 80 smallest squares which have a volume (space) of 1/50 mm3. We can also know the cell count in 1 smallest square, which has a volume (space) of 1/4000 mm3. We can now calculate the number of red cells in two ways as shown below:

C. Calculation of red cell count

i. Let x be the number of cells in 1/50 mm^3 of diluted blood.

Cells in 1 mm3 of diluted blood = x × 50

Dilution employed was = 1 in 200

∴ Number of cells in 1 mm^3 of

undiluted blood will be = x × 50 × 200

= x × 10000

Thus, adding, 4 zeros in front of x will give the RBC count per 1 cubic mm of undiluted blood.

8.Measurement of total count of WBC.

PRINCIPLE

A sample of blood is diluted with a diluting fluid which destroys the red cells and stains the nuclei of the leukocytes. The cells are then counted in a counting chamber and their number in undiluted blood reported as leukocytes/mm3.

APPARATUS AND MATERIALS

1. Microscope •Counting chamber with a heavy coverslip. •Blood lancet/pricking needle. •Sterile cotton/gauze swabs. •70% alcohol.
2. **WBC pipettes:** white bead in bulb, and markings 0.5, 1.0, and 11. Two such, clean and dry pipettes, with free-rolling beads are required.
3. **Turk's fluid.** This fluid is used for diluting the blood.

Glacial acetic acid = 1.5 ml (hemolyzes RBCs without affecting WBCs).
Gentian violet (1% solution) =1.5 ml (it stains the nuclei of leukocytes). Distilled water to 100 ml.

PROCEDURE:

1. Take 1 ml of Turk's fluid in a watch glass. Place the counting chamber on the microscope stage. Adjust the illumination, and focus the right upper group of 16 WBC squares. You will see all the squares in one field.
2. Observing all the aseptic precautions, get a finger-prick, discard the first 2 drops of blood, and let a good-sized drop to form.
3. **Filling the pipette:** Dip the tip of the pipette in the edge of the drop, draw blood to the mark 0.5 and suck Turk's fluid to the mark 11. Mix the contents of the bulb thoroughly for 3–4 minutes.
• Your partner can draw blood up to the mark 1.0 in the second pipette, followed by Turk's fluid to mark 11. This will give a dilution of 1 in 10.
4. **Charging the chamber:** Discard the first 2 drops of fluid from the pipette and charge the chamber on both sides, 1 in 10 dilution on one side and 1 in 20 dilution on the other. The chamber should neither be over-charged nor under-charged.
5. Allow the cells to settle for 3–4 minutes, then carefully transfer the chamber to the microscope. Use the fine adjustment again and try to identify the WBCs.

Under low magnification: The leukocytes appear as round, shiny (refractile), darkish dots, with a halo around them. These 'dots' represent the nuclei, which have been stained by gential violet. The cytoplasm is not stained.
• Do not confuse with dust particles which have varying sizes and shapes, often angular. They are usually opaque, with no 'halo' around them. They may be brown, black or yellow in color.
6. **Switch to high magnification** and study the leukocytes. By racking the microscope, you should be able to make out the morphology, of these cells—their round shapes, the clear unstained cytoplasm, and the deep blue-violet nuclei which appear lobed in some cells and single in others. You will also see the remnants of the red cell membranes; these are called 'ghost' cells since they are faintly visible.
7. **Counting the cells:** The procedure for counting the WBCs is similar to that employed for red cells.

• Count the cells under high power lens; once some practice is gained they can be counted under low power.
• You may count the WBCs in 16 squares under low power and then under high power and compare the results.
• Count the cells in the 4 groups of 16 squares each, i.e., in a total of 64 squares.
• Draw appropriate squares in your work-book for entering the counts.

OBSERVATIONS AND RESULTS

Note that the deep brown color of the diluted blood is due to the formation of acid hematin by the action of acetic acid on the Hb released from the ruptured red cells. However, hemolysis and formation of acid hematin (the principle used for estimation of Hb by the Sahli method) does not interfere with the counting of leukocytes.

Calculations

The leukocytes were counted in 64 squares, the volume of one square being 1/160 mm^3.
Volume of 64 squares = 1/160 × 64 = 4/10 mm^3.
Thus, the total volume of diluted blood in which WBCs were counted = 4/10 mm^3.
Let the count in 4/10 mm^3 be = x
Then 1 mm^3 of diluted blood will contain
= x × 10/4 white cells.
Since the dilution employed is 20 times (10 times in the 2nd pipette)
1 mm^3 of undiluted blood
will contain = x × 10/4 × 20
= x × 200/4
= x × 50
(x × 10/4 × 10 in the 2nd pipette)
This means that multiplying the number of cells in 64 squares with 50 will give the total leukocyte count (multiply the number of cells in 64 squares with 25 in the 2nd pipette).
Compare the two counts. The difference between the two should not be more than 10%. It will confirm the accuracy of your procedures in the two counting's.

9.Preparing a Peripheral Blood Film

A. DROP PRESENTATION

The first and obvious way to study the cells of the blood is to examine fresh blood under the microscope in the form of a drop preparation and in a thin blood film or smear made on a glass slide. In this experiment, the students will examine some features of blood cells and record their observations. They will also practice making (preparing) blood films and examine them without staining. In later experiments, they will prepare and stain blood smears and identify and count various blood cells, reticulocytes, and platelets.

• Anticoagulated blood obtained from a student volunteer, or spare blood obtained from the clinical laboratory may be provided to the students to avoid skin pricks at this time (a drop of blood can be put on the slide without touching it). Students may also use their own blood from skin pricks.

APPARATUS AND MATERIALS

1. Disposable, sterile blood lancet/pricking needle.
   - Sterile cotton/gauze swabs
   - 70% alcohol/methylated spirit.
2. 8–10 thin, absolutely transparent, grease-free standard glass slides (75 mm × 25 mm).
   - Vaseline • Toothpicks.

PROCEDURES

While you prepare the drop preparation, your work-partner can make blood films from the same finger-prick blood.

1. Get a finger-prick under aseptic conditions. Discard the first 2 drops and allow a good drop to form. Holding a coverslip by its edges between your thumb and finger, touch its center to the blood drop, thus forming a bead.
2. Invert and carefully drop the coverslip (along with the blood drop under it) in the center of a glass slide. Do not press. The blood drop will spread into a thick film by the weight of the coverslip.
3. Using a toothpick, apply a little vaseline all around the edges of the coverslip to seal the capillary space under it. This will prevent evaporation of water and drying up of the preparation.
4. Examine the preparation under low and high magnifications and record your observations.

OBSERVATIONS

Note the degree of separation of cells. Do they lie in a single layer or in 2, 3 or more layers? The red cells are non-nucleated, flat biconcave disks, round, oval or pear-shaped, thinner in the center and appear as colorless, or pale pink structures. (When stained with Leishman's stain, they appear dull orange-pink). Note if there is any rouleaux formation (cells lying on top of each other like a pile of coins) and the number of cells in a rouleaux. Observe if any leukocytes are seen, and their types if possible.

B. PREPARATION OF A BLOOD FILM (BLOOD SMEAR)

Blood films can be made from anticoagulated, or finger-prick blood. (See Expt 1-12)

PROCEDURES

1. Place 3 or 4 slides on a white sheet of paper on your work-table, the surface of which should be even and smooth.
2. Allow a medium-sized drop of blood to form on the finger-tip.
3. Steady the pricked finger of your partner with your left hand. Lift a slide from the table, holding it along its long edges. Then touch its center, about 1 cm from the narrow end, to the blood drop. (If anticoagulated blood is being used place a drop of blood in a similar position with a dropper). Do not apply the blood drop at the finger to the slide placed on the table. One cannot see the amount of blood placed on the slide.
4. Place the slide flat on the table, with the blood drop to the right side (neither your fingers, nor the skin of the subject's finger should touch the surface of the slide).
5. Support the left end of the slide with your thumb and fingers of your left hand. Now grasp the long edges of a second slide, the "spreader", between thumb and fingers of your right hand, so that its free left end extends downwards and to the left at an angle of about 40° to the horizontal.
6. Place the narrow edge of the spreader on the first slide, at an angle of 40°, just in front of the blood drop (step 1, **Figure**). Pull the spreader back gently so that it touches the front of the blood drop. Hold it there, (or move it a little from side to side) till the blood, moving along the junction of the two slides by capillarity, almost reaches the ends of the spreader, except the last 2 mm on each side, thus distributing the blood evenly across its width. (If the blood drop is too big, you may start to spread the smear before the whole of the blood spreads along the slide).
7. Steady the first slide with your left hand, and maintaining a light but even pressure and 40° angle (step 2, **Figure**), move the spreader forwards to the left in a single, smooth, fairly fast gliding motion, pulling the blood behind it in the form of a thin smear. The smear should be spread in about half a second. Any hesitation will result in striations in the film.
8. Make as many trials as possible to get acceptable films, keeping in mind the features of an ideal blood smear, as described below. Dry the film by waving the slide in the air (Do not try to blot-dry the film).

OBSERVATIONS

Examine the slides against diffuse light, with naked eye. What is the color of the smear? Does it appear thick, thin, or granular? Are there any striations— longitudinal or transverse? Are there any vacant places in the film? Is it uniformly distributed in the middle two-thirds of the slide? Is its head—the starting point, straight and about 1 cm from the end? Are its edges about 2 mm from the long sides of the slide? Is there a tail? Try to answer all these questions.
Examine the slides under low and high magnification. Describe what you see about the various cells and compare with what you saw in the drop preparation.

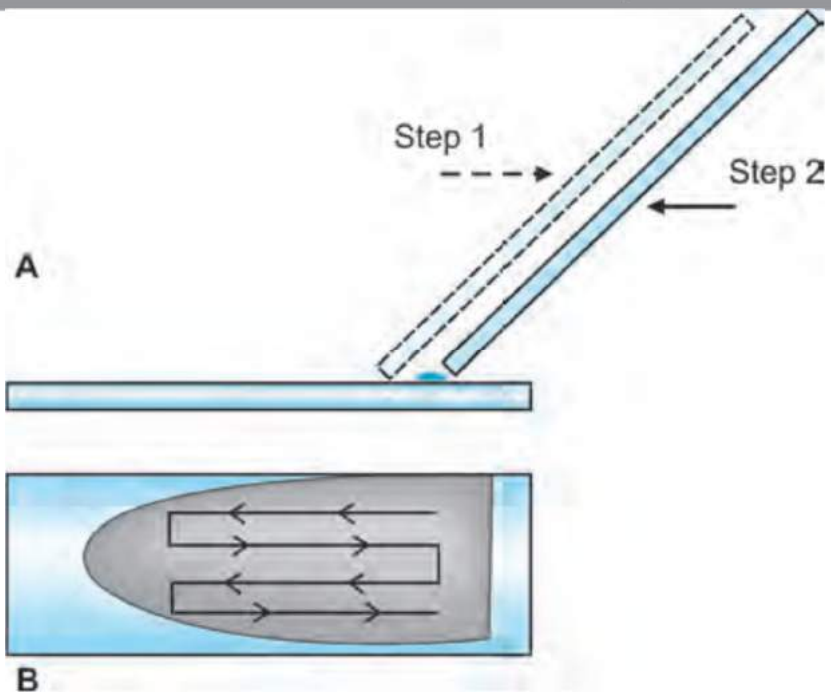


Figure: (A) Method of spreading a blood film. Step 1: The spreader is placed in front of the blood drop and pulled back till it touches the blood. Step 2: Spreader is pushed forwards to spread the film. (B) The appearance of a well-prepared film, showing the movement of the objective over it may start to spread the smear before the whole of the blood spreads along the slide).

PRECAUTIONS

1. The slides should be absolutely free from dust and grease, because blood will not stick to areas where oils from your fingers have been left. New slides should be preferred. But if old ones are to be used, they should be properly cleaned, as described below.
2. The edge of the spreader should be smooth and not chipped, otherwise the slide would leave striations along or across the smear. Leukocytes may also be caught in chipped places and be carried towards the tail.
3. When applying the slide to the blood drop from a finger-prick, do not touch the skin with the slide, but only the periphery (top) of the blood drop. This is to avoid taking up epidermal squames or sweat.
4. The blood should be spread immediately after taking it on the slide. Any delay will cause clumping of cells due to partial coagulation. This will give a 'granular' appearance to the blood film, which is visible to the naked eye.
5. The angle of the spreader should be 35° to 40°. The more the angle of the spreader approaches the vertical, the thinner the film, and the lesser the angle, the thicker the film.
6. The pressure of the spreader on the slide should be slight and even and the pushing should be fairly quick while maintaining a uniform pressure throughout.
7. The film should be dried by waving it in the air immediately after spreading it. A delay can cause not only clumping, but also crenation and distortion of red cells in a damp atmosphere (if

water is allowed to slowly evaporate from the blood plasma on the slide, crenation occurs due to gradual increase in the concentration of salts).
8. By turning the spreader over, you can use it to make 4 blood films.

Cleaning the Slides

Prepare acid-dichromate solution by mixing 1part of concentrated sulfuric or nitric acid with 9 parts of 2–3% potassium dichromate solution.

1. Wash the slides and coverslips with soap and water and rinse in running water. Then soak them overnight in the acid-dichromate solution. Follow this with a wash in running water, then in distilled water.

2. Dip the slides in 90–95% alcohol and dry with a clean, lint-free cloth.

• Another method is to use a good detergent for overnight soak in place of acid-dichromate solution.

• The acid-dichromate solution is best kept in a 1–2 litre, wide-mouth jar. After washing the used slides, they are put in this jar. The slides can then be treated as described above.

10. Measurement of differential count of WBC.

PRINCIPLE:

A blood film is stained with Leishman's stain and scanned under oil immersion, from one end to the other. As each WBC is encountered, it is identified until 200 leukocytes have been examined. The percentage distribution of each type of WBC is then calculated. Knowing the TLC and the differential count, it is easy to determine the number of each type of cell per mm3.

Special Importance of a Blood Smear

The special importance of a stained blood smear is that, unlike any other routine blood test, the smear can be retained and preserved as a permanent original record. The slide can be taken out and re-assessed whenever required after days, weeks, months or even years. The slide can also be conveniently sent to specialists for their opinion in doubtful cases.
The stained smears can also provide information about the morphology and count of red cells and platelets, and Hb status, besides detecting the presence of various parasites (e.g. malaria).

APPARATUS AND MATERIALS

1. Microscope. •5–6 Clean glass slides. •Sterile lancet. •Cotton and gauze swabs. •70% alcohol. •Glass dropper.
2. A drop bottle containing Leishman's stain.
3. A wash bottle of distilled water (or buffered water, if available). • Fluff-free blotting paper.

Leishman's stain. This stain is a simplification of Romanowsky group of stains. It is probably one of the simplest and most precise method of staining blood for diagnostic purposes. It contains a compound dye—**eosinate of methylene-blue** dissolved in acetone-free methyl alcohol.
i. Eosin. It is an acidic dye (negatively charged) and stains basic (positive) particles—granules of eosinophils, and RBCs a pink color.
ii. Methylene-blue. It is a basic dye (positively charged) and stains acidic (negatively charged) granules in the cytoplasm, nuclei of leukocytes, especially the granules of basophils, a blue-violet color.
iii. Acetone-free and water-free absolute methyl alcohol. The methyl alcohol is a fixative and must be free from acetone and water. It serves two functions:
a. It fixes the blood smear to the glass slide. The alcohol precipitates the plasma proteins, which then act as a 'glue' which attaches (fixes) the blood cells to the slide so that they are not washed away during staining.
b. The alcohol preserves the morphology and chemical status of the cells.
• The alcohol must be free from acetone because acetone being a very strong lipid solvent, will, if present, cause crenation, shrinkage, or even destruction of cell membranes. This will make the identification of the cells difficult. (If acetone is present, the stain deteriorates quickly).

• The alcohol must be free from water since the latter may result in rouleaux formation and even hemolysis. The water may even wash away the blood film from the slide.

Steps in Differential Leukocyte Counting

1. Getting a blood sample from a finger-prick and making blood smears. If blood is obtained from a vein, place a drop of blood (through the needle) on each of the 4–5 slides and spread blood films.
2. Examining the blood smears under LP and HP and choosing the ideal films for staining.
3. Fixing and staining the blood films.
4. Identification and counting of various leukocytes.

PROCEDURES:

A. Preparing the Blood Films

1. Prepare 4 or 5 blood films.
2. Air dry the slides immediately by waving them in the air.
3. Examine them under low and then under high magnifications, and choose the best for staining.

B. Fixing and Staining of Blood Films

While supravital staining is employed for living cells, the staining of blood films involves dead cells. **Fixation** is the process that makes the blood film and its cells adhere to the glass slide. It also preserves the shape and chemistry of blood cells as near living cells as possible. (See Q/A 8). **Staining** is the process that stains (colors) the nuclei and cytoplasm of the cells. Both these purposes are achieved by the Leishman's stain.

• Since the timings for fixing and staining of the films with the Leishman's stain vary with different batches of the stain, check the timings with the laboratory assistant.

1. Fixing the Blood Films. Place the slides, smear side up, on a 'staining rack' assembled over a sink (two glass rods placed across the sink, with the ends fitted into short pieces of rubber tubing). Ensure that they are horizontal.

2. Pour 8–10 drops of the stain on each unfixed slide by dripping it from a drop bottle, or use a dropper. This amount of stain usually covers the entire surface and "stands up" from the edges of the slides without running off. Note the time.

3. Allow the stain to remain undisturbed for 1–2 minutes, as advised.

• During this time, watch the stain carefully, especially during hot weather, and see that it does not become syrupy (thick) due to evaporation of alcohol. If the stain dries, it will precipitate on the blood film and appear as round, blue granules.

This can be prevented by pouring more stain on the slides as required.

4. Staining the blood film. After the fixing time is over, add an equal number of drops of distilled water (or buffered water, if available) to the stain. If the water is carefully dripped from a drop bottle or a dropper, the entire mixture will stand up from the edges of the slides (due to surface tension) without spilling over.

5. Mix the stain and water by gently blowing at different places on the slides through a dropper, without scratching the smear. A glossy greenish layer (scum) soon appears on the surface of the diluted stain. Allow the diluted stain to remain on the slide for 6–8 minutes, or as advised.
6. Flush off the diluted stain in a gentle stream of distilled water for about 30 seconds and leave the slides on the rack for about a minute with the last wash of water covering them. Drain the slides and put them in an inclined position against a support, stained sides facing downwards (to prevent dust particles settling on them) to drain and dry. The under sides of the slides may be blotted with filter paper.

OBSERVATIONS AND RESULTS

C. Assessment of Stained Blood Smears

Before starting the actual counting of WBCs, you should—
i. Take an assessment of all the blood films. Examine with naked eye first, and then under low and high magnifications. Choose the best stained films for cell counting.
ii. Make sure that you can identify all the leukocytes with certainty.
Ensure that you are examining the blood smear side of the slide. Hold the slide in bright light and tilt it this way and that to see if there are any reflections. The clean side shows reflections, while the side which has the blood smear appears dull and does not show any reflections.
1. Draw 200 squares in your workbook for recording various WBCs as they are encountered and identified one after another. Enter these cells by using the letters 'N' for neutrophils, 'M' for monocytes, 'LL' for large lymphocytes, 'SL' for small lymphocytes, 'E' for eosinophils, and 'B' for basophils.
• You can indicate these cells in a column (instead of the 200 squares), and as you identify a cell, put a short vertical stroke against that cell. In this way, you can place different types of cells in groups of 5, a horizontal stroke representing the 5th cell (e.g. Neutrophils = ~~IIII~~ III, etc.)
2. Place a drop of cedar wood oil on the right upper corner of the film, a few mm away from the head end. Bring the oil immersion lens into position till it enters the oil drop. Adjust the focus.
• Do not flood the entire surface of the slide with oil; as you move the slide, the oil will move with the objective lens.
3. Move the slide slowly to the right (the image will move to the left) and as you encounter a leukocyte, identify it, and enter it in your workbook. As you approach the end of the smear, move 2 fields down and scan the film in the opposite direction. As you near the head, again move 2 fields down and scan the film towards the tail. Traverse the film in this to and fro fashion till you have examined 200 cells (count 400 cells for good results). This "battlement" procedure, as shown in Figure 1-9, ensures that you do not count a leukocyte more than once.
• The possibility of WBCs sticking to the edge of the spreader should be kept in mind.
4. **Recount.** After you have, counted 200 (or 400) cells, count the leukocytes once more, starting from the lower left corner of the film, and going up in the "battlement" procedure.
Differential leukocyte count. When counting has been done, calculate the percentage of each type of cell in your count of 200 (or 400) white cells. The neutrophils are the prominent cells of the

blood and constitute about 50–60% of the WBCs. The next predominant cells are lymphocytes (20–40%), which may be small or large. The third cell in the order of population is the monocyte which constitutes 8–10% of the WBCs.

11. Haemoglobin estimation by Sahli's method.

PRINCIPLE:

The Hb present in a measured amount of blood is converted by dilute hydrochloric acid into acid hematin, which in dilution is golden brown in color. The intensity of color depends on the concentration of acid hematin which, in turn, depends on the concentration of Hb. The color of the solution (i.e. its hue and depth), after dilution with water, is matched against golden-brown tinted glass rods by direct vision. The readings are obtained in g%.

APPARATUS AND MATERIALS:

A. Sahli (Sahli-Adams) Hemoglobinometer (Hemometer). The set consists of:

1. Comparator. It is a rectangular plastic box with a slot in the middle which accommodates the calibrated Hb tube. Non-fading, standardized, golden-brown glass rods are fitted on each side of the slot for matching the color. An opaque white glass (or plastic) is fitted behind the slot to provide uniform illumination during direct visual color matching.

2. Hemoglobin tube. The square or round glass tube is calibrated in g Hb % (2–24 g%) in yellow color on one side, and in percentage Hb (20–160%) in red color on the other side. There is a brush to clean the tube.

3. Hemoglobin pipette. It is a glass capillary pipette with only a single calibration mark-0.02 ml (20 cmm, cubic millimeters; or 20 ml, micro liters). There is no bulb in this pipette (as compared to cell pipettes) as no dilution of blood is done. **Figure 1-10B** shows the Hb pipette.

Note The calibration mark 20 cmm indicates a definite, measured volume and not an arbitrary volume, as is the case with diluting pipettes.

4. Stirrer. It is a thin glass rod with a flattened end which is used for stirring and mixing the blood and dilute acid.

5. Pasteur pipette. It is a 8–10 inch glass tube drawn to a long thin nozzle, and has a rubber teat. Ordinary glass dropper with a rubber teat also serves the purpose.

6. Distilled water.

B. Decinormal (N/10) hydrochloric acid (0.1 N HCl) solution. Mixing 36 g HCl in distilled water to 1 liter gives 'Normal' HCl; and diluting it 10 times will give N/10 HCl solution.

C. Materials for skin prick.

- Sterile lancet/needle
- Sterile gauze and cotton swabs
- Methylated spirit/70% alcohol.

PROCEDURES:

1. Using a dropper, place 8–10 drops of N/10 HCl in the Hb tube, or up to the mark 20% or 3 g, or a little more till the tip of the pipette will submerge, and set it aside.
2. Get a finger prick under aseptic conditions, wipe away the first 2 drops of blood. When a large drop of free-flowing blood has formed again, draw blood up to the 20 cmm mark (0.02 ml).

Carefully wipe the blood sticking to the tip of the pipette with a cotton swab, but avoid touching the bore or else blood will be drawn out by capillarity.

3. Without any waiting, immerse the tip of the pipette to the bottom of the acid solution and expel the blood gently. Rinse the pipette 3–4 times by drawing up and blowing out the clear upper part of the acid solution till all the blood has been washed out from it. Avoid frothing of the mixture. Note the time.

4. Withdraw the pipette from the tube, touching it to the side of the tube, thus ensuring that no mixture is carried out of the tube. Mix the blood with the acid solution with the flat end of the stirrer by rotating and gently moving it up and down.

5. Put the Hb tube back in the comparator and let it stand for 6–8 minutes (or as advised by the manufacturer). During this time, the acid ruptures the red cells, releasing their Hb into the solution (hemolysis). The acid acts on the Hb and converts it into acid hematin which is deep golden brown in color.

• The color of acid hematin does not develop fully immediately, but its intensity increases with time, reaching a maximum, after which it starts to decrease. An adequate time, usually 6–8 minutes, must be allowed before its dilution is started. Too little time and all Hb may not be converted into acid hematin. And, waiting too long, may result in fading of color. In either case, the result will be falsely low.

6. **Diluting and matching the color.** The next step is to dilute the acid hematin solution with distilled water (preferably buffered water, if available) till its color matches the color of the standard tinted glass rods in the comparator.

7. Take the Hb tube out of the comparator and add distilled water drop by drop (or larger amounts depending on the experience), stirring the mixture each time and comparing the color with the standard.

8. Hold the comparator at eye level, away from your face, against bright but diffused light. Read the lower meniscus (lower meniscus is read in colored transparent solutions).

OBSERVATIONS AND RESULTS

Compare your colour matching with that of your work-partner and record the observations in your workbook. Take the average of 3 readings as shown below, and report your result as: Hb = …….g/dl.

1st reading, when the colour is slightly darker than the standard:……..…..g/dl.

2nd reading, when, after adding a few drops of distilled water, the colour exactly matches the standard: ……..…… g/dl.

3rd reading, when, after adding some more drops, the colour becomes a little lighter than the standard:……..…….. g/dl.

For report. Express your result as: Hb= ...….g/dl.

• **Oxygen carrying capacity:** Knowing your Hb concentration, and that 1.0 g of Hb can carry 1.34 ml of O2, calculate its oxygen-carrying capacity as …….……ml O2/dl.

• **100 % Saturation.** When blood is equilibrated with pure (100 %) oxygen at a PO2 of 120 mm Hg, the Hb gets 100 % saturated, i.e. it picks up as much O2 as it possibly can.

For report:

• Oxygen carrying capacity
• 100% saturation.

Normal Values

The levels of Hb in normal Indian adults, especially in the economically deprived population, are on the lower side of those reported from affluent countries. The reason may be the poor intake of grade 1 proteins and other nutrients. The average levels and their ranges are as follows:
Males: 14.5 g/dl (13.5–18 g/dl).
Females: 12.5 g/dl (11.5–16 g/dl).

Advantages of Sahli Method

The method is simple, fairly quick, and accurate. It does not require any costly apparatus, since it needs only direct color matching. Its running cost is minimal and can, therefore, be used in mass surveys.

Disadvantages of Sahli Method

Since the acid hematin is not in true solution, some turbidity may occur. The method estimates only the oxyHb and reduced Hb, other forms, such as carboxyHb and metHb are not estimated. Also the degree of error may be high if proper precautions are not taken.

12. Estimation of Hemoglobin by Drabkin's Method

- There are commercially available kits, which is easy to make the solution.

Manual Method

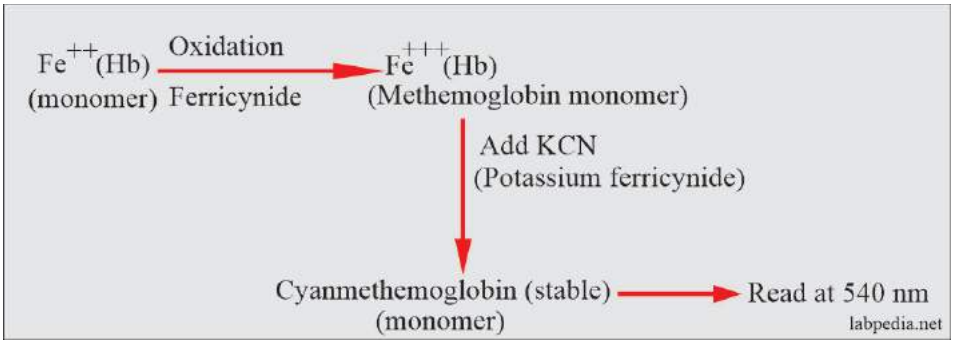
- Hemoglobin solution can be prepared in the laboratory. This is called Drabkin's solution.
    1. Drabkin's solution reagents needed are:
        1. Potassium ferricyanide = 200 mg
        2. Potassium cyanide = 50 mg
        3. Potassium dihydrogen phosphate = 140 mg
        4. Non-ionic detergent = 1 ml
        5. Distal water = Make up to 1000 ml (1 L)

Precautions

1. Keep the solution in a dark-colored bottle and in the dark to protect it from the light.
2. If the solution is cloudy after adding the blood, in that case, centrifuge before the reading, this may be due to nonhemolyzed RBCs or globulins.
3. Spectrophotometer cells should be free of fingerprints; otherwise will be high.

The Principle Of Drabkin's Solution:

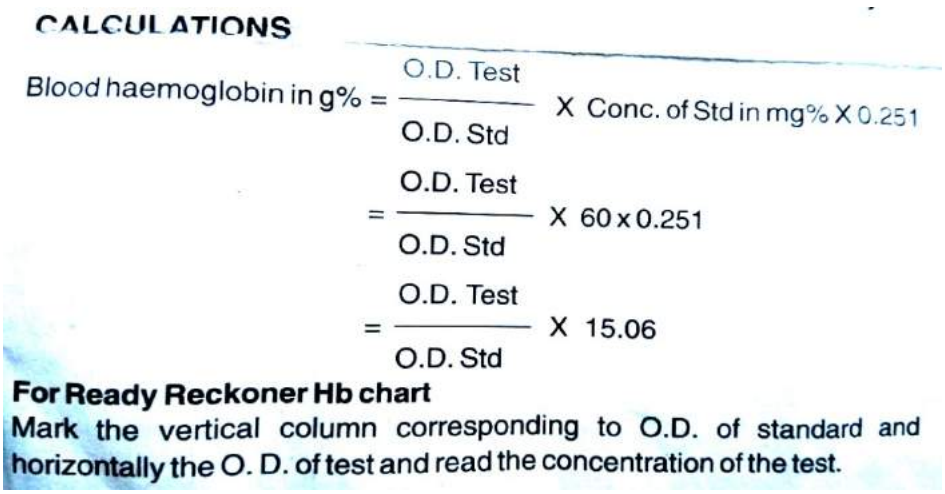
- This is based on the oxidation of Hb and its derivatives except sulfhemoglobin to form methemoglobin in the presence of alkaline K- ferricyanide.
- The methemoglobin reacts with K-cyanide to form a very stable compound, cyanmethemoglobin, and this complex has maximum absorption at 540 nm.



Drabkin's solution principle

Procedure:

1. Take 20 microliter of blood + Drabkin 5 mL = 1 : 251 dilution. (TEST)
2. Take 20 microliter standard solution + 5ml of Drabkin.
3. Now mix well.
4. Read within 6 hours of mixing on filter 540.
5. Read against blank of Drabkin solution (Drabkin solution can be used as blank).



Normal Hemoglobin:

1. Adult male = 14 to 18 g/dL
2. Adult female = 12 to 16 g/dL
3. 10 years old child = 12 to 14.5 g/dL
4. 3 months old infants = 9 to 14 g/dL
5. Newborn = 17 to 23 g/dL

Physiological Variation Of Hb:

1. Strenuous physical exercise.
2. There is a diurnal variation with the highest level in the morning and low in the evening.
3. High altitude increases the Hb concentration.

False Causes Of Raised Hb:

1. Hemoconcentration due to dehydration and burns.
2. Immediately after hemorrhage.
3. If taken during the I/V infusion, if it contains iron.

13. Determination of ESR

PRINCIPLE:

In the circulating blood the red cells remain uniformly suspended in the plasma. However, when a sample of blood, to which an anticoagulant has been added, is allowed to stand in a narrow vertical tube, the red cells (specific gravity = 1.095) being heavier (denser) than the colloid plasma (specific gravity = 1.032), settle or sediment gradually towards the bottom of the tube. The rate, in mm, at which the red cells sediment, called ESR, is recorded at the end of one hour.

Sedimentation of red cells

The settling or sedimentation of red cells in a sample of anticoagulated blood occurs in 3 stages:
i. In the ***first stage,*** the RBCs pile up (like a stack of coins), and form rouleaux that become heavier during the first 10-15 minutes.
ii. During the ***second stage,*** the rouleau (pleural of rouleaux) being heavier (see below) sink to the bottom. This stage lasts for 40–45 minutes.
iii. In the **third stage,** there is packing of massed bunches of red cells at the bottom of the blood column. This stage lasts for about 10–12 minutes.
Thus, most of the settling of the red cells occurs in the first hour or so.

WESTERGREN'S METHOD:

APPARATUS AND MATERIALS:

1. 2 ml disposable syringe with needle •Sterile cotton/gauze swabs moist with alcohol •Container (discarded penicillin bottle).
2. Sterile solution of 3.8 percent sodium citrate as the anticoagulant.
3. Westergren pipette (tube) and stand. It is 300 mm long and has a bore diameter of 2.5 mm. It is calibrated in cm and mm from 0 to 200, from above downwards in its lower two-thirds. The Westergren stand can accommodate up to 4 tubes at a time. For each pipette, there is a screw cap that slips over its top, and, at its lower end, the pipette presses into a rubber pad or cushion. When the pipette is fixed in position, there is enough pressure of the screw cap to prevent leakage of blood from its lower end. There is a spirit level to ensure vertical position of the pipette.

PROCEDURES:

1. Draw 2.0 ml of venous blood and transfer it into a vial containing 0.5 ml of 3.8% sodium citrate solution. This will give a blood: citrate ratio of 4:1. Mix the contents by inverting or swirling the vial. Do not shake, as it will cause frothing.
2. Fill the Westergren's pipette with blood-citrate mixture by sucking, after placing the tip of your finger over the top of the pipette to control the flow of blood into and out of it, or with a rubber bulb. Bring the blood column to exact zero mark. (If there is a difference of 1–2 mm, it should be noted and taken into account before giving the final report at the end of one hour).

3. Keeping your finger (or the rubber bulb) over the pipette, transfer it to the Westergren stand by firmly pressing its lower end into the rubber cushion. Now slip the upper end of the pipette under the screw cap. Confirm that there is no leakage of blood and that the pipette will remain vertical.
4. Leave the pipette undisturbed for one hour at the end of which read the mm of clear plasma above the red cells.

Express your results as:………mm 1st hour (Westergren).

Normal values

Males : 3–9 mm 1st hour

Females : 5–12 mm 1st hour.

14.Determination of Packed cell volume.

PRINCIPLE:

Measurement of hematocrit (Hct) or packed cell volume (PCV) is the most accurate and simplest of all tests in clinical hematology for detecting the presence and degree of anemia or polycythemia. In comparison, hemoglobin estimation is less accurate, and RBC count far less accurate. Also, if Hb, RBC count, and PCV are determined at the same time, various absolute corpuscular values (e.g., volume and Hb content of a single red cell) of a person can be determined. These values help in the laboratory diagnosis of the type of anemia in a person.

APPARATUS AND MATERIALS

1. Equipment for venepuncture.

• Sterile swabs, alcohol, syringe and needle

• Container (penicillin vial or bulb) with anticoagulant (double oxalate or sequestrene).

2. Wintrobe tube (hematocrit tube): It is 11 cm long, heavy, cylindrical glass tube, with a uniform bore diameter of 2 mm. Its lower end is closed and flat. The tube is calibrated in cm and mm from 0 to 10 cm from above downwards on one side of the scale (for ESR), and 10 to 0 cm on the other side (for PCV). The mouth of the tube can be covered with a rubber cap to prevent loss of fluid by evaporation.

3. Pasteur pipette: It is a glass tubing drawn to a long thin nozzle about 14 cm long. A rubber teat is provided to suck blood into the pipette by a slight pressure. It is used for filling the Wintrobe tube.

4. Centrifuge machine: lt packs the red cells in the Hct tube by centrifugal force. The magnitude of force produced by rotation of the tube depends on:

a. The radius, i.e., the distance between the center of the shaft and the bottom of the centrifuge tube when laid horizontally.

b. The number of revolutions per minute (rpm).

In terms of gravitational force (G), the value of this force should be 2260 units. This much force is created when the radius is 9 inches and the speed is 3000 rpm.

PROCEDURES:

1. Draw 5 ml of venous blood and transfer it to a container (penicillin vial or bulb) of anticoagulant. Rotate the bulb between your palms.

i. To ensure proper mixing of cells and plasma (inaccurate results are likely if this precaution is not taken).

ii. To oxygenate blood cells to remove CO2 (red cells are larger when CO2 is high, in venous blood).

2. Fill the pasteur pipette with blood and take its nozzle to the bottom of the Wintrobe tube. Expel the blood gently by pressing the rubber teat, and fill the tube from below upwards while withdrawing the pipette but always keeping its tip below the level of blood. Ensure that there is no air bubble trapped in the blood.

• Do not try to fill the tube from its top as blood will not flow down to its bottom because of air present in the tube.

3. Bring the blood column exactly to the mark 10 (or the mark 0 on the other side of the scale) at the top. There should not be any bubbles at the top of blood.

• lf less blood is available, note the level.

4. Close the mouth of the tube with its rubber cap and centrifuge it at 3000 rpm for 30 minutes (slower speed will not pack the red cells fully). Balance this tube with another tube filled with water, or another sample of blood placed in the opposite tube holder.

5. At the end of 30 minutes, take the reading of upper level of packed red cells on the side of the scale where zero is at the bottom. Replace the tube in the machine and centrifuge it again for 15 minutes. Read the packed cell height again; it should be the same as before. If the height is reduced, centrifuge it again for 5 minutes. To be reliable, at least 3 successive readings, at intervals of 5 minutes, should be the same.

• Note that unnecessarily prolonged centrifugation may cause mechanical hemolysis of red cells which must be avoided.

OBSERVATIONS AND RESULTS

Note that the blood has been separated into 3 layers:

i. A tall upper layer of clear plasma—amber or straw-colored. It should not be pink or red which would indicate hemolysis of red cells in the sample or within the body (i.e., before withdrawal of venous blood) in hemolytic diseases. If there is hemolysis, the test must be repeated on a fresh sample.

ii. A greyish-white, thin layer (about 1 mm thick) the so-called “buffy layer”, consisting of platelets above and leukocytes below it.

iii. A tall bottom layer of red cells which have been closely packed together. A greyish red line separates red cell layer from the layer of leukocytes above it. This line is due to the presence of reduced Hb in the red cells lying next to the leukocytes which reduce the oxyHb of the cells. The line marks the upper limit of the red cell layer.

The percentage of the volume of blood occupied by the red cells constitutes hematocrit or packed cell volume, i.e., the percentage of whole blood that is red cells

Hematocrit (Hct) = Height of packed red cells (mm) / Height of packed RBCs and plasma × 100.

Normal values. The average value of PCV is 42% when the RBC count is 5 million/mm3 and their size and shape are normal.

Males: 44 percent (38–50 percent)

Females: 42 percent (36–45 percent)

The PCV for new borns is about 50 percent.

15. Red Cell Indices

The calculation of the size and haemoglobin content of the red cells from the Hb, PCV and red cell count, have been widely used in the classification of anemia. The three most common indices are the MCV, the MHC and the MCHC.

Mean Corpuscular Volume (MCV)

The MCV is the average volume of a single red cell expressed in femtoliters (fl) or 10^{-15} L. It helps in determining the size of the RBC. The PCV and red cell volume are used in its calculation.

Calculation Formula

MCV = PCV % X 10 / RBC count (the number of millions not the actual count)

Example:

PCV = 0.45 L/L (45%), **RBC** = 5 x 10^{12}/L

MCV = PCV X 10/RBC = 45(%) x 10/5= 90 fl

RBC 5

Reference Range

The MCV reference range is 83-101 fl. The MCV is increased in macrocytic anemias (e.g. megaloblastic anemia) and decreased in microcytic anemias (e.g. iron deficiency, thalassemia)

MCV value is within the normal range Normocytic.

MCV value is above the normal range Macrocytic.

MCV value is below the normal range Microcytic**.**

Mean Cell Hemoglobin (MCH)

MCH is the average weight in picograns of Hb in one red cell. The Hb level and the RBC count are used

for calculation.

Calculation Formula

MCH = Hb (g/dl) X 10 / RBC (use the number of millions rather than the actual count)

Example:

Hb = 150g/l (15 g/dl), **RBC** = 5 x 10^{12} /L

MCH = 15(g/dl)x10 / 5= 30 pg

Reference Range

The MHC reference range is 27-32 pg. MHC is increases in macrocytic anemia and decreased in microcytic anemias.

MCH value is within the normal range Normochromic

MCH value is above the normal range Hyperchromic

MCH value is below the normal range Hypochromic

Mean Cell Hemoglobin Concentration (MCHC)

The MCHC is the concentration of haemoglobin per unit volume of red blood cells expressed as a percentage, g/dl or g/l. Hemoglobin and PCV are required to calculate MCHC.

Calculation Formula

MCHC = Hb(g/dl) x 100 / PCV%

Example:

If one liter of blood contains 0.45 liters of packed cells and 150g of Hb (150g Hb are contained in 0.45 liters of RBCs). What is the Hb concentration?
Hb = 15 g/dl (150 g/l), **PCV** = 45% (0.45 l/l)
MCHC = Hb (g/dl) x 100 / PCV%= 15 x 100/ 45 = 33.3% or 33.3 g/dl = 333.3 g/l

Reference Range
The reference range for MCHC is 32-36 g/dl or (320-360 g/l)**.**
A fully saturated red cell has a hemoglobin concentration of 36 g/dl. MCHC is a useful guide to the degree of hypochromia present in iron deficiency anemia. The Hb and PCV can be estimated reasonably accurately and the derived MCHC is therefore a reliable parameter.
Automated Estimation of Red Cell Indices
In automated counters, MCV is measured directly, but in semiautomated counters MCV is calculated by dividing the PCV by RBC. MCH is derived from the Hb divided by RBC. The MCHC is derived from the Hb and the PCV with instruments that measure the PCV and calculate the MCV, whereas when the MCV is measured directly and the PCV is calculated, the MCHC is derived from the Hb, PCV and RBC.

16. ***Absolute Eosinophil Count***

Relevance

Absolute eosinophil count is required when the differential count shows a high percentage of these cells. This is especially true in cases of bronchial allergy, asthma, urticaria, intestinal parasites, pulmonary eosinophilia, etc.

Counting Methods

The absolute count of eosinophils can be done by two methods:
1. ***Direct method.*** The cells are counted directly by employing hemocytometry.
2. ***Indirect method.*** As mentioned in DLC, the percentage of eosinophils is determined from a blood smear counting of leukocytes. If TLC is done simultaneously, the absolute count can be calculated.

APPARATUS AND MATERIALS

1. Microscope •Counting chamber •WBC pipette •Coverslips •Equipment for finger prick.
2. ***Pilot's diluting fluid*** for eosinophil counting:

Stock solutions:

a. Propylene glycol
b. Phloxine: 1 % solution in water (0.5% eosin may be used but phloxine is superior).
c. Sodium carbonate: 10 % solution in water.

Working solution: It is made by mixing and filtering the following:

Propylene glycol = 50 ml
Phloxine (1 %) = 10 ml
Sodium carbonate (10 %) = 1 ml
Heparin = 100 units
Distilled water = 40 ml

PROCEDURES

1. Get a finger prick under asepsis, discard the first 2 drops, and then fill 2 WBC pipettes with blood to the mark 1.0 (EDTA anticoagulated blood can be used).
2. Suck the diluting fluid to the mark 11 in both pipettes. Mix the contents of each for 2 minutes.
3. Place the pipettes on 2–3 layers of moistened filter papers and cover them with a petri dish. Allow to stand for 15 minutes for proper lysis and staining. (The purpose of moist filter papers is to prevent evaporation of water from the pipettes).
4. Take out the pipettes and mix the contents once again for 30 seconds. Discard the fluid in the stems and charge each side of the chamber from each pipette and bring the chamber into focus in the usual manner.

5. Using HP objective, count the eosinophils in the 4 corner groups of 16 squares each, i.e. in a total of 64 squares that were used for TLC. When the counting has been done, calculate the number of cells in 1 mm3 of undiluted blood. Enter your observations in appropriate squares drawn in your notebooks.

OBSERVATIONS AND RESULTS

Calculations

Volume of 64 squares
(each = 1/160 mm^3) = 1/160 × 64 = 4/10 mm^3
Let the eosinophil count in 4/10 mm^3
(i.e. 64 squares) be = x
Then, 1 mm3 of diluted blood will contain
= x × 10/4 cells
Since the dilution is 10 times, 1 mm^3
of undiluted blood will contain
= x × 10/4 × 10
= x × 25 cells.
Compare the count from the two pipettes. The difference should not be more than 10%.

Normal absolute eosinophil count = 10–400/ mm^3 (Eosinophil count of capillary blood is usually 10–15% higher).

Indirect Method of Counting Eosinophil

This method was mentioned in Expt 1-11. For this both TLC and DLC are required. For example, if the TLC is 8000/mm^3 and eosinophils are 2% in DLC, then the absolute count would be = 2/100 × 8000 = 160/mm^3 of undiluted blood. This method can act as a check on the result of direct method.

17. ***Study of Morphology of Red Blood Cells***

Alterations in the morphology of red cells (their size, shape structure, staining characteristics, etc.) are commonly seen in various types of anemia and other diseases. A careful examination of the peripheral blood film can, therefore, provide important information in the diagnosis of these conditions.

PROCEDURES

Prepare and stain a 'thin' blood film. If a suitable film is available from the previous experiments, it may be used. Study the cells in the area between the tail and the thicker head of the smear, away from the edges, where the RBCs are spread out. Note the following features of the red cells:

Size and shape. Note that there is a moderate variation in the size around the diameter of about 7.5 µm. Most cells are round, though a few may be slightly oval.

Staining. Note the size of the central pallor (it normally occupies the central third) and compare the depth of color of different cells. Note if there are any granules. Though reticulocytes are present (0.5 to 2%), their basophilic network does not take up Leishman's stain, and a special process called 'supravital' staining is required (see next Expt). The nucleated red cells are not normally present in the peripheral blood.

Demonstration slides. Stained slides showing reticulocytes and abnormal morphology of red cells (obtained from pathology department) will be set up on the demonstration table. Examine and compare these smears with your own blood film. Note the descriptions listed on the cards beside the microscopes and enter these in your workbook.

Abnormal red cells. The following terms express some abnormal morphological states and the conditions with which they are associated:

1. Anisocytosis: Abnormal variation in size; seen in iron deficiency and megaloblastic anemias.
2. Basophilic stippling (punctate basophilia): Bluish granules, seen in lead poisoning, thalassemia.
3. Burr cells: Irregularly shaped red cells, seen in uremia.
4. Cabot's rings and Howell-Jolly bodies: Bluish remnants of nuclei that persist in iron-deficiency and megaloblastic anemias; rarely leukemias.
5. Leptocytes (target cells, also called Mexican hat cells): Central staining, a ring of pallor, and an outer rim of staining, seen in liver disease, thalassemias, sickle cell disease.
6. Hypochromia: Less dense staining, wider central pallor; seen in iron deficiency anemia.

7. Hyperchromia: More dense staining red cells; seen in pernicious anemia.
8. Microcytes and macrocytes: Small or large cells; seen in iron deficiency and pernicious anemia.
9. Normoblasts: Immature, nucleated red cells; seen in hypoxia, hemolysis.
10. Pappenheimer bodies: Visible in lead poisoning, carcinomatosis, after splenectomy.
11. Poikilocytosis: Variable shaped cells, seen in iron deficiency anemia.
12. Polychromasia: RBCs of different ages stain unevenly, younger cells being bluer. This is a response to bleeding, hemolysis, hematinics (e.g. ferrous sulphate, B12).
13. Rouleaux formation: Stacking of red cells on each other; the "visual analog" of high ESR.
14. Schistocytes: Fragmented RBCs, sliced by fibrin bands; seen in intravascular hemolysis.
15. Spherocytes: Smaller, spherical cells, appearing more dense, seen in congenital hemolytic anemia or, rarely, in hereditary spherocytosis.
16. Sickle cells: The RBCs are shaped like a sickle; it is due to the HbS which alters the shape of red cells.

18. ***The Reticulocyte Count***

Introduction. Reticulocytes are the non-nucleated immediate precursors of red cells that develop in the red marrow from the PHSCs. They contain large amounts of **the remnants of RNA and ribosomes.** They are slightly larger (Diameter = 9.0 μm) than RBCs, and are present in large numbers in bone marrow and in small numbers in blood. Their cell membranes are sticky which plays an important role in their controlled release from the bone marrow. Most of them, because of their larger size and stickiness, are trapped in the trabeculae of the spleen. Here they ripen and mature in a day or two before entering the circulation once again.

Relevance

The reticulocyte count, which is 1–2% of circulating red cells, is an indicator of erythropoietic activity of red bone marrow. It is indicated in all conditions where high counts are expected, such as in hemolytic anemias. It can also help in assessing the effectiveness of a drug being used in the treatment of anemia.

PRINCIPLE

A mixture of blood and a dye (stain) is spread in the form of a thin smear on a glass slide and suitably counterstained to bring out their reticulum. They are then counted per 1000 red cells and their percentage calculated.

APPARATUS AND MATERIALS

1. Microscope. • Glass slides. • Equipment for finger prick. • Petri dish. • Blotting paper.
2. Reticulocyte stains (supravital stains). These stains are used for staining unfixed, "living" cells and tissues *in vitro* (outside the body).
i. *Brilliant cresyl blue.* 1.0 g of this dye dissolved in 100 ml of citrated saline (1.0 volume of 3.8% sodium citrate and 4 volumes of normal saline). The dye stains the RNA of reticulocytes, citrate

prevents clotting of blood, and normal saline provides tonicity (1.0% solution of the dye in methyl alcohol can also be used).

ii. ***New methylene blue.*** While methylene blue does not stain the reticulum, new methylene blue (which is chemically different) stains this material more deeply and uniformly. 1.0 g of the dye is dissolved in 100 ml of citrate saline.

Theory of Reticulocyte Staining

The basophilic remnants of RNA and ribosomes in the cytoplasm of reticulocytes cannot be stained by the basic dye methylene blue, which is a component of Leishman's stain. The material can only be stained with certain dyes such as brilliant cresyl blue. The dye enters the cells and stains the basophilic material to form bluish precipitates of dots, short strands, and filaments. This reaction can occur only in **supravitally (or vitally)** stained cells, i.e. in "unfixed" and "living" cells. The more the immature cells, greater is the amount of precipitable ribosomal material present in them.

PROCEDURES

1. Take 2–3 clean, grease-free glass slides and place a drop of reticulocyte stain in the center of each slide about 1 cm from its end.
2. Get a finger prick under aseptic precautions and add an equal-sized drop of blood to each drop of stain. Stir with a pin and put the slides on moist filter paper and cover with a petri dish. Allow the mixture to remain on the slides for 1 minute. (The slides may be incubated at body temperature for 15 minutes to simulate the living conditions so that the stain may better penetrate the reticulocytes).
3. Spread a smear of the blood-dye mixture on each slide, then counterstain with Leishman's stain in the usual manner. (This will stain all cells).

OBSERVATIONS AND RESULTS

1. Using oil-immersion objective, bring the blood cells into focus and identify reticulocytes. They stain lighter than the red cells and also contain dots, strands, and filaments, etc. of bluish-stained material.

Identification of Reticulocytes. These non-nucleated cells are slightly larger (diameter about 8 µm) than the red cells (average diameter = 7.5 µm). They also stain lighter than the red cells, and contain dots, strands, and filaments of bluish-stained material.

2. Count the reticulocytes in 100 alternate fields, i.e., move one uncounted field before counting them again. (In some fields you may not see any such cells.)
3. Count the red cells in every 5th field, for a total of 10 fields. (When counting red cells in a field, divide the field into 4 imaginary quadrants. This will make the counting easier).

Calculations

If, say, the number of reticulocytes in 100 fields is = 72 and the number of red cells in 10 fields is = 450 .The number of red cells in 100 fields = 450 × 10 = 4500
Percentage of reticulocytes = 72/4500 × 100 = 1.6%

Normal Values

1. Newborns = 30–40%. Their number decreases to 1–2% during the first week of life.
2 Infants = 2–6 %
3. Children and adults
    a. 0.2–2.0% (average = 1%)
    b. Absolute count = 20,000–90,000/mm3.

Absolute Reticulocyte Count

A direct reticulocyte counting by hemocytometry is not possible. An indirect absolute count can be obtained from the relative percentage by doing a total red cell count.
= Reticulocyte percent × red cell count/100
Normal value = 25,000 – 100,000 / mm^3

19. Determination of *Bleeding Time (BT) & Clotting time (CT)*

Bleeding Time (BT) is the time interval between the skin puncture and spontaneous, unassisted (i.e. without pressure) stoppage of bleeding. The BT test is an *in vitro* test of platelet function.
Clotting time (CT) is the time interval between the entry of blood into the glass capillary tube, or a syringe, and formation of fibrin threads.

[I] "Duke" Bleeding time (finger-tip; ear-lobe)

• Since the skin of the fingertip is quite thick in some persons, a small cut in the skin of the earlobe with the corner edge of a sterile blade gives better results. The earlobe method is the original "Duke" method for BT.
• Ask your partner to fill the capillary tube with blood from the same skin puncture from where you are doing the BT (see below for CT).
• **Materials** •Equipment for sterile finger-prick. • Clean filter papers. • Chemically clean, 10–12 cm long, glass capillary tubes with a uniform bore diameter of 1–2 mm. • Stopwatch.

PROCEDURES

1. Get a deep finger-prick under aseptic conditions to get free-flowing blood. Start the stop watch and note the time.
2. Absorb/remove the blood drops every 30 seconds by touching the puncture site with the filter paper along its edges, without pressing or squeezing the wound. Number the blood spots 1 onwards.
3. Note the time when bleeding stops, i.e. when there is no trace of blood spot on the filter paper. Encircle this spot and number it as well. This is the end point. (Do not keep the filter paper on the table and then press your wound on it).
4. Count the number of blood spots and express your result in minutes and seconds.
Normal bleeding time = 1–5 minutes.
• The test is simple and quite reliable in spite of the fact that the depth of the wound cannot be controlled.

• The BT is prolonged in purpura (platelet deficiency, or vessel wall defects) while it is usually normal in hemophilia.
• Lack of several clotting factors may prolong BT, though it is especially prolonged by lack of platelets.

PRECAUTIONS

1. The skin site chosen for BT should be scrubbed well with alcohol to increase the blood flow.
2. The skin should be dry and the puncture should be 3–4 mm deep to give free-flowing blood. Do not squeeze.
3. Do not press the filter paper on the puncture site.
4. If bleeding continues for more than 10–12 minutes, stop the test and press a sterile gauze on the wound. Inform your teacher about the bleeding.

[II] Another method is to get a finger-prick and dip the finger in a beaker containing normal saline at 37°C. The blood drops will be seen falling to the bottom in a continuous stream. Note the time when bleeding stops.

[III] "Ivy" Bleeding Time (Hemostasis Bleeding Time).

This method is more reliable than the "Duke" method. However, it requires some practice to apply the BP cuff and maintain the pressure.

Procedure

1. Clean the skin over the front of the forearm with 70% alcohol.
2. Apply a blood pressure cuff on the upper arm, raise the pressure to 40 mm Hg and maintain it there till the end of the experiment.
3. Clean the skin area once again. Grasp the underside of the forearm tightly, make a 1–3 mm deep skin puncture, about 5–6 cm below the cubital fossa. Note the time.
4.Remove the blood every 30 seconds by absorbing it along the edges of a clean filter paper by gently touching the wound with it, till the bleeding stops. This is the end-point.

Normal bleeding time with this method is upto 9 minutes.

CAPILLARY BLOOD CLOTTING TIME (WRIGHT'S CAPILLARY GLASS TUBE METHOD)

(While your partner is doing BT on your finger prick, you can proceed with your CT.
1. Absorb the first 2 drops of blood on a separate filter paper and allow a large drop to form. Now dip one end of the capillary tube in the blood; the blood rises into the tube by capillary action. This can be enhanced by keeping its open end at a lower level.
2. Note the time when blood starts to enter the tube. This is the zero time.
3. Hold the capillary tube between the palms of your hands to keep the blood near body temperature (in winter, you may blow on it).

4. Gently break off 1 cm bits of glass tube from one end, at intervals of 30 seconds, and look for the formation of fibrin threads between the broken ends. The end-point is reached when fibrin threads span a gap of 5 mm between the broken ends ("rope formation"). Note the time.

Normal clotting time = 3–6 minutes.

20. Determination platelet count.

Despite their small size (2–4 μm) and being non-nucleated fragments of cytoplasm, the platelets contain a wide variety of chemical substances that play an important role in vasoconstriction, hemostatic plug formation, activation of factor X, conversion of prothrombin to thrombin, and in clot retraction that results in permanent sealing of a ruptured vessel. Thus they take part in almost all stages of hemostasis.

Platelet counting. There are two methods for this count: *direct method* and the *indirect method.* Automated counters are also available.

A. DIRECT METHODS

You will require: • Microscope • RBC pipette • Counting chamber with cover slip • Equipment for fingerpick • Rees-Ecker diluting fluid—OR—Freshly prepared 1.0% ammonium oxalate solution.

PROCEDURES

I. Ammonium Oxalate Method. This fluid destroys red cells but preserves platelets; it also acts as an anticoagulant.

1. Get a finger- prick and draw blood up to the mark 1.0. Suck the diluting fluid to the mark 101.
2. Mix the contents thoroughly and wait for 20 minutes. The red cells will be hemolyzed, leaving only the platelets. Mix the contents once again and charge the chamber on both sides.

Place the charged chamber on wet filter paper and cover it with a petri dish to avoid evaporation.

3. Focus the RBC square under HP; adjust the diaphragm and position of condenser till you see the platelets – which appear as small, round or oval structures lying separately, highly refractile bodies with a silvery appearance. Rack the microscope continuously and count the platelets in 5 groups of 16 squares each, as was done for red cell count.

Knowing the dilution (1 in 100) employed and the dimensions of the squares, calculate the number of platelets in 1 mm^3 of undiluted blood.

21. Determination of prothrombin time

The patient's blood is quickly oxalated (or citrated) to remove calcium ions so that prothrombin cannot be converted to thrombin. The sample is then centrifuged. Then to the oxalated plasma, a large excess of calcium ions (as calcium chloride solution) and rabbit brain suspension (to provide tissue thromboplastin; tissue factor, TF) is added. The excess calcium neutralizes the

effect of oxalate and the TF converts prothrombin to thrombin via the extrinsic clotting pathway (i.e. factor VII).

The time required for clotting to occur is called the prothrombin time (PT).

Normal PT = 15–20 seconds.

Clinical Significance. Since the potency of tissue thromboplastin (TF) may vary, blood from a normal person is used as a control when the test is used for controlling anticoagulant dose, or in a hemorrhagic disease. Bleeding tendency is present when the prothrombin level falls below 20% of normal (normal plasma prothrombin = 30–40 mg/dl). Prolonged PT suggests the possibility of deficiency of factors II (prothrombin), V, VII and X. Prothrombin level is low in vitamin K deficiency and various liver and biliary diseases.

22. ***Osmotic Fragility of Red Blood Cells***

Relevance

In certain hemolytic anemias, the red cells become more fragile, i.e., they are likely to burst and release their hemoglobin into the plasma. The osmotic fragility test assesses their ability to withstand hypotonic saline without bursting. It is employed as a screening test for hemolytic anemias.

PRINCIPLE

The normal red cells can remain suspended in normal saline (0.9% NaCl solution) for hours without rupturing or any change in their size or shape. But when they are placed in decreasing strengths of hypotonic saline, they imbibe water (due to osmosis) and finally burst. The ability of RBCs to resist this type of hemolysis can be determined quantitatively.

APPARATUS AND MATERIALS

1. Wood or metal test tube rack with 12 clean, dry, 7.5 cm × 1.0 cm glass test tubes. •Glass marking pencil. •Glass dropper with a rubber teat.
2. Sterile swabs moist with alcohol. •2 ml syringe with needle.
3. Freshly prepared 1 percent sodium chloride solution. •Distilled water.

PROCEDURES

1. Number the test tubes from 1 to 12 with the glass-marking pencil and put them in the rack.
2. Using the glass dropper, place the varying number of drops of 1% saline in each of the 12 test tubes as shown in Table 1.6. Then, after thorough rinsing of the same dropper with distilled water, add the number of drops of distilled water to each of the 12 tubes.

Mix the contents of each test tube by placing a thumb over it and inverting it a few times. Mark the tonicity of saline on each of the test tubes. Note that tube # l contains normal saline, which is isotonic with plasma, while tube # 12 contains only distilled water which has no tonicity.

3. Draw 2 ml of blood from a suitable vein and gently eject one drop of blood into each of the 12 tubes. (The blood may be put into a container of anticoagulant, and a drop can be put into each tube with a pipette). Mix the contents gently by placing a thumb over it and inverting the tube only once.
4. Leave the test tubes undisturbed for one hour. Then observe the extent of hemolysis in each tube by holding the rack at eye level, with a white paper sheet behind it.

OBSERVATIONS AND RESULTS

While judging the degree or extent of hemolysis from the depth of the red color of supernatant saline, tube # 1 (normal saline), and tube # 12 (distilled water) will act as controls, i.e. no hemolysis in normal saline (# 1) and complete hemolysis in distilled water (# 12).
a. The test tubes in which no hemolysis has occurred, the RBCs will settle down and form a red dot (mass) at the bottom of the tube, leaving the saline above clear.
b. If there is some hemolysis, the saline will be tinged red with Hb, with the unruptured cells forming a red dot at the bottom. The color of the saline will be seen to be increasingly deeper with decreasing tonicity of saline.
c. The test tubes in which there is complete hemolysis, the saline will be equally deep red with no red cells at the bottom of these tubes.

Results. Carefully observe each tube for depth of red color of the supernatant and the mass of red cells at the bottom.
• Note the start of hemolysis (also called onset of fragility) and record the test tube number. Express your result in % saline.
• Note the start of complete hemolysis, i.e. the test tube in which there are no red cells at the bottom (hemolysis will be complete below this saline strength). Express your result in % saline.
Hemolysis begins in ..... % saline.
Hemolysis is complete in ..... % saline.
If there is doubt about the presence of intact RBCs at the bottom of a test tube, the solution can be centrifuged and the sediment examined under the microscope.

Normal Range of Fragility

Normally, hemolysis begins in about 0.48% saline (tube # 6 in this case). No cells hemolyze in solutions of 0.5% saline and above.
Hemolysis is complete at about 0.36 % saline (tube # 9). It is also complete in tubes 10, 11, and 12.

Modified Experiment

In this test, the red cell fragility is tested by counting the cells in a hemocytometer, using 0.45% saline for diluting the blood in one pipette, and using Hayem's fluid for diluting blood in a second pipette. Both pipettes are shaken for about 2 minutes and counts are made from both pipettes. The percent of red cells hemolysed in 0.45% saline is thus determined. Less than 20% of normal RBCs

are hemolyzed by this method. In hereditary spherocytosis, the abnormal increase in fragility may cause hemolysis of more than 70% of red cells.

23. Staining of Heinz bodies

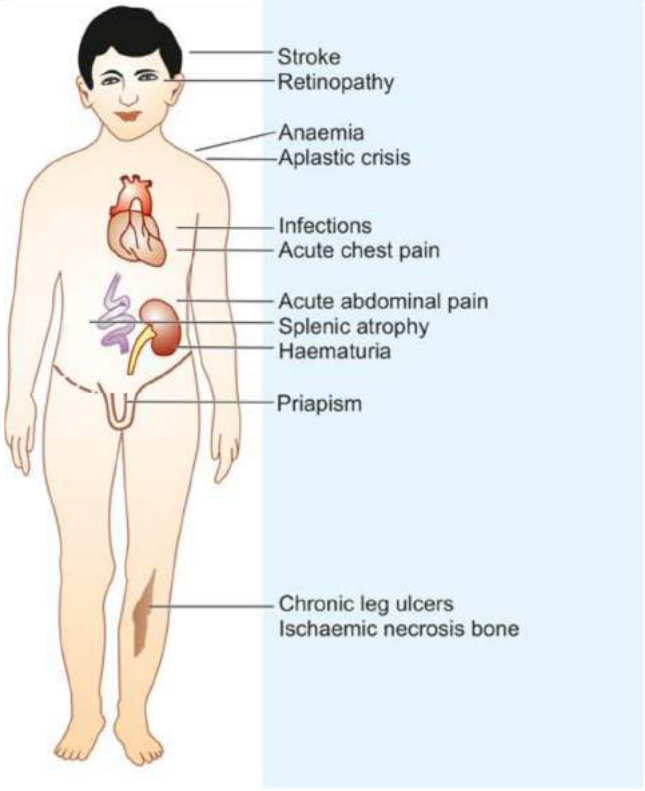
Heinz bodies do not appear on a peripheral blood smear stained with a Wright or Wright-Giemsa-stain. A supravital stain, such as brilliant green, crystal violet, or new methylene blue is necessary for Heinz bodies to be observed.

Heinz bodies are composed of denatured hemoglobin. This occurs when an individual is exposed to oxidizing chemicals. Heinz bodies are commonly associated with glucose-6-phosphate dehydrogenase (G6PD) deficiency and other red cell enzyme disorders.

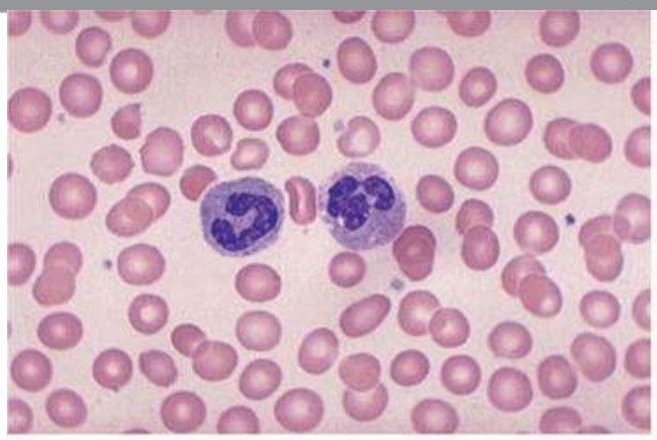
24. Screening test for sickle cell anemia

The diagnosis of SS is considered high in blacks with haemolytic anaemia. The laboratory findings in these cases are as under -

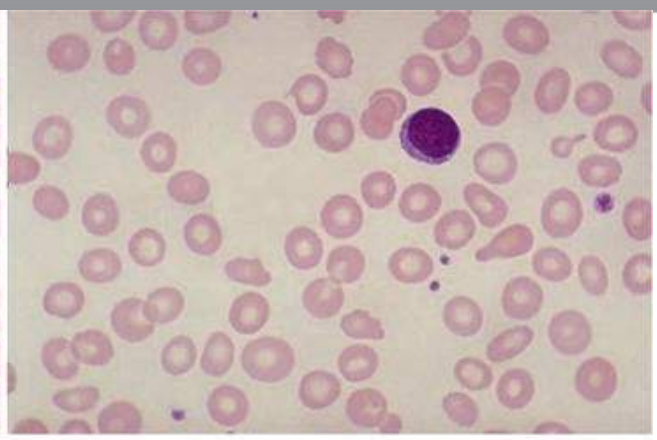
1. Moderate to severe anaemia (haemoglobin concentration 6-9 g/dl).
2. The blood film shows sickle cells and target cells and features of splenic atrophy such as presence of Howell-Jolly bodies.
3. A positive sickling test with a reducing substance such as sodium metabisulfite.
4. Haemoglobin electrophoresis shows no normal HbA but shows predominance of HbS and 2-20% HbF.



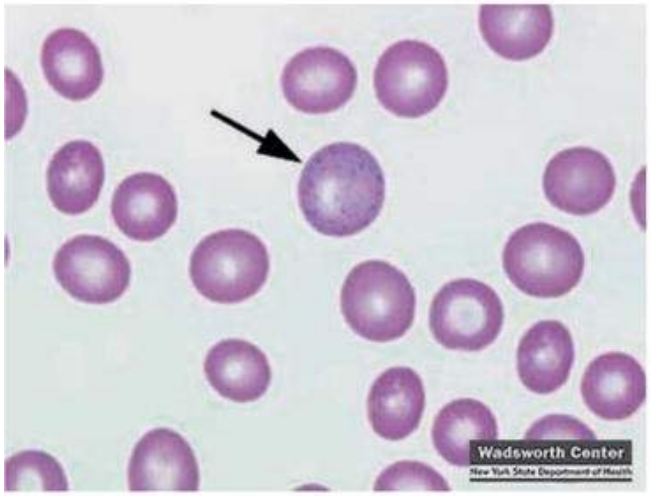
25. Slide identification of thalassemia



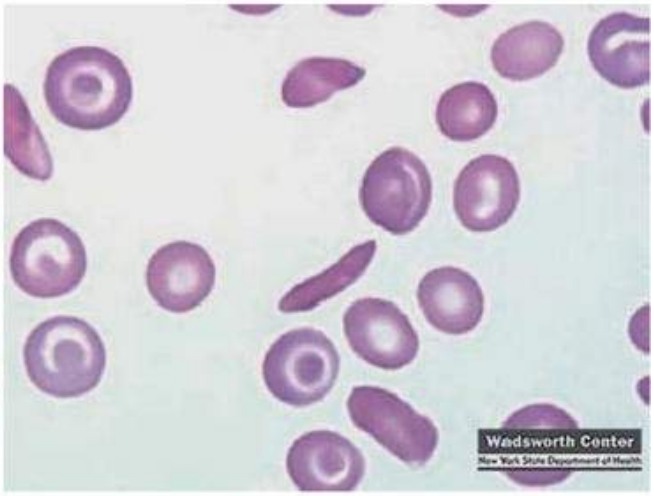
Slide 1: Normal Red Blood Cell



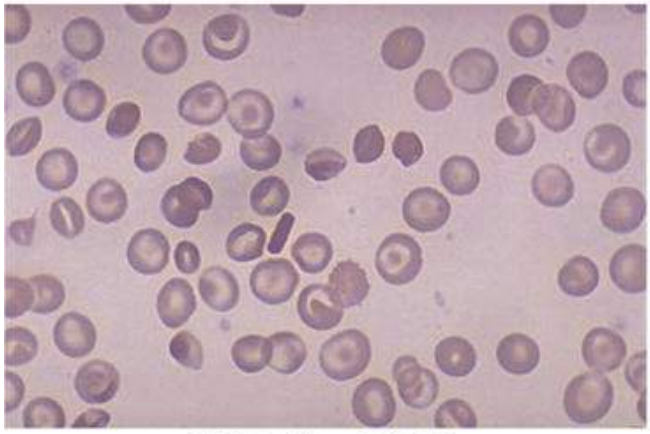
Slide 2: Iron Deficiency Anemia



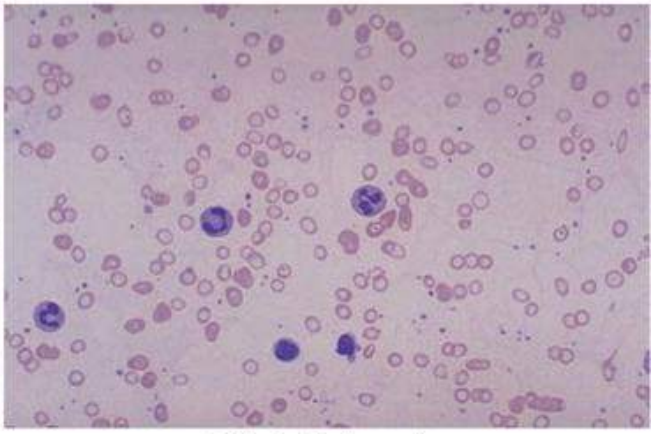
Slide 3: Lead Poisoning



Slide 4: Sickle Cell Anemia



Slide 5: Hemoglobin SC



Slide 6: Thalassemia

Clinical Biochemistry, Serology and Immunology

Code: MMLT 196

Biochemistry Laboratory Instruments :

PHOTOMETRY :

When light is passed through a coloured solution, certain wavelengths are selectively absorbed giving a plot of the absorption spectrum of the compound in solution. The wavelength at which maximum absorption is called the absorption maximum (λmax) of that compound. The light that is not absorbed is transmitted through the solution and gives the solution its colour.

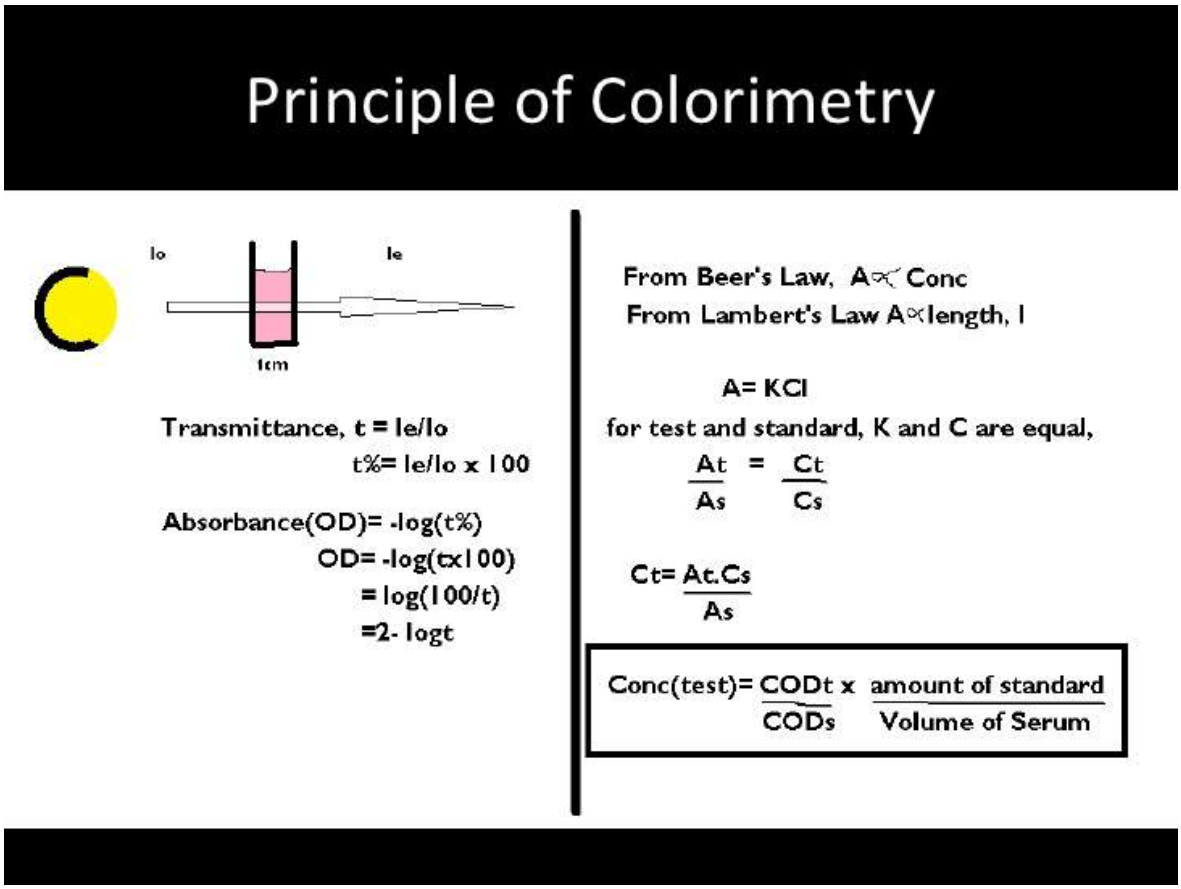


Fig. Principle of colorimetry

Transmittance (T) = Intensity of the emergent (or transmitted) light (le)/ Intensity of the incident light (lo)

Transmittance is usually expressed on a range of 0 to 100%.

Absorbance (A) = log1/T = log (le/lo)

Absorbance has no units. Photometric instruments electronically convert the measured transmittance to absorbance values.

Much of photometry is therefore based on two laws:

When a parallel beam of monochromatic light passes through a solution, the absorbance (A) of the solution is directly proportional to concentration(c) of the compound in the solution. This is Beer's law.

Each successive layer of the solution absorbs a constant proportion of the light entering the solution, although the absolute amount entering each layer diminishes progressively. Therefore, absorbance is directly proportional to the thickness or length of the light path (l) through the solution. This is Lambert's law.

$A \propto c$ (Beer's law) ---------- 1)

$A \propto l$ (Lambert's law) ----------2)

By combining 1) and 2), we get

$A = \varepsilon l\, c\, l$

εl, the proportionality constant is termed the molar absorption coefficient.

It is specific for a given substance at a given wavelength. It is the absorbance of a one molar solution of a substance with a light path of one centimetre (if c is expressed in mol/L). But Beer's law applies to only dilute solutions and in practice the concentrations of the solutions that are used in photometry are usually in the mmol/L range. In colorimetry, the absorption coefficient is not usually used. Concentration of an unknown solution can be determined by using equation 1, which is derived as follows:

Absorbance of test sample (At) = ε x concentration of test (Ct) x l

Absorbance of standard sample (As) = ε x concentration of standard (Cs) x l

Hence,

Conc. of Test (Ct) = (Absorbance of Test sample (At)/ Absorbance of Standard sample (As)

The light path, l, is usually kept constant in photometric measurements at 1 cm. This is the diameter of the tube (called the cuvette) containing the solution.

A standard (or calibrator) is representative of the substance whose concentration is sought to be determined. The concentration of the compound in the test sample is obtained by comparing its absorbance with that of a known concentration of a standard solution. Ideally, a series of standards of known concentration are prepared to obtain a standard (calibration) curve. This helps to determine the range of concentrations over which Beer's law is obeyed.

Appropriate blanks to exclude the absorbance contributed by the solvents and reagents used- i.e. by anything other than the compound of interest- are also essential for any photometric measurement.

COLORIMETRY

Colorimetry uses the basic principles of photometry but the solutions have to be coloured, i.e. they must absorb light in the visible range. Colourless compounds are converted into coloured compounds using chemical reactions. Under defined reaction conditions, the quantity of colour formed is proportional to the quantity of the original colourless compound.

PHOTOMETRIC INSTRUMENTS

Colorimeter

It is used to measure the intensity of light transmitted through a coloured solution. It uses light only in the visible range. Ordinary light from a tungsten lamp is passed through a suitable filter to obtain light of a desired wavelength, which is then passed through the solution.

Transmitted light falls on the sensitive surface of selenium photocell which generates a current proportional to the light intensity. The cell is connected to a galvanometer, which is used to read out percentage transmission or absorbance.

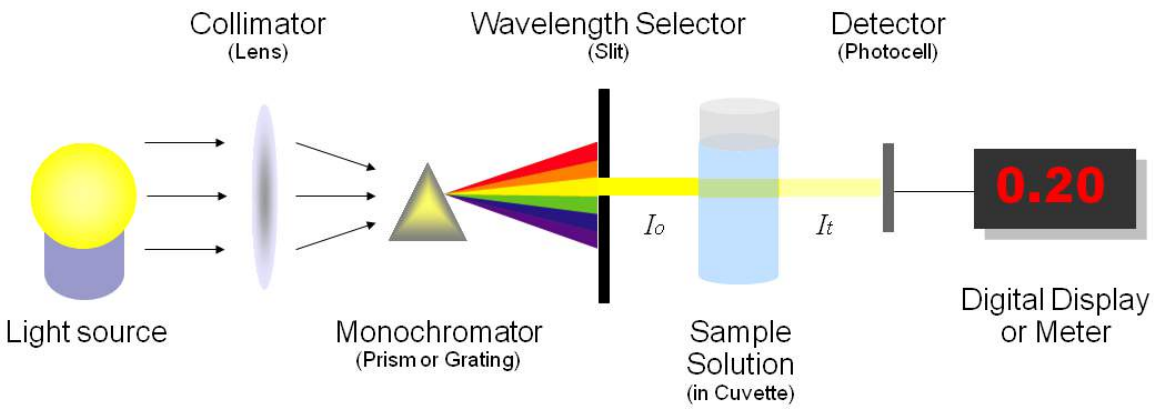


Fig. Components of spectrophotometer

Spectrophotometer

A spectrophotometer works on the same principle as a colorimeter but it is more sensitive and sophisticated. There are light sources that emit light in the ultraviolet, visible and infrared regions of the spectrum. The wavelength is selected using a prism or diffraction grating and narrower bandwidths can be selected. Since light in the ultraviolet and infrared ranges is also emitted, the compound to be estimated does not necessarily has to be coloured and can be measured directly if they significantly absorb even at these wavelengths. This offers a significant advantage over the colorimeter, which is restricted only in the visible range.

Types of spectrophotometers:

Grating spectrophotometers : In these spectrophotometers, the monochromator is a diffraction grating, which disperses the white light into a continuous spectrum. By turning the wavelength adjustment knob, the grating is rotated and different parts of the spectrum are allowed to fall on the photocell. In this manner, the desired wavelength can be selected.

Prism spectrophotometers: The prisms may be made of glass or quartz. Light from a tungsten filament is focussed on the entrance slit and it passes through a glass prism that forms an extended spectrum. Only the light that falls on the exit slit can pass through the cuvet and

illuminate the photocell. The monochromatic light obtained here is much more pure than that produced by light filters, i.e. a much narrower band of wavelength is present in the incident beam of light. Different photocells are used, one sensitive to the short wavelength range of the instrument (360-525nm), while the other is used for the longer wavelengths (600 to 1000nm). The total wavelength range at which measurements can be made includes the visible spectrum (400 to 750nm) and extends on each side into the near ultraviolet (360 to 450 nm) and the near infrared (750 to 1000nm). It is therefore possible to estimate substances that are more or less colourless in the visible region, but which absorb light in the ultraviolet or infrared regions.

Quartz prisms help in extending the wavelength range below 350nm down to about 190nm. A deuterium lamp with a quartz envelope is provided as an additional light source. This is a rich source of UV radiation. Quartz cuvets will also be needed if one has to work in the UV range.

Uses :

A spectrophotometer can be used for routine analysis in clinical chemistry. By using a narrower bandwidth than is available with ordinary filters, the absorbance is often higher and the relation between absorbance and concentration remains linear over a wide range. If very dilute colour is to be measured (e.g. in serum iron determinations), then a spectrophotometer is usually required.

Analytical methods depending on ultraviolet absorption are commonly used in clinical chemistry and research. Examples include serum enzyme assays, assays of glucose, urea, uric acid, etc, which take advantage of the UV absorption of the coenzymes NADH and NADPH at 340nm. For, such methods a quartz spectrophotometer is essential.

ELISA Reader

ELISA stands for "enzyme linked immuno-sorbent assay." The ELISA reader or microplate reader is modification of the spectrophotometer, which enables quantitation of upto 96 samples per ELISA microplate. Microplates have a 12 x 8 well format. A set of 8 lamps, 8 filters and 8 detectors enable the sequential determination of 96 samples.

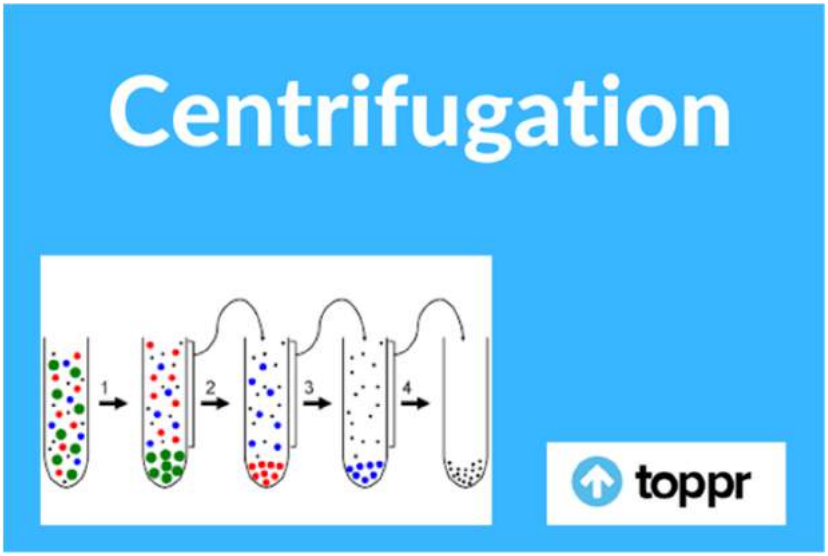
Automated clinical chemistry analyzers:

These usually incorporate mechanized versions of basic manual laboratory techniques and procedures. They help in the processing, transport and testing of a large number of clinical

specimens in an efficient manner. Work is simplified by using robotic and computer technology to undertake repetitive tasks like pipetting, dispensing and mixing. Advanced versions of the spectrophotometer help in increasing specimen throughput.

Centrifugation :

Centrifugation is a process which separates or concentrates materials suspended in a liquid medium. The theoretical basis of this technique is the effect of gravity on particles in suspension. 2 particles of different masses will settle in a tube at different rates in response to gravity. The centrifugal force is proportional to the rotation rate of the rotor. The centrifuge consists of a rotor and closed in a refrigerated chamber buy an electric motor.



Definition of Centrifugation

It is a unit operation working for separation separating the consequent present in a dispersion with the help of centrifugal force for example centrifugal force includes the earth revolves around the sun. It is a technique which involves the application of centrifugal force to separate particles from a solution according to their size, shape, density, the viscosity of the medium and rotor speed.

Principle of Centrifugation

1) The centrifuge involve the s principle of sedimentation.

2) The principle of the centrifugation technique is to separate the particles suspended in liquid media under the influence of a centrifugal field. These are placed either in tubes or bottles in a rotor in the centrifuge.

3) Sedimentation is a phenomenon where suspended material settles out of the fluids by gravity. The suspended material can be particles such as clay or powder. Example, tea leaves falling to the bottom in a teacup.

4) The particles having size more than 5 micrometres are separated by simple filtration process while the particles having size 5 micrometre or less do not sediment under gravity. The central force is useful to separate those particles.

Objectives of Centrifugation

1) To separate the immiscible liquids

2) To purify the component by removing impurities in the supernatant liquid.

3) To separate crystalline drugs from the mother liquor.

4) To test the emulsion and suspensions for creaming and sedimentation at an accelerated speed.

Application of Centrifugation

1) Production of bulk drugs.

2) Production of biological products.

3) Evaluation of suspensions and emulsion.

4) Determination of molecular weight of collides.

6) Removing fat from milk to produce skimmed milk.

8) Biopharmaceutical analysis of drugs.

Theory of Centrifugation

In centrifugation, centrifugal force is used as the driving force for the separation of particles. Centrifugal force is replacing a gravitational force which is responsible for the sedimentation of two particles. Thus centrifugation is useful when the ordinary filtration methods do not apply to the separation of particles. When two particles having different sizes but same densities are suspended in any liquid medium then they may not be able to separate by a simple filtration method. In such kind of cases, centrifugation method is useful.

The particle size above 5-micrometre sediment at the bottom with the help of gravity but the particles having a size less than 5 micrometres, start Brownian motion and do not sediment because of the gravity that's why they require the centrifugal force to separate properly. The centrifugal force causes the denser dense particle direction of the radical where writer practical moves to the centre. The ratio of the force acting on radical the direction to the gravitational force is the centrifugal effect.

Process of Centrifugation

1) The centrifuge consists of a container in which a mixture of solid and liquid or two liquids is placed. Then this container is rotated at very high speed. When this container is rotated at high-speed mixture separated into its constituent parts by the action of centrifugal force on their density.

2) The solid or liquid of higher specific gravity is thrown out word with greater force.

Types of Centrifugation Techniques

1) Density gradient centrifugation

It allows separation of many or all components in a mixture and allows for measurement also. There are 2 forms of density gradient centrifugation one is rate zonal centrifugation and the second is I saw pyknic or sedimentation equilibrium centrifugation.

A) Rate zonal centrifugation – In rate zonal centrifugation the solution has a density gradient. The sample has a density therefore greater than all the layers the solution. The sample is applied in a thin zone at the top of the centrifuge tube on a density gradient. Under centrifugal force, the particles will begin segmenting through the gradient. The particles will begin segmenting in separate zones according to their size, shape and density.

B) Isopycnic or sedimentation equilibrium centrifugation –centrifugation separation of particles occur into the zone based on their density difference, In this type of centrifugation, the solution contains a greater range of densities. The density gradient contains the whole range of densities of the particle in the sample pool stop each particle with sediment only to the position in the centrifuge tube at which the gradient density is equal to the phone density. In sedimentation independent of time.

2) Differential Centrifugation

Differential centrifugation is a common procedure in microbiology and cytology useful to separate certain organelles for further analysis of specific parts of cells. In the process, a tissue temple is first homogenised generalized to break the cell membranes and mix up the cell

contents. The homogenate is then subjected to repeated centrifugation, each time remove in the palate and increasing the centrifugal force.

3) Ultracentrifugation

Svedberg coined the term “ultracentrifugation”. He was a chemist. He used the ultra fuse to determine the MW and subunit structure of emo globin. The first commercial ultra fuse was produced in 1940. An important tool in biochemical research is the centrifuge, which through rapid spinning imposes hi centrifugal forces on suspended particles, or even molecules in solution, and causes separation of such matter on the basis ultracentrifugation of differences in weight. For example, red cells may be separated from the plasma of blood, nuclei from mitochondria and cell homogenate, and one protein from another in complex mixtures.

Laboratory water bath :

A water bath is laboratory equipment made from a container filled with heated water. It is used to incubate samples in water at a constant temperature over a long period of time. Most water baths have a digital or an analogue interface to allow users to set a desired temperature, but some water baths have their temperature controlled by a current passing through a reader. Utilisations include warming of reagents, melting of substrates or incubation of cell cultures. It is also used to enable certain chemical reactions to occur at high temperature.

Precautions :

- Use with caution.
- It is not recommended to use water bath with moisture sensitive or pyrophoric reactions. Do not heat a bath fluid above its flash point.
- Water level should be regularly monitored, and filled with distilled water only. This is required to prevent salts from depositing on the heater.
- Disinfectants can be added to prevent growth of organisms.
- Raise the temperature to 90 °C or higher to once a week for half an hour for the purpose of decontamination.
- If application involves liquids that give off fumes, it is recommended to operate water bath in fume hood or in a well ventilated area.

Types of water bath :

Circulating water baths - Circulating water baths (also called stirrers) are ideal for applications when temperature uniformity and consistency are critical, such as enzymatic and serologic experiments. Water is thoroughly circulated throughout the bath resulting in a more uniform temperature.

Non-circulating water baths –

This type of water bath relies primarily on convection instead of water being uniformly heated. Therefore, it is less accurate in terms of temperature control. In addition, there are add-ons that provide stirring to non-circulating water baths to create more uniform heat transfer**.**

Shaking water baths

This type of water bath has extra control for shaking, which moves liquids around. This shaking feature can be turned on or off. In microbiological practices, constant shaking allows liquid-grown cell cultures grown to constantly mix with the air.

Some key benefits of shaking water bath are user-friendly operation via keypad, convenient bath drains, adjustable shaking frequencies, bright LED-display, optional lift-up bath cover, power switch integrated in keypad and warning and cut-off protection for low/high temperature.

Water bath technologies

The bath is a fundamental product in any laboratory. Over the years, water baths have evolved from basic analog tools to advanced digital machines capable of sophisticated and programmable controls, functions, and capabilities.

Key features in water baths often include:

- Multi-language operation
- User-settable limit values
- "Eco modes" which save energy after set programs are completed
- User-settable alarms: audible, visible or both
- Displays of actual and/or set point temperatures
- Programmable pre-sets for frequently used temperatures
- Integrated timers
- Hinged gable covers
- Calibration off-set capabilities
- Stainless reservoirs
- Reservoir drains
- Primary and automatic safety thermostats
- Compatible with waterless alloy bath beads

Temperature control knob of a water bath

Inside water bath with micro-centrifuge rack

Professionalism in biochemistry laboratory

Medical laboratory professional

Introduction:

With technical sophistication and innovation in the field of medical science, a considerable proportion of medical diagnosis now rely on laboratory analyses, which emphasises the crucial role of laboratory physicians in patient care. In laboratory medicine, there is usually no director minimal contact with patients; however, the laboratory physicians' first and for most duty is to act in the best interests of the patient who is often "just a number" (as coded).Although personal knowledge of the patient is often lacking, the laboratory physician does have intimate knowledge of at least a part of the patient – "the labelled specimen"– and an unusual but traditional three-way contract is made between clinician, laboratory physician and patient. This is the case in particular where a life-altering event may occur on the basis of the definitive decision of a laboratory physician and the act of faith with which this is accepted by the patients.

The evolution of medical ethics over the years is well documented and evolved Bangladesh Journal of Bioethics 2019; 10(3): 5-106 through the Nuremberg Code from 19474, the Declaration of Geneva from September 1948.

Moreover, 'The Belmont Report' remains one of the key milestones concerning ethics in biomedical research. Created in 1978 by the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research of the United States, it outlines ethical principles and guidelines for the protection of human subjects. It identifies three core principles; those are as follows:

a) Respect for persons:

To ensure autonomy of the subjects/patients as well as to protect autonomy of those with diminished capacity to consent and make decision by themselves.

b) Beneficence: Acting in the best interests of patients or study subjects as well as maximize benefits and minimize harm. It is also termed as non-maleficence.

c) Justice: Moral obligation to treat all the patients equally disregarding age, sex, and race, and to ensure fair allocation of resources, e.g. treatment facilities and medications/vaccines, what is rightly due in terms of benefits, risks and cost.

As practicing physicians in the field, we have felt that sustaining high ethical standards remains crucial in both clinical biochemistry and laboratory medicine, and several ethical dilemmas are faced by laboratory physicians in day-to-day practice.

Ethical Issues in the Pre-analytical Phase:

Usually it is assumed that the referring clinician has ordered the appropriate tests to support his/her provisional diagnosis, not for any financial gain, discussed the risks and benefits of the tests to the patients, referred testing to an appropriate laboratory (one that is that is properly certified and approved by the corresponding authority and in which the referring individual has no financial interest) and practiced those three ethical principles to the best of his/her ability.

Ethical Issues in the Analytical Phase:

Confidentiality, quality assurance and competence are vital for all laboratories and settings. In the analytical phase, confidentiality may be maintained through automation that uses automated bar code readers, automated analysis, and auto verification, as the patient names are deemed by codes. Nevertheless, challenges of ensuring confidentiality are often greater in small laboratories with low-resources that perform manual testing and in operations that conduct near-patient (point-of-care) testing.

Ethical Issues in the Post Analytical Phase:

The post analytical phase includes reporting and interpretation of results, residual specimen storage, and access to data. Laboratories should have a policy for specimen storage and data protection. Archiving of results in either electronic or hard copy format is an important aspect of good laboratory practice. Archiving of results in either electronic or hard copy format is an important aspect of good laboratory practice.

Calibration of pipettes and other volumetric apparatus

Calibration of Graduated Pipette

Graduated pipettes are used for measuring the amounts of liquid between 1 mL to 100 mL. A pipette bulb is put on one side of the equipment to create a suction for the liquid. There are four reasons why one needs to clean the pipette properly every once in a while.

- Clean apparatus will offer more accurate outcomes.
- It can be calibrated conveniently.
- The cross-contamination risk gets reduced.
- Well-maintained pipettes last longer.

The outer part of the apparatus can be easily cleaned using general lab cleaners. But one must check the instruction manual before doing so. To clean the equipment from inside, distilled water can be used.

Along with this, proper contamination cleaning must be done from time to time. This ensures that none of the liquids gets contaminated during experiments.

The importance of pipette calibration increases due to the use of this equipment. Experts require the most accurate results from it. Thus, it must be calibrated. For the purpose of calibration of graduated pipettes, these steps can be followed:

Graduated Pipette

- At time of calibration, ensure no air bubbles are sticking to the glass walls and no water droplets exist above the graduation mark

- Deliver water from calibrated mark till it is emptied or in case of graduated pipette till the calibrated mark.

- Repeat 3-4 times till consistent reading is obtained for weight \((W_2)\), volume and delivery time.

Volume collected = (W_2 – W_1) / D,

where D is density of water at 25°C.

Calibration of Burette :

A burette is a narrow and long glass tube that comes with a stopcock at the base. The liquids are poured from the above end, and then the stopcock can be used to dispense a certain amount of it. Titration experiment comes under one of the primary burette laboratory apparatus uses.

It is extremely vital to keep the apparatus clean before using. Otherwise, the droplets of the liquid will stick to its walls, and it won't give out accurate results. Besides this, the tip and stopcock of the burette must be in place and working appropriately. It will help in getting the exact amount of liquid.

For calibration purposes of this equipment, these steps can be followed:

- Weigh a clean dry beaker (W_1)
- Deliver water from 0 mark to the point of calibration with tip touching the beaker wall.
- Weight of the collected water + beaker (W_2)
- Take note of the water temperature with a calibrated thermometer
- Repeat process at least 3 times

Volume collected = (W_2 – W_1)/ D where D is density of water at recorded temperature.

Record the delivery time and volume for consistent results.

Calibration of Volumetric Flask

Unlike other glassware equipment on the list, a volumetric flask is used for mixing or preparing a certain amount of any solution. It is crucial to clean this apparatus properly so that appropriate quantities can be measured, and no contamination happens with the liquid.

In general cases, volumetric flasks can be cleaned with water or other cleaning agents. But a proper procedure must be followed to avoid contamination. Plus, they are kept in an inverted position to let the liquid at the bottom drain away. A clean air stream can also be used to dry the equipment faster.

Volumetric Flask

To calibrate these, one can follow these steps:

- Dry the flask after rinsing with acetone
- Take weight (W_1)
- Fill the flask up to the graduation mark with distilled water and weigh the filled flask (W2)
- Note the temperature of water using the calibrated thermometer

- Volume = (W_2 – W_1)/D , Where D is density of water at recorded temperature
- Repeat 3 – 4 times to ensure consistency of volumes

It is necessary to clean, calibrate, and maintain all the volumetric glassware to ensure appropriate results and better durability.

Micropipettes for Highest Accuracy

Micropipettes are used to accurately measure and dispense small volumes of liquids in µl to ml range i.e., 1µl to 1000µl (1ml)

Advantages of using micropipette

- Fast
- Accurate
- Reproducible
- Disposable tips
- Do not require washing or rinsing
- Reduction of pipetting errors associated with normal pipettes

Precautions while using Micropipettes

- Never use the micropipette without a tip.
- Never lay or invert a loaded micropipette down or tilt it as this could allow liquid to run into the pipette barrel.
- Return the button to the top position smoothly and DO NOT snap back.
- Use the micropipette for only the specified pipetting range.
- Wipe droplets if any from the sides of tip but never wipe the tip opening.

- Never use a micropipette without a tip as it will contaminate the shaft assembly.
- Store micropipette set to its maximum volume. This releases pressure in the spring inside the micropipette.
- If trying to pipette organic solvents make sure they are compatible with the tip and also allow solvents to come to room temperature before pipetting.

Calibration of Micropipettes

These are used for the same purpose as a regular pipette. The only difference between the two is the amount of liquid they can hold. Micropipettes are used just for measuring quantities of less than 1.00 mL. They also come with disposable tips. So you can easily maintain their cleanliness. To calibrate them, one can follow these simple steps:

- Label and weigh five centrifuge tubes.
- Using single tip to transfer same volume in each centrifuge tube with single low range volume.
- Weigh each tube and record weight of water transferred into each tube.
- Calculate volume of water in each tube
- V= W/D
- Where V = volume in µl
- W = weight in mg
- D = Density of water in mg/ µl at the recorded temperature
- Calculate the standard deviation based on the five volumes.
- Determine the accuracy of micropipette by calculating the percentage (%) error.
- Repeat above steps with single upper range volume of micropipette.

Choice of Balance for Micropipette Calibration

Weighing of transferred volumes requires different types of balances for the required accuracies.

Micropipette capacity	Balance to be used
0.5 -10 µl	Micro Balance
10 – 200 µl	Semi Micro Balance
200-1000 µl	Analytical Balance

General Principles of Specimen Collection and Transport

Michael L. Wilson

From the Department of Pathology and Laboratory Services, Denver Health and Hospitals, and Department of Pathology, University of Colorado School of Medicine, Denver, Colorado

In this issue of Clinical Infectious Diseases, *we present the first article in a series entitled "Diagnostic Microbiology Updates." Although clinical microbiology is included in the curricula of virtually all infectious disease fellowships, the degree of emphasis on this subject varies considerably. Infectious disease physicians—even those who have direct responsibilities or consulting responsibilities for the microbiology laboratories of the institutions in which they practice—may be hard pressed to keep up with the rapidly changing content of the primary literature in clinical microbiology. The purpose of this series, therefore, is at least in part to fill this void and to provide concise updates for clinicians. The first article, written by Dr. Michael L. Wilson, reviews current concepts in specimen collection and transport. A key issue for all clinicians (which is not always sufficiently emphasized) is the quality of the specimen submitted to the laboratory. It is an axiom that if specimens of poor quality are submitted, the results generated by the laboratory will have little or no clinical utility. Dr. Wilson's article describes some of the methods available to assure that only specimens of good quality, i.e., those most likely to be useful clinically, are processed in the microbiology laboratory. Future articles will address specific types of specimens, groups of pathogens, and diagnostic techniques, including molecular methods. We hope this series will be informative and valuable to the readers of* Clinical Infectious Diseases, *and we look forward to your comments.*

Melvin P. Weinstein and L. Barth Reller

Departments of Medicine and Pathology, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, and the Microbiology Laboratory, Robert Wood Johnson University Hospital, New Brunswick, New Jersey; and Departments of Pathology and Medicine, Duke University School of Medicine, and the Clinical Microbiology Laboratory, Duke University Medical Center, Durham, North Carolina

Specimens submitted for microbiological testing require proper handling from the time of collection through all stages of transport, storage, and processing. Issues common to all clinical specimens submitted for microbiological testing include not only proper identification but also collection techniques that maximize recovery of microbial pathogens and minimize contamination. For specimens such as sputum and urine, the relative proportions of microorganisms present in vivo must be preserved, or culture results may be misleading. If specimens are handled properly, culture results are easier to interpret, patient care is improved, and costs are potentially decreased. Although most guidelines for specimen handling remain unchanged, a recent emphasis has been placed on modifying traditional practices to decrease or eliminate unnecessary work, increase laboratory efficiency, and make microbiological testing more cost effective.

Proper handling of specimens is crucial for obtaining microbiological test results that are both timely and clinically relevant. Proper handling of specimens is also one of the most important factors—along with appropriate use of tests—in maximizing the cost-effectiveness and clinical relevance of microbiological testing. The purpose of this article is to review recent changes in specimen handling, particularly specimen collection, that can be used to modify traditional practices in clinical microbiology [1].

General information on the collection, transport, and storage of specimens from different body sites for microbiological testing is presented in table 1. More-specific information is presented in the references from which this table was derived [2–6].

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Reprints or correspondence: Dr. Michael L. Wilson, Medical Laboratories #0224, Denver General Hospital, 777 Bannock Street, Denver, Colorado 80204.

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Table 1. Guidelines for collection, transport, and storage of specimens for microbiological testing.

Specimen or site	Container or method	Volume (mL)	No. of specimens	Transport (temperature, time)	Storage (temperature)	Comments
Blood						
Type of test						
Routine	Blood culture vials (aerobic and anaerobic) or lysis-centrifugation tube	20–30 for adults, 1–5 for children	2–3	25°C	25°C or 35°C	Avoid delays in processing lysis-centrifugation tubes
Fungal	Aerobic blood culture vials, BACTEC HBV-FM* vials, or lysis-centrifugation tube	20–30 for adults	2–3	25°C	25°C or 35°C	...
Mycobacterial	BACTEC 13A or lysis-centrifugation tube	10–20	1	25°C	25°C or 35°C	Submit one specimen initially; repeat if negative but mycobacteremia is clinically suspected
Bone	Sterile vial	NA	NA	25°C	4°C	Only infected bone should be cultured; avoid contamination from sinus tracts or skin
Catheters						
Type of catheter						
Urinary	NA	NA	NA	NA	NA	Specimen is inappropriate for culture
Vascular	Catheter tip in sterile vial	NA	NA	25°C	4°C	For detection of line sepsis, draw peripheral blood for cultures
CNS						
Abscess fluid	Anaerobic vial	1–5	NA	25°C	4°C	...
CSF	Sterile vial	1–5	NA	25°C (4°C for viral cultures)	4°C for ≤24–48 h (-70°C for >48 h for viral cultures)	Cytomegalovirus loses infectivity if stored at −20°C and with freeze-thaw cycles
Shunt/catheter fluid	Sterile vial	1–5	NA	25°C	4°C	...
Tissue	Anaerobic vial	NA	NA	25°C	4°C	...
Eye	Sterile vial	NA	NA	25°C	4°C	For bacterial cultures, handle in same way as other tissues
Fluid						
Abdominal	Anaerobic vial	1–10	NA	25°C	4°C	...
Pericardial	Anaerobic vial	1–10	NA	25°C	4°C	...
Pleural	Anaerobic vial	1–10	NA	25°C	4°C	...
Synovial	Anaerobic vial	1–10	NA	25°C	4°C	...
Other	Anaerobic vial	As appropriate	NA	25°C	4°C	...
Genitourinary tract (STDs)						
Organism to be cultured						
Candida albicans	Swab	NA	NA	NA	NA	...
Chlamydia trachomatis	Swab (transfer contents to 2-sucrose phosphate solution)	NA	NA	4°C	4°C	Avoid use of cotton swabs
Haemophilus ducreyi	Swab	NA	NA	NA	NA	...
Herpes simplex virus	Swab	NA	NA	NA	4°C	Avoid use of calcium alginate swabs and swabs on wooden applicator sticks
Mycoplasma/ Ureaplasma	Swab (transfer contents to 2-sucrose phosphate)	NA	NA	NA	NA	Avoid use of calcium alginate swabs and swabs on wooden applicator sticks
Neisseria gonorrhoeae	Insulate medium immediately	NA	NA	25°C	None; incubate immediately	Various transport systems are available; avoid calcium alginate swabs
Treponema pallidum	Scraping or aspirate of lesions on slide	NA	NA	Transport immediately	NA	Perform darkfield microscopic examination immediately
Trichomonas vaginalis	Swab for culture, smears for rapid tests	NA	NA	NA	NA	...

catch method), and that laboratory personnel are not inadvertently and unknowingly exposed to highly pathogenic microorganisms.

with the exception of that collected for viral cultures, should be transported at room temperature; and specimens submitted for culture of *Neisseria* species, which should be transported

Table 1. (*Continued*)

Specimen or site	Container or method	Volume (mL)	No. of specimens	Transport (temperature, time)	Storage (temperature)	Comments
Genitourinary tract (diseases other than STDs)						
Specimen or site of specimen collection						
Amniotic fluid	Anaerobic vial	1–10	NA	25°C	4°C	...
Cervix	Swab	NA	NA	NA	NA	Specimen is unacceptable for anaerobic culture
Endometrium	Anaerobic vial	1–5	NA	25°C	4°C	...
Pelvic fluid (culdocentesis fluid/abscess)	Anaerobic vial	1–5	NA	25°C	4°C	...
Prostate	Obtain secretions via prostatic massage (use sterile vial)	1–5	NA	25°C	4°C	Specimen is unacceptable for anaerobic culture
Vagina	Swab	NA	NA	25°C	4°C	Specimen is unacceptable for anaerobic culture
Hair	Sterile vial or Petri dish	NA	NA	NA	NA	...
Oral cavity	Anaerobic vial	NA	NA	25°C	4°C	Submit tissue or fluid collected from site of infection; collect specimen in such a way as to eliminate or minimize contamination with oral flora
Respiratory tract						
Specimen or site of specimen collection						
Bronchoscopy fluid	Sterile vial	NA	NA	25°C		Specimen is unacceptable for anaerobic culture unless collected with protected catheter
Expectorated sputum	Sterile vial	NA	NA	25°C	4°C	Specimen is unacceptable for anaerobic culture; screen for contamination with saliva
Nasopharynx	Swab	NA	NA	25°C (plate immediately)	4°C	Specimen is unacceptable for anaerobic culture
Sinuses	Aspirate transferred to anaerobic vial	NA	NA	25°C	4°C	...
Throat	Swab	NA	NA	25°C (plate immediately)	4°C	Specimen is unacceptable for anaerobic culture
Skin and soft tissues						
Site of collection or organism to be cultured						
Deep wound/abscess	Anaerobic vial, syringe	NA	NA	25°C	4°C	...
Dermatophytes	Sterile Petri dish	NA	NA	25°C	4°C	...
Superficial wound	Anaerobic vial, syringe	NA	NA	25°C	4°C	Do not submit swabs of specimens from the surface of decubitus ulcers, diabetic foot ulcers, margins of nonviable amputations, or other wounds
Stool						
Type of test						
Culture	Sterile screw-capped jar or container	NA	1–3	Immediately	4°C for fresh specimen	Do not submit specimens from patients who develop diarrhea after 3–4 days of hospitalization; submit specimen for detection of *Clostridium difficile*
Ova and parasite examination	Sterile screw-capped jar or container	NA	1 (initially)	Immediately for fresh specimen; commercial system for preserved specimens	4°C for fresh specimen	

Table 1. (*Continued*)

Specimen or site	Container or method	Volume (mL)	No. of specimens	Transport (temperature, time)	Storage (temperature)	Comments
Urine						For all specimens, submit >20 mL of urine for mycobacterial or fungal cultures
Specimen or site						
Clean-catch	Sterile vial	1–20	NA	Immediately at 4°C or 25°C	4°C	Specimen is unacceptable for anaerobic culture
Indwelling catheter	Not acceptable for culture	NA	NA	NA	NA	. . .
Straight catheter	Sterile vial	1–20	NA	Immediately at 4°C or 25°C	4°C	Specimen is unacceptable for anaerobic culture
Suprapubic aspirate	Anaerobic vial	1–20	NA	Immediately at 4°C or 25°C	4°C	Specimen is acceptable for anaerobic culture

NOTE. Data are from [2–6]. NA = not applicable; STDs = sexually transmitted diseases.
* BACTEC high-blood-volume fungal medium (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD).

in an atmosphere with sufficient CO_2 and humidity and in a manner that prevents wide temperature fluctuations.

Written guidelines. Clinical microbiology laboratories should distribute written guidelines for proper specimen collection, transport, and storage. These guidelines should be complete, explicit, and up-to-date. Copies should be available to all personnel who handle specimens. Identical guidelines should be included in the laboratory procedure manual, with copies available for laboratory staff who answer telephone queries. In laboratories with sufficient computer resources, particularly those with a computerized order-entry system, guidelines can appear as a prompt when a test is ordered. In teaching institutions, new house staff should be given written instructions on handling specimens as part of their orientation.

Rejection criteria. Accredited laboratories must specify rejection criteria for specimens that are collected, transported, or stored under improper conditions prior to processing. Examples of specimens that are unacceptable for processing are listed in table 3. Clinical laboratories should be nearly inflexible regarding this issue; there is no benefit—and there is the potential for harm—to patients when specimens that have been improperly collected or improperly transported are processed and test results are reported. Correct labeling is of particular importance for ensuring that patient misidentification does not occur and that appropriate testing is performed. In all instances, the physician who ordered the test, as well as the person who collected the specimen, should be notified when a specimen is rejected.

Table 2. Principles of specimen collection for microbiological testing.

- To minimize contamination, use strict aseptic technique when collecting specimens
- Collect specimens from anatomic sites most likely to yield pathogens and least likely to yield contaminants
- Tissue or fluid submitted for culture is always superior to material on swabs
- Submit adequate volumes of specimens
- Provide complete information on specimen requisition forms or during entry of electronic orders
- Notify microbiology laboratory and surgical pathology laboratory when there is a need for both culture and histopathologic examination

Blood

Accurate and timely detection of bacteremia and fungemia remains one of the most important functions of clinical microbiology laboratories. For more than any other type of specimen, pre-laboratory (pre-analytic) variables affect microbial recovery, contamination rates, and the ability of physicians to interpret test results. Although a subsequent article in this series will be devoted entirely to the subject of blood cultures, it is important to emphasize that proper collection and transport of blood for culture are crucial.

Collection. The clinical interpretation of blood culture results, as well as the cost-effectiveness of blood cultures, depends on many variables; of these variables, the most important is the proportion of blood cultures that are contaminated by skin flora [7, 8]. Since only 8%–9% of blood cultures yield microorganisms that are ultimately judged to be the cause of an episode of bacteremia or fungemia, it is imperative that

Table 3. Specimens that are unacceptable for microbiological testing.

- Unlabeled or improperly labeled specimens
- Specimens received in leaking, cracked, or broken containers
- Specimens with obvious (visually apparent) contamination
- Unpreserved specimens received >12 hours after being collected
- Specimens not appropriate for a particular test

Table 4. Comparisons of disinfectants used for blood cultures.

Reference	Disinfectant	No. of cultures	No. (%) of pathogens	No. (%) of contaminants
[13]	Isopropyl alcohol (applied twice)	1,609	159 (9.9)	18 (1.1)
	Tincture iodine/ isopropyl alcohol	179	15 (8.4)	2 (1.1)
[15]	Isopropyl alcohol/ povidone iodine	181	12 (3.3)*	8 (4.4)
	Isopropyl alcohol	181		6 (3.3)
[16]	Povidone iodine	4,139	626 (7.4)*	259 (6.3)
	Tincture iodine	4,328		162 (3.7)†
[14]	Isopropyl alcohol/ povidone iodine	763	104 (13.6)	35 (4.6)
	PREP‡	783	114 (14.6)	17 (2.2)§

* Total no. (%) of pathogens recovered during both phases of each study.
† $P < .00001$.
‡ Consists of 2% iodine tincture and isopropyl alcohol swabs (Mediflex Hospital Systems, Overland Park, KS).
§ $P < .01$.

contamination rates be minimized. Even with good collection technique, 1%–3% of blood cultures are found to be contaminated. In some clinical settings (e.g., teaching hospitals and emergency departments), blood culture contamination rates are much higher, compromising the ability of physicians to distinguish between contaminants and clinically important isolates. Blood culture contamination rates can be minimized by strict adherence to aseptic collection technique and, whenever possible, collection of peripheral blood via venipuncture rather than via indwelling vascular catheters [9].

Although the results of a recent meta-analysis [9a] suggest that changing needles prior to inoculating blood culture bottles results in a statistically significant decrease in contamination rates, the results of several other studies do not support this conclusion [10–12]. Moreover, switching needles increases the likelihood of a needle-stick injury [12]. In the absence of an unequivocal benefit that outweighs the potential risk of needle-stick injury, this practice should be proscribed.

At most institutions, an iodophor or tincture of iodine, alone or in combination with isopropyl alcohol, is used for disinfecting skin. Despite the clinical importance of the choice of disinfectant, few controlled comparisons of different disinfectants have been performed (table 4) [13–16]. Even though two studies [14, 16] have shown statistically significant differences in the effectiveness of disinfectants, taken together the results of all the evaluations suggest that the specific disinfectant used may be less important with regard to contamination of blood cultures than is good disinfection technique.

Number of cultures. If sufficient blood (20–30 mL) is drawn from each venipuncture site, virtually all septic episodes can be detected with two-to-three blood cultures [17, 18]. This is true both for septic episodes characterized by intermittent bacteremia and those characterized by continuous bacteremia. For patients with suspected infective endocarditis that is likely to be caused by indigenous bacteria, the performance of three or four cultures may be necessary before the physician can be certain of the clinical importance of any isolates that are recovered [7]. As cultures of blood drawn from patients with continuous bacteremia almost always yield microorganisms, it is rarely necessary to perform more than three or four cultures. The once-common practice of ordering "blood cultures times six" in cases of suspected infective endocarditis should be abandoned, as this practice contributes nothing to patient care, and it is costly and wasteful of resources and needlessly contributes to nosocomial anemia. On the other hand, drawing only a single sample of blood for routine bacterial and fungal cultures is inappropriate [19]; single blood cultures will not reliably result in detection of all septic episodes, and such a practice compromises the ability of physicians to interpret blood culture results. Receipt of single blood samples in the laboratory should result in notification of the physician that additional samples are needed [19].

In contrast, when blood for cultures is drawn for the purpose of detecting disseminated *Mycobacterium avium* complex (MAC) infection, single samples should be drawn initially [20]. Although drawing two samples has been shown to increase yield [20, 21], a second blood sample is unnecessary since the first culture yields the result (positive or negative) for 98% of specimens [20]. Lack of a final result for the other 2% of specimens is generally acceptable in terms of patient care, since the clinical course of MAC bacteremia is more indolent than that of bacteremia caused by other bacteria or fungi; a second blood culture is not needed to interpret mycobacterial blood culture results (i.e., mycobacteria are virtually never contaminants); and second specimens can be collected easily. Therefore, a second specimen should be drawn only if the first culture is negative and the clinical findings continue to suggest disseminated MAC infection.

Volume of blood cultured. Because of the low number of microorganisms present in the blood of adults who are bacteremic or fungemic, the most important variable in recovering bacteria or fungi from adults is the volume of blood cultured [22, 23]. For adults, the recommended volume of blood to be drawn for *each* blood culture (i.e., from each venipuncture site) is 20–30 mL. For infants and small children, the number of organisms in the blood can be, but is not always, higher [24]. Therefore, although bacteremia or fungemia can be reliably detected when small volumes of blood ($\leq$1 mL) are cultured, microbial recovery is enhanced when larger volumes of blood are cultured [25]. For infants and small children, the recommended volume is 1–5 mL. For older children, the volume of blood to be drawn per culture should be appropriate for the age and weight of the patient.

Culturing an adequate volume of blood also ensures that the proper ratio of blood to broth medium is attained within each bottle. Although this ratio is probably less important than volume per se in optimizing microbial recovery, maintaining a

Table 5. Criteria for rejecting specimens of expectorated sputum.

Bartlett*

Neutrophils per field (magnification, ×10)	Grade
<10	0
10–25	+1
>25	+2
Presence of mucus	+1
Squamous epithelial cells per field (magnification, ×10)	
10–25	−1
>25	−2
	Total†

Murray and Washington‡

	Squamous epithelial cells per low-power field	Neutrophils per low-power field	Perform culture
Group 1	>25	<10	No
Group 2	>25	10–25	No
Group 3	>25	>25	No
Group 4	10–25	>25	No§
Group 5	<10	>25	Yes

* Data are from [34].

† The numbers of neutrophils and squamous epithelial cells are averaged based on examination of 20–30 separate fields (magnification, ×10). The total is then calculated; final scores of ≤0 suggest contamination with saliva and/or absence of acute infammation.

‡ Data are from [35].

§ Some laboratories set up cultures of specimens in group 4.

preted with use of criteria such as those shown in table 5 [34, 35]. If a sputum specimen is rejected, another specimen should be collected and screened in the same manner. Specimens submitted for mycobacterial culture should not be screened with use of these criteria, as the results do not reflect the likelihood that mycobacteria will be recovered [36]. Similarly, specimens submitted for culture should not be screened on the basis of the relative numbers of neutrophils and alveolar macrophages [37]. Morris et al. [38] studied the use of gram staining in screening endotracheal aspirates; if a gram stain reveals no bacteria or reveals >10 squamous epithelial cells per low-power field, the specimen should be rejected. Other respiratory tract specimens (e.g., bronchial lavage fluid) should not be rejected on the basis of criteria used for other specimens such as sputum.

Transport and storage. Because most respiratory tract specimens are likely to contain at least a few contaminating microorganisms, specimens should be transported quickly to the laboratory to minimize overgrowth of contaminants. If transportation or processing is delayed, specimens should be refrigerated. For fungal and mycobacterial cultures, prompt processing and refrigeration help prevent overgrowth of normal flora in the specimens, which complicates the recovery of pathogens.

Stool

The laboratory diagnosis of enteric infections is challenging. Problems include the number of potential pathogens; the biologic diversity of these organisms; the emergence of new pathogens; and the fact that accurate, reliable, and practical diagnostic tests have yet to be developed for many pathogens [5]. Moreover, international travel has become so common that, in some instances, epidemiological clues as to likely etiologic agents may not be as helpful as they once were. At the least, there are many more potential causes of diarrhea in travelers than in patients who have not traveled. Consequently, microbiology laboratorians must have the expertise, experience, and resources to recover and identify a variety of potential pathogens. For many laboratories, the problem is the provision of such a service in a cost-effective manner. This problem is best solved by close collaboration between laboratorians and clinical staff, with development of test utilization strategies that are appropriate for the patient population being served.

Collection. Numerous studies have verified the observation that there is minimal value in routinely performing stool cultures or microscopic examinations of stool for ova and parasites in patients who develop diarrhea after 3 or 4 days of hospitalization [39–48]. This observation holds true for both adults and children. Although the exact cutoff time (3 days vs. 4 days of hospitalization) may vary slightly depending on the specific health care setting, it is neither beneficial to patients nor cost-effective to routinely process specimens for these tests after the 4th day of hospitalization. Stool specimens collected from patients who develop diarrhea in the hospital should be tested for the presence of *Clostridium difficile*. Manabe et al. [49] have published guidelines for using clinical data and laboratory data to guide evaluation of patients with suspected *C. difficile* disease.

Similarly, there is little value in routinely testing three or more stool specimens as part of an evaluation of acute diarrhea, as the majority of published studies indicate that most pathogens are detected in the first specimen [41, 45, 47, 48, 50] (this is true for both cultures and examinations for ova and parasites). However, some studies have not supported this observation, a finding suggesting that in some settings it may be appropriate to routinely collect two or three specimens [51, 52].

This issue is a difficult one to resolve because the failure to detect pathogens in stool specimens relates partly to intermittent shedding of some intestinal pathogens as well as to current diagnostic limitations [5]. Although the testing of multiple specimens may eventually overcome the former circumstance, it cannot overcome the latter. Rather than submitting many specimens from the same patient, clinicians should consult the

laboratory for alternative diagnostic tests for specific pathogens. For example, a significant number of cases of strongyloidiasis are missed when stool specimens are examined for the presence of *Strongyloides* with use of traditional methods (direct fecal smear, formalin-ether concentration, or culture on a filter-paper strip); this organism can be detected more reliably by coproculture with use of agar plates [53].

More widespread use of newer diagnostic products such as enzyme immunoassays for *Giardia lamblia* may also obviate the need to routinely test more than one stool specimen. In most settings—and for detection of most common enteric pathogens—awaiting results for the first specimen before collecting subsequent specimens reduces the amount of unnecessary testing.

Transport. Optimal test results are obtained when microbiological testing is performed on fresh stool specimens. Because testing fresh specimens is impractical in most clinical settings (particularly outpatient settings), most stool specimens are collected and then placed in vials containing different transport media and fixatives. A variety of such products are commercially available; each system typically includes a transport medium for culture and 10% neutral-buffered formalin and polyvinyl alcohol for ova and parasite examination. For most purposes, these transport systems work well, are convenient and easy to use, and are relatively inexpensive [54].

Storage. Stool specimens submitted for culture typically are not stored for any length of time, since most laboratories set up all appropriate cultures at the time of receipt of the specimen. Only rarely is it necessary to retrieve a specimen for additional testing; such specimens should be refrigerated. Stool specimens submitted for ova and parasite examinations are typically stored at room temperature in a fixative. Specimens stored in 10% neutral-buffered formalin remain stable for many months, even when tested with some enzyme immunoassays for *G. lamblia.* Because trophozoites can deteriorate quickly in stool, even when refrigerated, fresh stool specimens submitted for ova and parasite examination should be examined within 2 hours.

Summary. For patients with acute diarrhea, one specimen should be submitted for culture and examination for ova and parasites. If these tests are negative and symptoms persist, additional specimens should be submitted for testing. For patients who develop diarrhea after the third or fourth day of hospitalization, a stool specimen should be tested for the presence of *C. difficile.*

Specimens Collected for Viral Cultures

Many commercial products are available for the collection and transport of viral culture specimens. For most of these systems, specimens are collected on swabs that are then rinsed in a broth medium (viral transport medium). Calcium alginate swabs should not be used, since they are known to adversely affect recovery of herpes simplex virus [6]. Although most viruses survive well at ambient temperature while in common transport media, recovery of viruses from specimens containing low numbers of viruses may be decreased following prolonged holding under these conditions. Therefore, it is advisable to transport specimens on ice or to keep them refrigerated. Specimens should never be exposed to temperatures higher than room temperature. Johnson [55] has reviewed the details regarding collection and transport of specimens for recovery of specific viruses. A subsequent article in this series will include a more detailed review of diagnostic virology.

Specimens Submitted for Detection of Microorganisms by Molecular Diagnostic Techniques

A variety of molecular diagnostic assays have been developed for use in clinical microbiology laboratories. Despite the potential for improving the diagnosis of infectious diseases, several important issues need to be resolved before many of these assays can be recommended for routine use. First, it is not known whether specimens submitted for molecular diagnostic testing should be screened with use of the same methods and criteria as are used for specimens submitted for culture. Second, even though molecular diagnostic assays are analytically more sensitive (i.e., they detect smaller quantities of analyte), it is not known whether they are diagnostically more sensitive (i.e., they generate fewer false-negative test results). Third, many of these tests, particularly nucleic acid amplification assays, are not yet commercially available and thus have not been standardized. "Home-brew" assays, in particular, remain poorly standardized. Last, the clinical relevance of results obtained from many assays has yet to be defined.

Controlled clinical trials to establish performance characteristics will be possible once these assays become commercially available and are more widely used. Until that time, it would be prudent to test only those specimens that are appropriate for culture, since they have already been shown to yield more pathogens with fewer contaminants. A subsequent article in this series will include a more-detailed review of molecular diagnostic techniques.

Specimens Collected for Serological Diagnosis of Infectious Diseases

For most diseases, serological testing is not a surrogate for culture or other diagnostic tests. Physicians should order serological tests sparingly, since many serological assays have limitations that often are not appreciated. The most important of these limitations include technical issues such as cross-reactivity, turnaround time (for some assays, test results are not available in a clinically relevant time frame), and the inability to distinguish between acute disease and past exposure to infectious agents on the basis of single assays. Serological testing certainly plays an important role in the treatment of patients

with infectious diseases, but only when the tests are used appropriately.

Collection. Specimens obtained for serological analysis should be collected either in sterile evacuated tubes or in serum separator tubes. Strict aseptic technique should be used during venipuncture. Adequate volumes of blood should be drawn for anticipated tests. In many cases it is prudent to draw a small additional volume of blood that can be stored for future testing.

Most clinical laboratories perform limited serological testing, forwarding many specimens to commercial reference laboratories, state laboratories, the Centers for Disease Control and Prevention, and other public health laboratories. Because of the multiplicity of assays and testing laboratories, serological testing is best facilitated when the laboratory procedure manual contains the following data for each reference laboratory: detailed information regarding infectious agents and/or diseases for which tests are available; specific assays for each agent and/or disease; types of specimens that will be accepted for testing; minimum and optimal volumes of specimens for testing; reference ranges; limitations of assays; recommendations for specimen collection; guidelines for test interpretation; and special considerations for specimen collection and transport.

It is inadvisable to collect specimens other than serum for most serological tests. Since most commercial serological tests were not developed to test specimens other than serum, standardized controls are not available, reference ranges have not been defined, and the performance characteristics (i.e., sensitivity, specificity, and positive and negative predictive values) of the tests are unknown. In addition to these technical limitations, the clinical relevance of test results for specimens other than serum is usually unknown.

Transport and storage. To prevent loss of immunologic reactivity and growth of contaminating microorganisms, specimens should be transported promptly to the laboratory and centrifuged, and the serum should be poured off and refrigerated or frozen immediately. Serum specimens that will be used within 1 week after collection can be refrigerated. Specimens that need longer storage should be frozen at −70°C. Storage of specimens at −20°C is not recommended, since some antibodies deteriorate at an unpredictable rate at that temperature. Most assays of specimens that are properly stored at −70°C yield accurate results for many months.

Safety During Handling of Specimens Collected for Microbiological Testing

Collection. To minimize the potential exposure of personnel to infectious agents, specimens should be collected with use of standard (universal) precautions [56]. In particular, blood collection should be performed in strict accordance with guidelines to prevent needle-stick injuries [57]. Collection of specimens from patients with communicable diseases, particularly those transmitted via the respiratory route, should be done under appropriate isolation conditions.

Transport. Specimens should be transported with use of standard precautions. Additional measures should be taken to ensure that specimens are not damaged during transportation, which can result in contamination or leakage of the specimen. In particular, specimens in glass containers should be transported in such a way that the risk of breakage is minimized. This is especially important when pneumatic tube systems are used for transport, as cleanup of leaked specimens within these systems is difficult and expensive.

Microbiological specimens transported via mail or other interstate couriers is subject to federal regulations; McVicar and Suen [58] have recently reviewed these regulations. If laboratory staff are uncertain as to whether shipping a given agent is regulated or as to what constitutes appropriate packaging and labeling, they should consult with the appropriate agency before packaging the agent.

Storage and processing. Once received in the laboratory, specimens should continue to be handled with use of standard precautions. Strict adherence to such precautions decreases the likelihood of exposure to blood-borne pathogens as well as pathogens being sought in specimens and those that are clinically not suspected. Although most cultures can be plated safely on a standard laboratory bench, many microbiologists prefer setting up cultures in a biological safety cabinet. This is mandatory for specimens that may contain *M. tuberculosis.* Once pathogens are propagated in the laboratory, there is further risk of developing laboratory-acquired infection [56]; etiologic agents of particular risk include *M. tuberculosis, Brucella, Francisella tularensis, Yersinia pestis, Histoplasma capsulatum,* and *Coccidioides immitis* [56, 59]. Cultures containing (or suspected of containing) one of these agents should be processed only in Class II biological safety cabinets under Biosafety Level 3 conditions [59].

Summary

Obtaining accurate and cost-effective microbiological test results is possible only when specimens are collected, transported, and stored properly. When proper procedures are followed, cultures of specimens are less likely to be contaminated and more likely to yield pathogens. Not only does this make interpretation of test results easier, but it also reduces unnecessary work and, as documented for some specimens, reduces health care costs. Proper collection includes submitting the appropriate number of specimens. It is increasingly evident that for most specimens, submission of more than the recommended number of specimens does not improve the physician's ability to interpret test results.

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Anticoagulants :

Anticoagulants, commonly known as blood thinners, are chemical substances that prevent or reduce coagulation of blood, prolonging the clotting time.

Anticoagulants are closely related to antiplatelet drugs and thrombolytic drugs by manipulating the various pathways of blood coagulation. Specifically, antiplatelet drugs inhibit platelet aggregation (clumping together), whereas anticoagulants inhibit specific pathways of the coagulation cascade, which happens after the initial platelet aggregation but before the formation of fibrin and stable aggregated platelet products.

Common anticoagulants include **warfarin** and **heparin**.

Medical uses :

The use of anticoagulants is a decision based upon the risks and benefits of anticoagulation. The biggest risk of anticoagulation therapy is the increased risk of bleeding. In otherwise healthy people, the increased risk of bleeding is minimal, but those who have had recent surgery, cerebral aneurysms, and other conditions may have too great of risk of bleeding.

Some indications for anticoagulant therapy that are known to have benefit from therapy include:

- Atrial fibrillation — commonly forms an atrial appendage clot[19]
- Coronary artery disease.
- Deep vein thrombosis — can lead to pulmonary embolism[21]
- Ischemic stroke.
- Hypercoagulable states (e.g., Factor V Leiden) — can lead to deep vein thrombosis.
- Mechanical heart valves.
- Myocardial infarction.
- Mechanical heart valves
- Myocardial infarction
- Pulmonary embolism
- Restenosis from stents
- Cardiopulmonary bypass (or any other surgeries requiring temporary aortic occlusion
- Heart failure
  In these cases, anticoagulation therapy can prevent formation of dangerous clots or prevent growth of clots.

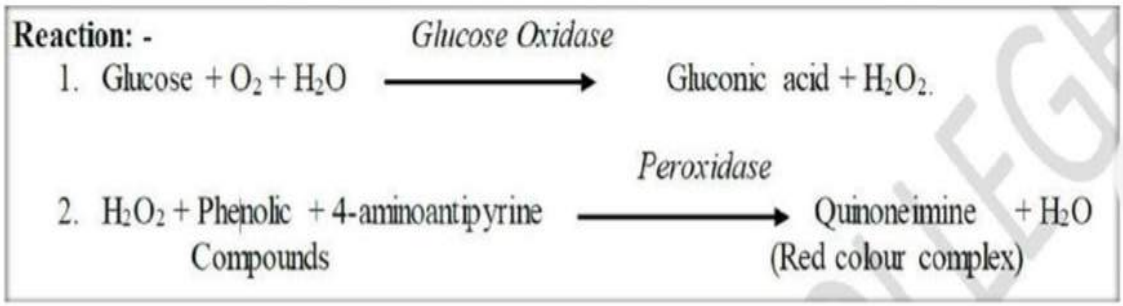
Estimation of blood sugar (GOD-POD METHOD)

Principle:

Glucose oxidase (GOD) oxidizes the specific substrate β-D- glucose to gluconic acid and hydrogen peroxide (H2O2) is liberated. Peroxidase (POD) enzyme acts on hydrogen peroxide to liberate nascent oxygen (O2), then nascent oxygen couples with 4- amino antipyrine and phenol to form red quinoneimine dye.

The intensity of the colour is directly proportional to the concentration of glucose present in plasma. The intensity of colour is measured by colorimeter at 530 nm or green filter and compared with that of a standard treated similarly. Final colour is stable for at least 2 hours if not exposed to direct sunlight.

Reaction:



Reagents:

1. Glucose colour reagent; it contains GOD, POD, 4- amino antipyrine, phenol & phosphate buffer (pH 7.5)

2. Glucose standard solution, Concentration = 100 mg/dl. 100 mg of anhydrous glucose is dissolved in 100 ml of distilled water.

Procedure:

Pipette into clean, dry test tube labelled as Blank (B), Standard (S) and Test (T).

Then add the solution in each of test tubes separately as shown in table below

	BLANK	STANDARD	TEST
Glucose colour Reagent	**1000 µl**	**1000 µl**	**1000 µl**
Distilled Water	**10 µl**	-----	-----
Standard	-----	**10 µl**	-----
Plasma	-----		**10 µl**
Mix thoroughly and keep the tubes at 37°C for 15 minutes.			
OD at 530 nm	0.02	0.45	0.58

Data

Plasma Glucose standard concentration is 100 gm/dl

CALCULATION

Concentration of Glucose =

$$\frac{O.D.of\ Test - O.D.of\ Blank}{O.D.\ of\ Standard - O.D.of\ Blank} \times Concentration\ of\ Standard$$

Result

Plasma glucose concentration in given unknown blood sample = mg/dl.

Normal Range:

	Fasting	After eating	2-3 hrs after eating
Normal	80 - 100	170 - 200	120 - 140
Pre Diabetic	101 - 125	190 - 230	140 - 160
Diabetic	126+	220 - 300	200+

Interpretation:

• Hyperglycemia:

• It is found in following conditions

• I. Physiological:

• 1. Alimentary : After high carbohydrate diet.

• 2. Emotional: Stress, anger, anxiety etc.

• II. Pathological:

• 1. Diabetes mellitus

• 2. Hyperadrenalism

• 3. Hyperpituitarism

• Hypoglycemia:

• It is found in following conditions:

• I. Physiological:

– During starvation

– After Severe Exercise

• II. Pathological:

– Prolonged fasting

– Due to excess of insulin e.g.

• Excessive dose of insulin

• No food intake after insulin administration

• Tumours of pancreas (insulinoma)

– Glycogen storage disease

– Hypoactivity of adrenal and pituitary gland

Glucose tolerance test

GTT short for Glucose Tolerance Test is a test designed to assess the body response to glucose. In GTT, the patient is given a glucose solution and blood samples are drawn afterword at intervals to measure how well the body cells are able to absorb glucose. There are several variations to the glucose tolerance test used in different conditions but, the most common one of them is the Oral glucose tolerance test or OGTT.

The OGTT is mainly used in the diagnosis of gestational diabetes. For OGTT the patient is required to fast for 8 hours and then a fasting plasma glucose is tested, after that oral glucose solution is given. After that blood samples can be drawn up to 4 times at different intervals to measure the blood glucose. A OGTT is usually performed in the morning as glucose levels usually fall by afternoon.

OGTT used to be the gold standard in the diagnosis of diabetes type 2 but, is now being replaced with other GTT methodology. The GTT is primarily used for the diagnosis of diabetes, insulin resistance, impaired beta cell function, carbohydrate metabolism disorder and also reactive hypoglycaemia and acromegaly.

The GTT is usually given to pregnant women during 24th and 28th week of pregnancy. This test is also given to pregnant who has diabetes symptoms or have the risk of developing diabetes prior to the pregnancy.

Besides that the GTT is also given to other patients who are experiencing symptoms of varied diseases that can cause high glucose levels in the blood stream or restrict the proper absorption of glucose by the body cells.

Principle:

A glucose tolerance test is the administration of glucose in a controlled and defined environment to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes, insulin resistance, and sometimes reactive hypoglycemia. The glucose is most often given orally.

Preparation for GTT

TT is an elaborate blood test, that requires frequent testing and as the special requirements need for GTT are as follows;

- Have a normal diet like any other day.
- Inform the doctor about the varied prescription drugs you are taking, as certain drugs like corticosteroids, diuretics and anti- depressants can cause false results.
- Fasting is required for 8 to 10 hours prior to the test and only water is allowed during this period.
- You might want to avoid using the washroom prior to testing as urine samples might be needed.
- On the morning of the test do not smoke or have coffee or caffeine based product.
- The GTT is not to be done on a sick person.

GTT procedure

The GTT procedure is as follows;

- At first a zero -time or baseline blood sample is drawn.
- Then the patient is given a specific dose of glucose solution to drink.
- After that the blood samples are drawn at regular intervals to measure the blood sugar levels and also insulin levels in certain cases. The blood sampling can be done as requested by the doctor and could involve up to 6 hours of testing.

GTT normal values:

- The GTT normal value is lower than 140 mg/dL and if the blood glucose level is between 140 and 199 mg/dL then it is a strong indication of prediabetes.

- The OGTT normal range for fasting results is between 100 – 125 mg/dL for prediabetes, 126 mg/dL or greater for diabetes and greater than 92 mg/dL for gestational diabetes.

- The OGTT normal range for after 2 hour test results is between 140 – 199 mg/dL for prediabetes, 200 mg/dL or greater for diabetes and greater than 153 mg/dL for gestational diabetes.

GTT result interpretation

For Gestational diabetes no further test is required and proper medication and treatment can start. In case of diabetes, further testing is advised to confirm the diagnosis. In case of prediabetes doctors start the treatment with medication and dietary changes along with lifestyle changes.

Analyte: Glycohemoglobin

Matrix: Whole Blood

Method Tosoh A1c 2.2 Plus

Glycohemoglobin Analyzer

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Hemoglobin subfractions formed by the glycation of the alpha or beta chains of hemoglobin A1 (HbA) are collectively known as glycosylated or glycated hemoglobins. Hemoglobin A1c, the best-defined of these, is formed by the reversible condensation of the carbonyl group of glucose and the amino group at the N-terminus of the beta chain of hemoglobin A, resulting in a labile aldimine or Schiff base. As the red cell circulates, some of the aldimine undergoes a slow, irreversible conversion (Amadori rearrangement) to a stable ketoamine form (HbA1c). As blood glucose levels rise, the increase in glycated hemoglobin is proportional to both the level of glucose and the lifespan of the red cell. Hemoglobin A1c measurements are used in the clinical management of diabetes to assess the long-term efficacy of diabetic control. The glycated hemoglobin result is a reflection of the mean daily blood glucose concentration and the degree of carbohydrate imbalance over the preceding two to three months.

In the past, accurate measurement of stable HbA1c was possible only after removing labile HbA1c by pretreatment. In this assay, the stable (SA1c) and labile (LA1c) forms can be individually resolved on the chromatogram without manual pretreatment, allowing accurate measurement of the stable form of HbA1c. The analyzer dilutes the whole blood specimen with Hemolysis & Wash Solution, and then injects a small volume of the treated specimen onto the HPLC analytical column. Separation is achieved by utilizing differences in ionic interactions between the cation exchange group on the column resin surface and the hemoglobin components. The hemoglobin fractions (A1c, A1b, F, LA1c, SA1c, A0 and H-Var) are subsequently removed from the column material by step-wise elution using Elution Buffers 1, 2 and 3, each with a differing salt concentration. The separated hemoglobin components pass through the photometer flow cell where the analyzer measures changes in absorbance at 415 nm. The analyzer integrates and reduces the raw data, and then calculates the relative percentages of each hemoglobin fraction. Analysis requires three minutes. Elution using Elution Buffers 1, 2 and 3, each with a differing salt concentration. The separate hemoglobin components pass through the photometer flow cell where the
analyzer measures changes in absorbance at 415 nm. The analyzer integrates and reduces the raw data, and then calculates the relative percentages of each hemoglobin fraction. Analysis requires three minutes.

2. SAFETY PRECAUTIONS

Follow all procedures and policies in the Fairview-University Medical Center Laboratory Safety Manual. Consider all specimens as potentially infectious.

Sodium azide can react with copper and lead plumbing to form explosive metal azides. On disposal, flush reagents with a large volume of water to prevent the buildup of azides.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

NHANES SA1c% results are entered unto a spreadsheet provided electronically by WESTAT, Inc for NHANES.

To access the spreadsheet click on My Computer → Z drive → User → Dep Labs → Collab Studies → NHANES → Glyhb 004.

Choose the file named with the corresponding box number.

Enter the analysis date, run number, technologist's initials, SA1c%, and result comment code.

The spreadsheet will be sent electronically by the contact person.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Samples are collected and processed in mobile examination centers according to NHANES protocols.
Specimens are packaged and shipped on cold packs or dry ice according to the established schedule.

Specimens are shipped via Federal Express for delivery directly to Collaborative Studies Clinical Laboratory.

Shipments for NHANES will arrive on Tuesdays and/or Wednesdays. The shipments will consist of two boxes, one with frozen gel packs containing HbA1c specimens and one with dry ice containing frozen glucose and insulin specimens. These shipments will be recorded on the shipping log located in a blue 3 ring binder labeled NHANES Shipping Log in the receiving area.

Included in the shipping box for HbA1c (glycohem) specimens are a shipping manifest, a Federal Express airbill for return shipment, frozen gel packs, and a box or boxes of HbA1c(glycohem) specimens (vessel/vial number 004). Record the appropriate information on the shipping log. Check the specimen numbers in the box against the manifest. Write the received date on top of the box. Bring the specimens to the HbA1c desk. File the manifest in the blue 3 ring binder labeled NHANES Shipping Manifests located in the receiving area. Remove all labels from the shipping box and attach the provided airbill for return shipment. Weigh the boxes on the scale in L237 to complete the information on the airbill. Bring the boxes to the Fairview dock.

A venous whole blood specimen collected in EDTA is required. Tubes containing heparin, potassium oxalate or sodium flouride are acceptable. Whole blood specimens are stable up to fourteen days stored at 2-8 degree C or up to eight hours at room temperature before analysis. Prior to analysis, mix each patient specimen by gentle inversion to ensure homogeneity.

Fingerstick capillary specimens collected using the Bio-Rad Sample Preparation Kit are an acceptable alternative to venous whole blood collection and provide enhanced stability during sample storage and transportation. Samples prepared as directed are stable for 2 weeks stored at room temperature or four weeks stored at 2-8 degree C.

Optimum sample volume: 1 mL whole blood

Minimum sample volume: 50 uL whole blood (for specimens of volume less than 1 mL whole blood, a manual pre-dilution (1:250) must be prepared)

5. **Procedures for Microscopic Examinations**

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLs

A. Instrumentation

(1) A1c 2.2 Plus Glycohemoglobin Analyzer. Part # 018379, with 90 sample

loader, Par # 018442. Tosoh Medics, Inc., 347 Oyster Pt. Blvd., Suite

201, So. San Francisco, Ca 94080.

2) Labquake Rotator. Catalog no. 415-110, Labindustries, Inc., 620 Hearst

Avenue, Berkeley, CA 94710-1992.

3) Auto Dilutor, model AD-7, Catalog No. 196-7393, Bio-Rad clinical

Division, 4000 Alfred Noble Drive, Hercules, CA 94547.

B. Materials

(1) TSKgel Glyco HSi Variant Column. Part # 018577, Tosoh Medics, Inc.

Guaranteed for 2500 counts; replace as necessary (as indicated by

appearance of chromatograms). Stable indefinitely when stored at 4-15o

C away from direct sunlight. Use only with column-matched buffers (first letter

of buffer lot must match last letter of column lot). When a new column is installed, analyze 5 duplicates after calibrating and analyzing controls. Also record the previous results on the protocol page. The results must agree within established duplicate range.

(2) Filter element, 5/pkg. Part # 018550, Tosoh Medics, Inc. Replace at or before 400 injections (do not exceed 400 injections) or when pressure rises above 150 kg/cm2 (15 Mpa).

(3) Thermal paper for A1c 2.2 Plus, 10 roll/box. Part # 018417. Tosoh Medics, Inc.

(4) Sustaining tube, Dilution Sample, 10 mL, 50/pkg, Part # 017093. Tosoh Medics, Inc.

(5) Adapter Ring, Dilution Sample, 5/pkg, Part # 017094. Tosoh Medics, Inc.

(6) Adapter Ring, Sample Rack: 12 mm, Part # 018496; 13 mm, Part # 018433; and 14 mm, Part # 018497.

(7) Sample vials, 1.8 mL (no caps), 1000/pkg. Part # 007419. Tosoh Medics, Inc. (For preparing dilutions of whole blood samples).

(8) Microcentrifuge tubes, 0.6 mL polypropylene (with caps), 500/bag, Stock # CX15559. University Stores. (For preparing aliquots of controls for frozen storage.)

(9) DIAMAT HbA1c Sample Preparation Kit, Cat. No. 196-1026, Bio-Rad Laboratories, Clinical Division, 4000 Alfred Nobel Drive, Hercules, CA 94547. Samples prepared as directed in the Instruction Manual are stable for 2 weeks at room temperature or 4 weeks at 2-8 degree

C. Includes supplies sufficient for 100 test samples:

(10) Sample Preparation Vials, 100/kit, each contain 1 mL of an aqueous solution

of EDTA and potassium cyanide (0.25 mmol/L). Store at 5-30o

C.

(11) Capillaries, one glass dispenser vial containing 100 sodium-heparinized

capillary tubes (5 uL). Reorder box of 20 vials (50 capillary tubes/vial), Cat.

No. 195-1053, Bio-Rad Laboratories, Clinical Division.

(12) Capillary tube holder, one holder for manipulating 5 uL capillary tubes.

Reorder box of 20 holders, Cat. No. 196-1054. Bio-Rad Laboratories,

Clinical Division.

(13) Labels, 4 sheets of 25 blank labels each.

(14) Instruction Manual.

C. Reagent Preparation

1) Elution Buffer HSi Variant No. 1, (S) Part # 018572 (1 x 800 mL). Tosoh Medics, Inc. Succinic acid buffer, contains less than 0.06% sodium azide as a preservative. Unopened buffer is stable until expiration date printed on label. Once open, (S) buffer is stable for three months. Store at 4-25oC. Use only with other column-matched buffers (first letter of buffer lot matches last letter of column lot). When a new lot number of buffer is installed, analyze 5 duplicate samples at the beginning of the run. Record the previous results on the protocol page also. The results must agree within the established duplicate range.

(2) Elution Buffer HSi Variant No. 2, (S) Part # 018573 (1 x 800 mL). Tosoh Medics, Inc. Succinic acid buffer, contains less than 0.06% sodium azide as a preservative. Unopened buffer is stable until expiration date printed on label. Once open, (S) buffer is stable for three months. Store at 4-25 degreeC.

(3) Use only with other column-matched buffers (first letter of buffer lot matches last letter of column lot). When a new lot number of buffer isinstalled, analyze 5 duplicate samples at the beginning of the run. Record the previous results on the protocol page also. The results must agree within the established duplicate range.

4) Hemoolysis & Wash Solution, (S) Part # 018430 (1 x 800 mL. Tosoh Medics, Inc. Contains deionized water, EDTA and Triton X and contains less than 0.12% sodium azide as a preservative. Once open, (S) buffer is stable for three months. Store at 4-25 degree C.D.
Standards Preparation

HbA1c Calibrator Set: Calibrator 1 (5 x 4 mL) and Calibrator 2 (5 x 4 mL). Part # 018767, Tosoh Medics, Inc. Buffered human red blood cells, 2 mg/mL human hemoglobin, and 0.5 mM EDTA as preservative. Un-reconstituted calibrator set is stable stored at 4-8 degree C until expiration date printed on label.

Reconstitute Calibrators 1 and 2 by adding 4 mL Milli-Q water to each vial then

mix gently by inversion. Record dates of reconstitution and expiration on vial

labels, then promptly store upright at 4-8 degree C. Always return calibrators promptly to refrigerator--do not leave vials at room temperature for an extended period.

Calibrator Lot Validation: Each new lot of calibrators must be evaluated against the current lot prior to putting into use. (Evaluation against whole blood calibrators may be performed as needed – see Note 1). Analyze each level induplicate within the same run over a period of two to three days (include both instruments) to verify that manufacturer-assigned values are valid. First, calibrate the run using the current lot of calibrators and analyze the controls.

Analyze the new lot calibrators as unknowns immediately after the controls, running each level in duplicate. Record the values obtained including analyses from both instruments. When the tally is complete, calculate a mean to confirm the assigned bottle value or to determine new assigned values. Prior to analyzing patient specimens, verify that analysis of current lot of controls against the new lot calibrators produces results that fall within established control ranges. New lots of calibrator may be evaluated as necessary against whole blood calibrators obtained from the NGSP CPRL at the University of Missouri (UMO calibrators). Perform this procedure when validation of new lot calibrators against current lot calibration does not confirm manufacturer-assigned values.

(1) Obtain aliquots of UMO whole blood calibrators and UMO controls. First calibrate both instruments with UMO calibrators using their assigned values. Analyze the UMO controls, the current lot of in-house controls and both levels of new lot calibrator in duplicate as unknowns. Verify that the controls fall within their respective QC limits (preferably within 1 SD). Evaluate the results of the new lot calibrators against their manufacturer-assigned values.

(2) Next calibrate both instruments with the new lot of Tosoh calibrators using the manufacturer-assigned values. Analyze the UMO controls and in-house

controls and verify that they fall within their respective QC limits (preferably within 1 SD). If control results are acceptable, the assigned values may be used. If controls do not fall within established ranges, repeat analysis of new lot calibrators against UMO calibrators to establish new assigned values.

(3) Additionally, the most recent set of NGSP Monthly Monitoring samples may be thawed and analyzed on both instruments and the results compared with results obtained using the current lot calibrators.

E. Preparation of Quality Control Material

Two levels of glycated hemoglobin control (Normal and Elevated) are analyzed in duplicate (or more) with each batch. See QC charts for controls currently in use and established ranges. Controls are prepared from whole blood drawn from a normal (Normal) and a diabetic (Elevated) individual. Stable indefinitely Collect six 10-mL potassium-EDTA tubes from one normal or one diabetic stored at –70 degree C.

volunteer depending on the control level to be prepared. Mix well by gentle inversion then pour blood into a 100-mL beaker containing a small magnet and place the beaker into a bucket containing wet ice. Place bucket on a magnetic stirrer set on low speed. Aliquot ~ 100 uL into 0.6 mL polypropylene microcentrifuge tubes with caps. Continue to add ice to the bucket as needed to keep beaker chilled. During preparation, aliquots may be held in an insulated bucket filled with ice until placed into boxes to be stored at –70 degree C (chest freezer).

At the start of each week, take one week's supply of controls from the stock supply and place in the working -70 degree C freezer.

Evaluate the new lot of controls according to QC/QA guidelines to establish temporary ranges prior to placing into clinical use.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Perform weekly calibration of the instrument on Mondays prior to analysis of controls and patient samples. Calibration must also be performed after repeated control failure, major maintenance or service has been performed or whenever a new column is installed.

The analyzer utilizes a two-point automatic calibration function. Select SET from the Status screen. Press DAILY CALIB so that it appears reverse-highlighted. The STATUS screen will display 'CALIB. : YES '. Check the parameter printout posted inside the analyzer door to be sure the current calibrator values are entered.

If necessary to change the current calibration values, press SET on the Status screen, then CALIB-1. An asterisk (*) will appear next to the parameter to be changed. Input the assigned value for Calibrator 1. Your entry will appear along the bottom of the screen; press the 'left-hand arrow' key on the screen to enter this value. Then press CALIB-2 and enter the assigned value for Calibrator 2 in the same manner. Press the 'up' arrow to return to the Status screen.Pipette 950 uL (minimum volume 800 uL) of each calibrator into each of two sample vials. Place Calibrator 1 in position 1 (on the left) and Calibrator 2 next to it in position 2 in the rack using adapter rings and sustaining tubes. Place dilutions made from the current lot of controls in positions 3 and 4. Place the rack into the left compartment of the sample loader. Then place a second (empty) rack behind this rack. (The analyzer senses the end of a run after it detects 10 sequential empty positions.) Press the START key to begin the calibration. The analyzer measures Calibrator 1 three times and Calibrator 2 two times. The analyzer discards the first measurement and uses the remaining four measurements to calculate factors A (slope) and B (intercept). Record the new calibration parameters on the protocol page. Allow the instrument to analyze the controls (see step 7.. 'Prepare samples for analysis') and evaluate them before placing more specimen racks in the sample loader. If controls exceed acceptable limits, re-calibrate.

How the Analyzer Calculates Calibration Factors

$$\text{(slope)} = \frac{(\text{Cal2A} - \text{Cal1A})}{(\text{Cal2M} - \text{Cal1M})} \qquad \text{B(intercept)} = \text{Cal2A} - (\text{Cal2M} \times \text{A})$$

where Cal1A and Cal1M are assigned and measured values for Calibrator 1, and

Cal2A and Cal2M are assigned and measured values for Calibrator 2.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Procedure

(1) Turn on the autodilutor. On Mondays, prime the system with Hemolysis & Wash Solution by placing the inlet tubing into the reagent reservoir and secure the cap. (The autodilutor is stored in deionized water over the weekend.) Remove the spacers under each syringe. Set the mode switch to CONT and tap the remote switch on the probe to initiate continuous reagent dispensation. Let

syringes cycle 4-5 times to purge tubing and syringes of air and to prime the system with reagent. When the syringes are on the upstroke, set mode switch back to MAN mode (manual dispense). Install the 5% spacer for the 100 µL sample syringe and the 50% spacer for the 2.5 mL reagent syringe. For specimens with a low hematocrit, dilute the specimen using the 10% sample syringe spacer**.**

(2) Daily Setup procedure:

Consult both the Daily Maintenance and the As Needed Maintenance Logs prior to starting analysis each day (see Maintenance instructions at end of procedure). Check off each required task as it is performed and initial the log.

Both analyzers are currently programmed to warm-up automatically at 7:00 AM Monday through Friday. However, if necessary to manually initiate warm-up, press the POWER key located on the operation panel (located at lower left under the screen) to switch on the analyzer. This will initiate an 18-minute warm-up sequence. The analyzer Status screen displays the analyzer's current operating mode (WARMING-UP). Check for leaks and record pump pressure during the 18-minute WARMING-UP sequence. To check flow rate at a time other that during the WARMING-UP sequence, first verify that the analyzer is in STAND-BY mode. Press MENU then MAINTE on the Status screen. The valves operate as toggle switches.

Press the SV-1, Sv-2 and or SV-3 valve keys appropriately until valve/degasser status line appears as follows: 1/2/3/D OP/CL/CL/OP. Wait a few seconds for flow rate to stabilize, then record. Press the 'up arrow' at lower right of the screen display to return to the Status screen.

Note: The main power switch is located on the lower left side of the analyzer and must remain ON at all times. If the main power has been interrupted or switched off, the application software and default parameters must be re-loaded by inserting the System Disk in the floppy drive with the main power switch on and pressing the analyzer's POWER key on the bottom of the operation panel. Remove the current data disk from the analyzer and insert the analyzer's System disk. The analyzer automatically loads the program and begins the WARMING-UP sequence. After the LED on the drive goes out, remove the System Disk and return to a safe place. Replace the current data disk in the drive. Be sure to re-enter the current calibration parameters (and re-calibrate if necessary).

(3) Prime the system daily by running five whole blood specimens prior to calibration. First, turn off calibration by selecting SET on the Status screen then press DAILY CALIB so that it is no longer reverse-highlighted. Return to the Status screen by pressing the 'up arrow' key. Press the START key (located on the operation panel under the screen) so that once the WARMING-UP

sequence is complete, the analyzer will immediately begin sampling the priming specimens. Once analysis of the priming specimens is complete, use the paper feed button (located behind the screen) to advance the paper to reveal the entire chromatogram.

(4) Generate a parameter printout to bracket the run by pressing MENU on the Status screen, then press UTILITY, then PARAM PRINT. Verify that parameters are set correctly by comparing them with the example posted inside the left door of the analyzer. Pay close attention to the current values posted for CALIB-1 and CALIB-2. Press the 'up arrow' to return to the Status screen. After equilibration, the analyzer will enter the STAND-BY mode unless it has been programmed to advance to analysis of the priming specimens as directed in the previous step. In this instance, after analysis of the priming specimens is complete, the analyzer will enter the 'delayed' WASH mode.

(5) If instrument calibration is not required, run both calibrators as unknowns in positions 1 and 2, followed by dilutions made from the current lot of controls in positions 3 and 4. Place the rack in the left compartment of the sample loader. Place a second (empty) rack behind this rack. Press the START key. Allow the instrument to analyze the calibrators and controls and evaluate them before more specimen racks in the sample loader. If controls exceed acceptable limits, re-calibrate.

(6) Protocol pages:

A day's analysis load may consist of one or more runs. It acceptable to analyze samples in one continuous run or in several shorter runs. The 3-character Batch ID ('NO.') appears in upper left corner of chromatographic printout. A new batch is initiated after the daily WARMING-UP sequence is completed or any time the POWER button on the Operation panel is turned off then on again (such as after a power failure).

(a) Controls: Begin with analysis of both controls in the first run. Alternate analysis of control levels in each subsequent run and at least once on each protocol page. Controls must be analyzed in duplicate (at least) each day. Evaluate against established limits.

(b) Patient samples: Record accession numbers and/or CIDs on the protocol page in the order in which the samples are to be analyzed.

(c) Calibrators (as unknowns): Analyze Calibrator 1 and Calibrator 2 as unknowns at least once per day within the batch and again to bracket all samples at the end of the day's batch. Acceptable range for calibrators analyzed as unknowns are + 0.2 of assigned values.

(d) Batch duplicate: Analyze a specimen from the previous day as a duplicate at some point in the current day's run. Result must agree within ± 0.2 of the previous value.

(e) Within batch duplicate: Analyze a specimen from the beginning of the run again at the end of the run. Result must agree within established duplicate range.

NOTE: The analyzer is programmed to permit an 84-minute lag time between entering 'delayed' WASH mode and going into 'active' WASH mode (see Step 9). During this 84-minute period, you may initiate a new batch of analyses by pressing START. However, once the 'active' WASH cycle begins, you must allow it to proceed to completion (approximately 15 minutes). If washing is insufficient, column lifespan will be reduced and the result for the next sample could be affected. After the 'active' WASH cycle is completed, re-run controls prior to analyzing samples.

The 84-minute lag period can be circumvented by pressing the STOP button (located on the operation panel) ONCE before this time period has elapsed. This will place the analyzer in STAND-BY mode. If there is no further input from theoperation panel while the analyzer is in STAND-BY mode, after 3 hours (Off Time setting), the analyzer will shut down automatically.

(7) Prepare samples for analysis:

Whole Blood. Ensure that the stopper on the tube is properly seated and that the barcode label is vertically aligned; re-affix barcode label vertically on tube if necessary. Mix each patient specimen several times by gentle inversion (or place briefly on a rotator). Then place the specimen tube in the rack in order from left to right according to its rack and position number as recorded on the protocol page. Align its barcoded label so that it faces the barcode reader (i.e., facing away from you in the rack as it's loaded on the instrument). Note: Blood cells will begin to settle out as the tubes sit on the instrument waiting to be measured. This cell sedimentation over a period of approximately 5 hours does not affect the HbA1c result.

Low-volume samples (< 1.0 mL whole blood in tube). Using the autodilutor, prepare hemolysates by diluting 5 uL (5% sample spacer) of each low-volume patient sample with 1.25 mL Hemolysis & Wash Solution (50% reagent spacer). Wipe the probe tip after drawing up sample and again after dispensing into labeled sample vial. Place the sample vial in the rack using an adapter ring and sustaining tube. (Minimum dilution volume dispensed or pipetted into a sample vial is 300 µL.) WARNING: The sustaining tube must always be used with an adapter ring. First place the adapter ring into the sustaining tube, then put the sample vial into the ring; otherwise a rack position error will occur.

HbA1c Sample Preparation Vials. Remove caps prior to sampling! Place the Prep vial in the rack using an adapter ring and sustaining tube. WARNING: The sustaining tube must always be used with an adapter ring. First place the adapter ring into the sustaining tube, then put the prep vial into the ring, otherwise a rack position error will occur.

B. Quality Control Materials

Controls: Thaw aliquots and vortex briefly. Using the autodilutor, prepare hemolysates by diluting 5 µL (5% sample spacer) of each control with 1.25 mL (50% reagent spacer) Hemolysis & Wash Solution. Wipe the probe tip after drawing up sample and again after dispensing into a labeled vial. Place the sample vials in the rack using adapter rings and sustaining tubes. WARNING: The sustaining tube must always be used with an adapter ring. First place the adapter ring into the sustaining tube, then put the sample vial into the ring; otherwise a rack position error will occur. Adapter rings limit the maximum vertical movement of the sampling needle. Sampling needle may bend or break as it pierces bottom of vial if an adapter ring is not used.

C. Operation

(1) Place racks in ascending order into the left compartment of the sample loader with the first rack nearest you (this rack will advance first to the sampling station). Place an empty rack after the last rack to be processed. Press START key to begin analysis. The racks will be moved automatically along the sample loader. The analyzer will prime the fluid lines with buffer for 4.4 minutes, and then analyze samples at 3-minute intervals.

IMPORTANT: Keep tubes in the sample rack until the whole rack is processed and printed reports are available and have been reviewed.

(2) Processing automatically stops when the analyzer detects an empty rack (or 10 sequential empty spaces). When measurement ends, the analyzer will enter the ‘delayed’ WASH mode. Tosoh parameters have been modified so that the end of run wash is delayed for 84 minutes. This allows the operator to program and analyze samples out of sequence (‘stat’ or urgent samples) or perform additional runs after the current run has ended. If no further runs are performed within 84 minutes from entering ‘delayed’ WASH mode, the analyzer will enter the ‘active’ WASH mode in which it washes the column by pumping buffer for 15 minutes, then enters STAND-BY mode again. Once it has started, always allow the ‘active’ WASH sequence to go to completion!

3) If there is no further input from the operation panel while the analyzer is in STAND-BY mode, after 3 hours (Off Time setting), the analyzer shuts itself off automatically.

D. Special Method Notes

(1) Barcoded samples are scanned automatically by the analyzer and the CID number appears on the chromatographic printout in the ‘SAMPLE ID’ field. If a barcode is unreadable or unavailable, the rack and position numbers of the sample appear in this field instead. In such cases, always record the accession number or Lab ID on the chromatogram. Record %SA1C value from the tape onto the protocol page. Be sure to note any abnormal peak(s) (abnormal variants or POO peaks) on the protocol page.

(2) Dilution studies demonstrate that the assay is linear from a Total Area of 800 to 8000. In general, review and question any chromatogram with the following characteristics:

(a) The SA1C value is below 3.4%. Repeat the sample to confirm. Consult a supervisor before reporting (< 3.4 %).

(b) Total area reported is less than 800 or greater than 8000. Repeat dilution using appropriate reagent or sample syringe spacer to obtain area results within this range.

The SA1C peak is not detected. Repeat the sample to confirm. Do not report results. Consult a supervisor.

An unidentifiable peak (P00, P01 ...) peak appears before the A1A or between the A1A and the A0 peaks. Do not report results. Check for clots in the sample; re-analyze. Consult a supervisor before reporting results.

(3) SA1C - Report % HbA1c (SA1C) to one decimal place.

F – Observe elevated HbF peak between A1B and LA1c+ peaks. Levels of fetal hemoglobin (HbF) up to 22% do not affect test results because HbF is completely resolved by the analyzer. For adult patients with HbF > 22.0%, send to the V.A. Report the V.A. result with the comment C7671.

HHbS (heterzygous) – HbS appears as an H-VAR peak following the A0 peak and there is no carryover observed in the chromatograms that follow. HbS (heterozygous) does not interfere with quantitation of HbA1c. Report the HbA1c result with the following coded comment: C7672 (Abnormal hemoglobin variant observed).

HbC – HbC also appears as an H-VAR peak that follows the A0 peak however carryover is observed in one or more chromatograms that follow. Carefully examine the next 8-10 chromatograms! Observation of residual variant hemoglobin may be delayed for as many as 4-5 samples.

IMPORTANT: You must repeat all specimens that follow this type of H-VAR chromatogram up to and including the samples that exhibit the carryover. This may require repeating as many as 8-10 sequential specimens. Consult a supervisor. The SA1C result is reportable in the presence of HbC (look for the classic chromatographic pattern).

- Other hemoglobin variants – may appear as a POO peak or H-VAR. Consult a supervisor. Send the POO/H-VAR sample to the Minneapolis VAMC for analysis (see below).

8. REPORTABLE RANGE OF RESULTS

REPORTABLE RANGE: 3.4 – 18.8 % (manufacturer's insert)
Report results falling outside this range as <3.4 or >18.8 %.

10. QUALITY CONTROL (QC) PROCEDURES

Two levels of control are assayed each time the glycohemoglobin method is performed. Westgard rules are followed as outlined in the general laboratory Quality Control and Quality Assurance procedure. Controls are analyzed at the beginning of a run, periodically throughout, and at the end of a run.

Quality control evaluation:

Calibrators (as unknowns): Acceptable range for calibrators analyzed as unknowns are +0.2 of assigned values.
Controls: Values must fall within established ranges for each level.
Batch duplicates: Results must agree within + 0.2 of the previous batch's value.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If control values are out of the acceptable range, recalibration is required. Reanalyze any patient samples after recalibration.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Icterus (as indicated by free and conjugated bilirubin concentrations up to 200 mg/dL), lipemia (as indicated by triglyceride concentrations up to 3600 mg/dL), and hemoglobin (concentrations up to 4500 mg/dL) do not interfere with the assay.

13. REFERENCE RANGES (NORMAL VALUES)

REFERENCE RANGE: 4.3 – 6.0 % (DCCT/EDIC normal range)

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Early Reporting Results for NHANES:
Notify the NHANES Medical Officer of any SA1c% results greater than 6.9%. The contact person will report these results as soon as possible.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Any specimens not analyzed on the day of arrival in the laboratory are stored in the refrigerator (4°C - 8°C). Upon completion of analysis, specimens are stored for 1 week. NHANES specimens are frozen at -70°C and discarded after 1 year.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

The laboratory has 2 instruments for performing glycohemoglobins. If neither instrument is available for use, the specimens are stored at 4°C until testing can be performed.

17. TEST RESULT REPORTING SYSTEM; PROTOCOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

NHANES SA1c% results are entered unto a spreadsheet provided electronically by WESTAT, Inc for NHANES**.**

To access the spreadsheet click on My Computer → Z drive → User → Dep Labs →Collab Studies → NHANES → Glyhb 004**.**

Choose the file named with the corresponding box number.

Enter the analysis date, run number, technologist's initials, SA1c%, and result comment code.

The spreadsheet will be sent electronically by the contact person.

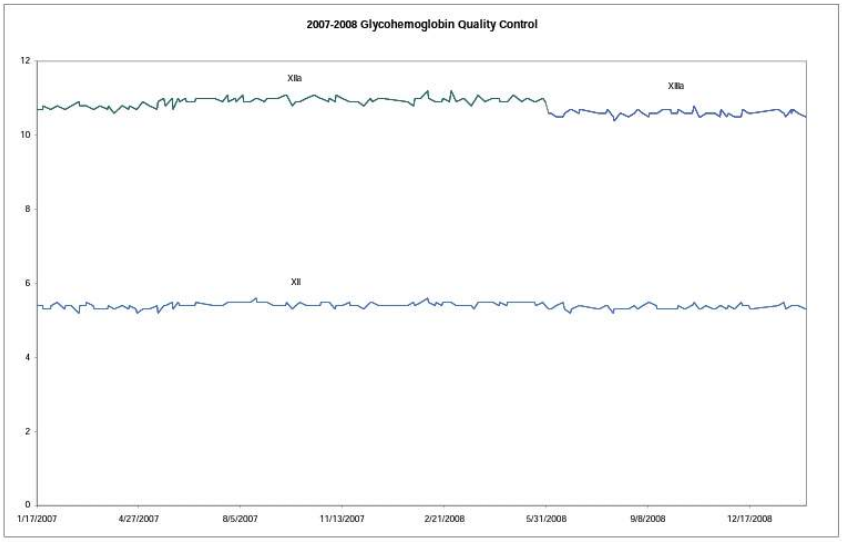
Early Reporting Results for NHANES:

Notify the NHANES contact person of any SA1c% results greater than 6.9%. The contact person will report these results as soon as possible.

18. SUMMARY STATISTICS AND QC GRAPHS

Summary Statistics for Glycohemoglobin by Lot

Lot	N	Start date	End Date	Mean	Standard Deviation	Cov
XII	111	1/17/2007	5/30/2008	5.422	0.081	1.5
XIIa	111	1/17/2007	5/30/200	10.905	0.122	1.1
XIII	56	6/3/2008	2/11/2009	5.357	0.074	1.4
XIIIa	56	6/3/2008	2/11/2009	10.598	0.080	0.8



Lipid determination of serum lipids

CHOLESTEROL KIT

(CHOD / PAP method)

For the determination of Cholesterol in serum or plasma.
(For Invitro 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 hypercholesterolaemia, hyperlipidaemia, hypothyroidism, uncontrolled diabetes, nephrotic syndrome, and cirrhosis. Decreased levels are found in malabsorption, malnutrition, hyperthyroidism, anemias and liver diseases.

Principle

Cholesterol esterase hydrolyses 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 fom a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of cholesterol present in the sample.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{4Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye} + H_2O$$

Nomal 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	75ml	2 x 75ml	2 x 150 ml	2 x 250 ml
L1: Enzyme Reagent 1	60 ml	2X60 ml	2x120 ml	2x200 ml
L2: Enzyme Reagent 2	15ml	2x15ml	2x30ml	2x50ml
S : Cholesterol Standard (200mg/d)	5ml	Sml	Sml	5ml

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 colour 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/R.T.
Light path	1cm

Pipette into clean dry test tubes labelled 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 5min. or at R.T. (25°C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank, within 60 Min.

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 flourides 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.

References

Trinder, P., (1969) Ann. Clin. Biochem. 6:24
Allain, C.C., etal, (1974) Clin. Chem. 20 :470
Flegg, H.M., (1972) Ann. Clin. Biochem. 10: 79

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TRIGLYCERIDES KIT

(GPO / PAP method)

For the determination of Triglycerides in serum or plasma.

(For Invitro 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 coronaiy disease and peripheral vascular disease. Decreased levels are found in malnutrition and hyperthyroidism.

Principle

Lipoprotein lipase hydrolyses triglycerides to glycerol and free faky acids' The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidised by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxidefurther reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directlyproportional totheamountof triglycerides presentinthesample.

$$\text{Triglycerides} \xrightarrow{\textit{LipoproleinLipase}} \text{Glycerol} + \text{Free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\textit{GlycefiolKinase}} \text{Glycerol 3 Phophate} + \text{ADP}$$

$$\text{Glycerol 3 Phophate} + O_2 \xrightarrow{\text{G/ycero/3PO}} \text{Dihydroxyacetonephos.} + H_2O_2$$

$$H_2O_2 + \text{4Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye} + H_2O$$

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	is=i	7smi	2x75ml	2x150ml
L1 : Enzyme Reagent 1	20 ml	60 ml	2 x 60 i lx	120 ml
L2 : Enzyme Reagent 2	5 ml	15 ml	2 x 15 l2ik	30 ml
5 : Triglycerides Standard (200 mg/dl)	5ml	5 ml	5 6fril	

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 colour however this does not affect the performance of thereagent.

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) & 1 part of L2 (Enzyme Reagent 2). Alternatively0.8 ml ofL1 and 0.2 ml ofL2 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 Sdayswhenstoredat 2-8°C.

Procedure

Wavelength/filter	505nm(Hg 546 nm)/Green
Temperature	37 C/R.T.
Light path	1cm

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test(T):

Additiion Sequence	B (ml)	S (ml)	(ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.01		
Triglycerides Standard(S)		0.01	
Sample			001

Mix well and incubate at 37 C for 5 min. or at R.T. (25'C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank, within 60 Min.

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 (NaCl 0.9%) and repeat the assay.

Note

Fasting samples of 12 to 14 hrs. are preferred. Fatty meals and alcohol may cause elevated results. Patient should not drink alcohol for 24 hrs. before the test

Liver function tests

Total protein kit

(Biuret method)

For this determination of total proteins in serum and plasma

(For invitro 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. Consisiting mainly of albumin and globulin the fractions vary

independenty and widely in diseases. Increased levels are found mainly in dehydration. Decreased levels are found mainly in malnutidion, impared synthesis, protein lásses as in hemorthage or excessive protein calabolism.

Principle

Proteins, in an alkaline medium, bind with the cupric lons present in the biuret reagent to form a blue-violet coloured Linearity complex. The intensity of the colour fommed is direcly propordonal to the amount of Proteins present in the This procedure is linear upto 15 g/dl. If values exceed this sample.

Proteins + Cu2+ = Blue Violet Coloured Complex

Normal reference values

Serum & Plasma : 6.0-8.0g/dl

It is recommended that each laboratory establish its own nomal range representing its patient population.

Contents	150 ml	2x150 ml	3x150 ml
Carton 1			
L1: Biuret Reagent	150 ml	2x 150 ml	3x 150m
Carton 2			
S : Protein Standard (8 g/dl)	5ml	5ml	5ml

Storage/ stability

Carton 1: Biuret Reagent is stable at RT. dil the expiry

mentioned on the label.

Carton 2: Protein Standard is stable at 2-8'C il 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/flter : 550 nm(Hg 546nm)Yellow-Green

Temperature : R.T./37 degree C

Light path : 1cm

Pipette into clean dry test tubes labelled as Blanks (B), Slandard (S).and Test (T)

Addition Sequence	B	S	T
Biuret reagent(L1)	1.0	1.0	1.0
Distilled water	0.02	-	-
Protein Standard (S)	-	0.02	0.02
Sample			

Mix well and incubate at 37C for 10 min. or at R.T. for 30 min. Measure the absorbance of the Standard (Abs.S), and Test Sample(Abs.) against the Blank, within 60 Min.

Calculations

Total Proteins in g/dl = (Abs.T/Abs.S)*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.

Estimation of Bilirubin – total and conjugated

INTRODUCTION

1. AutoZyme Bilirubin is a reagent set for determination of total and direct Bilirubin based on Jendrassik and Grof method, , using Diazolized Sulphanilic Acid with DMSO as an activator for total bilirubin.

2. AutoZyme Bilirubin is a two reagent system using one step procedure.

3. AutoZyme Bilirubin is linear upto 20 mg%.

4. AutoZyme Biliubin can be used on any Coloimeter. Spectrophotometer, Discrete semi-automated and Automated analyzer. Progamme can be designed for any speclc analyzer upon request.

5. AutoZyme Bilinibn has one step reconstitution.

6 AutoZyme Biinbin can be determned in just 5 minutes.

7. AutoZyme Bilrubin method Is ielatively free of interference from

haemoglobin and other commonly occuring substances in serum or

plasma.

8. This insert covers technical matter for the complete range of AutoZyme

Bilirubin kits available viz.

Product	**Pack-Size**
Bilirubin T& D (Total and Direct) 200	1 x 200 ml
Bilirubin Total 1000	1x 1000 ml
Bilirubin Direct 1000	1x 1000 ml

PRINCIPLE

Bilirubin reacts with diazotized sulphanilic acid to produce azobilirubin (violet colour). DMSO catalyzes the formation of azobilirubin from free bilirubin. The violet colour is proportional to bilirubin concentration measured at 546 nm. (530-550 nm.).

TOTAL BILIRUBIN

Bilirubin + Sulphanilic Acid + Sod. Nitrite Azobilirubin

PROCEDURE

Reaction type	**End-Point**
Reaction time	**5 mins. At R.T. (25-300C)**
Wavelength	**546 nm. (530 550 nm.)**
Zero setting with	**Serum Blank**
Sample	**50 mico liter**
Reagent	**1.02 ml**
Factor	**20.2**
Linearity	**20 mg%**

Manual assay procedure

Perform the assay as given below:

TOTAL BILIRUBIN

NOTE - The reagents T1, T2 and sample requires proper mixing.

	Serum Blank	Test
Reagent T1	**1.0 ml (500µL)**	**1.0 ml**
Reagent T2	**-**	**0.02 ml**
Serum/ Plasma	**0.05 ml (0.25ml)**	**0.05 ml**

DIRECT BILIRUBIN

	Serum Blank	**Test**
Reagent D1	**1.0 ml**	**1.0 ml**
Reagent D2	**-**	**0.02 ml**
Serum/ Plasma	**0.05 ml**	**0.05 ml**

Mix, and incubate the assay mixture at Room Temperature (25-30°C) for 5 minutes. Read before 8 minutes the absorbance the test against their respective blanks at 546 nm (530-550 nm)

Calculation:

Bilirubin mg% = (Abs. of Test -Abs. of Blank) x 20.2 (Total or Direct)

Estimation of NPN Substances

UREA KIT

(Mod. Berthelot method)

For the deteminalion of urea in seum, plasna and urie (or Invitro Diagnostic Use Only)

Summary

Urea is the end product of protein metabolism. It is synithesised 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 excreled. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart tailure and burns. Decreased levels are found in liver failure and pregnancy.

Principle

Urease hydrolyses urea to ammonia and CO2. The ammonia further reacts with a phenolic chromogen and hy Hypochlorite to form a green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Urea+ H2O = Ammonia+ CO2

Ammonia + Phenolic chromogen = Green coloured complex + hypochlorite

Normal reference values

Serum/Plasma : 14- 40 mg/dl

Urine: Upto 20g/L

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	75 Assays	3x75Assays	2x150Assays
L1:Buffer Reagent	75 ml	3*75ml	2*150 ml
L2:Enzyme Reagent	7.5 ml	3*7.5ml	30 ml

L3:Chromogen Reagent	15 ml	2*22.5ml	2*30 ml
S :Urea Standard (40 mg/dl)	5 ml	5 ml	5 ml

Storage / stability

Contents are stable at 2-8 degree C till the expiry mentioned on Urea Nitrogen in mg/dl the tabels.

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 ofL1 (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 Reagerit) wih 4 parts of fresh ammonia free distilled/ deionised water.Use 1 ml of workineg chrornogen inslead of 0.2 ml in the assay. The working chromogen reagent is stable for atleast 8 weeks when stored at 2-8 degree C in a tightly stoppered plastic bottle.

Sample material

Serun, plasma, Urine. Dilute urine 1+ 49 with distilled waler before the assay (Results x 50). Urea is reported to be stable in the serurm for 5 days when stored at 2-8 C.

Procedure

Wavelength/filter : 570 nm (Hg 578 nm)/ Yellow

Temperature : 37 degree C/R.T

Light path : 1 cm

Pipette into clean dry test tubes labelled as Blank (B),

Standard (S), and Test (T)

Addition Sequence	B	S	T
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 & incubate for 5 min. at 37"C or 1ú min. at R.T. (25 degree C)

Chromogen Reagent (L3)	B	S	T
	0.2	0.2	0.2

Mix well and incubate for 5 min. at 37 C or 10 min. at R.T

(25C). Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the blank, within 60 Min.

Calculations

Urea in mg/dl = Abs.T/Abs.S * 40

Urea Nitrogen in mg/dl = Urea in mg/dl x 0.467

Linearity

This procedure is linear upto 250 mg/dl. Using the working chromogen reagent (1 mi) the linearity is increased to 4000 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.

Notes

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 at 2-8 degree C immediateley after use.

CREATININE KIT

(Alkaline Picrate method)

For the determination of Creatinine in serum and urine.
(For Invitro 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 coloured complex with the alkaline picrate. Intensity of the colour formed is directly proportional to the amount of creatinine present in the sample.

Creatinine + Alkaline Picrate - Orange Coloured Complex

Reference values

	Serum	Urine, 24hrs. collection
Males	0.6-1 .2mg%	1.1- 3 .0gm
Females	0.5-1 .1mg%	1.0- 1 .8gm

It is recommended that each laboratory establish its own normal range representing its patient population

Contents	15 Tests	35 Tests	70 Tests
L1 : Picric Acid Reagent	60 ml	140 ml	2 x 140ml
L2 : Buffer Reagent	5ml	12 ml	25 ml
S : Creatinine Standard (2 mg/di)	5ml	5ml	10ml

Storage / stability

All reagents are stable at R.T. 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	520nm (Hg 546 nm)/ Green
Temperature	R.T.
Light path	1cm

Deproteinization of specimen:

Pipette into a clean dry test tube

Picric acid reagent (L1)	2.0ml
Sample	0.2ml

Mix well and centrifuge at 2500 - 3000 rpm for 10 min. to obtain a clear supernatant

Colour development :

Pipette into clean dry test tubes labelled 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 R.T. for exactly 20 minutes. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank.

Calculations

$$\text{Creatinine in mg\%} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 2.0$$

$$\text{Urine Creatinine in gm/Lit.} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 1.0$$

Urine Creatinine gm/24Hrs. = Urine Creatinine in gm/L x Vol of urine in 24 Hrs.

Linearity

The procedure is linear upto 8 mg/di 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 min. 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 temperatur.e

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 dilutionfactor.

Note

Plasma should not be collected with Flouride or Heparin salts as contamination by ammoniaorammonium salts lead toerroneousresults.

Reaction		Fixed Time Kinetic
Wavelength		340 nm
Zero Setting		
Incub. Temp.		Distilled Water
Incub. Time		
Delay Time		30 sec.
Read Time No. of read.	:	60 sec.
Interval		2
Sample Vol.		
Reagent Vol.		60 sec.
Standard		
Factor React.		Decreasing
Slope		250 mg/dl
Linearity		mg/dl

Coral

Gitanjali, Dr. Antonio Do Rego Bagh, Bambo
Complex P.O. GOA - 403 202, INDIA

URIC ACID KIT

(Uricase / PAP method)

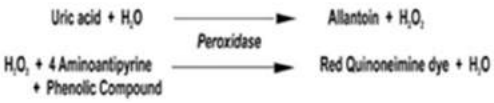
For the determination of Uric Acid in serum or plasma.
(For Invitro Diagnostic Use Only)

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'sdisease, Fanconis 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 coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.



Normal reference values

Serum/Plasma	(Males)	3.4 - 7.0 mg/dl
	(Females)	2.5 - 6.0 mg/dl

It is rec0mmended that each laboratory establish its own normal range representing its patient population.

Contents	25 ml	75 ml	2 x 75 ml	2 x 150 ml
L1 : Buffer Reagent	20 ml	60 ml	2 x 60 ml	2 x 120 ml
L2 : Enzyme Reagent	5 ml	15 ml	2 x 15 mg	x 30 ml
S : Uric Acid Standard (8 mg/dl)	5 ml	5 ml	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 (Buger 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 colour however this does not a8ect 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 (Buffer Reagent) and 1 part of L2 (Enzyme 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. Uric Acid is reported to be stable in the sample for 3-5 days when stored at 2-8°C.

Procedure

Wavelength / filter	520 nm (Hg 546 nm) / Yellow Green
Temperature	37°C / R.T.
Light path	1 cm

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test (T):

Addition Sequence	B (ml)	5 (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 min. or at R.T. (25°C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank, within 30 Min.

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.

Enzymes

SGOT (ASAT) KIT

(Mod. IFCC Method)

((For invitro diagnostic use only)

INTENDED USE

SGOT (ASAT) Kit is used for the determination of SGOT (ASAT) Activity in serum.

SUMMARY

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injuny 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 (ASAT) catalyzes the transfer of amino group between L-Aspartate and alpha-Ketoglutarate to form Oxaloacetate and glutarate. 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 (ASAT) activity in the sample.

$$\text{L-Aspartate + alpha- Ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate + L-Glutamate}$$

$$\text{Oxaloacetate + NADH+H+} \xrightarrow{\text{MDH}} \text{Malate +NAD+}$$

EXPECTED VALUES

Serum (males) upto 37 U/L at 37°C

(Females) upto 31 U/L at 37°C

It is recommended that each laboratory establish its own normal

range representing its patient population.

PRESENTATION

REF	1102200025	102200075	102202150	102201000
Pack Size	25 ml	75 ml	2x 150 ml	1000 ml

L1 Enzyme Reagent	20 ml	60 ml	2x120 ml	4 x 200 ml
L2 Starter Reagent	5 ml	15 ml	2*30 ml	4*50 ml

COMPOSITION

Tris Buffer 80mM; pH 7.8; L Aspartate 200mM; LDH 1000U; NADH 0.18mM; Ketoglutarate 12mM; Non Reactive : stabilizers, Detergents and Preservatives.

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 botle 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 (ASAT) is reported to be stable in

serum for 3 days at 2-8°C.

SAMPLE WASTE AND DISPOSAL

Do not reuse the reagent containers, boitles, caps or plugs due to the risks of contarnination and the potential to compromise reagent performance.

This product requires the handling of hurman specimens. It is recommended that all human sOurced material are considered potentially hazardous and are handled in accordance with the OSHA standard on blood borne pathogens.

Appropriate biosafety practices should be used for materials that contain or are suspected of containing infectious agents.

Handle specimens, solid and liquid waste and test components in accordance with local regulations and NCCLS guidelines M29, or other published biohazard safety guidelines.

MATERIALS REQUIRED BUT NOT PROVIDED

Photometer analyzer with standard thermostatic cuvette holder, micropipette and appropriate laboratory equipment.

PROCEDURE

Wavelength /filter : 340 nm

Temperature : 37'C/30'C/25'C

Light path : 1 cm

Substrate Start Assay

Pipeile into a clean dry test tube labelled 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	0.1ml

Incubate at the assay temperature for 1 min. and add

Starter Reagent (L2)	(T) 25°C/30°C 0.2 ml	(T) 37°C 0.2 ml

Mix well and read the initial absorbance A, after 1min. & repeat the absorbance reading after every 1, 2, & 3 mins. Calculate the mean absorbance change per min. (ΔA /min.).

Sample Start Assay:

Pipette into a clean dry test tube labelled as Test (T) :

Addition Sequence	(T)	(T) 37°C 25°C/30°C
Working Reagent	1.0 ml	1.0 ml

Incubate at the assay temperature for 1 min, and add

Sample	0.2 ml	0.1 ml

Mix well and read the initial absorbance A, after 1 min. & repeat the absorbance reading after every 1, 2, & 3 mins. Calculate the mean absorbance change per min. (ΔA /min.).

CALCULATIONS

Substrate/Sample start

SGOT (ASAT) Activity in U/L 25°C/30°C = ΔA/min. x 952

37°C = ΔA /min. x 1746

SGPT (ALAT) KIT

(Modified IFCC Method)

(For in vitro diagnostic use only)

INTENDED USE

SGPT (ALAT) Kit is used for the determination of SGPT ((ALAT) Activity in serum.

SUMMARY

SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive and other jaundice hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction.

PRINCIPLE

SGPT (ALAT) catalyzes the transfer of amino group between L Alanine and o Ketoglutarate to form Pyruvate and Glutamate. The Pyruvate formed reacts with NADH in the presence of Lactate measured as a decrease in absorbance which is proportional to the SGPT (ALAT) activity in the sample.

$$\text{L-Alanine} + \alpha\text{- Ketoglutarate} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

EXPECTED VALUES

Serum (Males) : upto 40 U/L at 37°C

(Females) : upto 31 U/L at 37°C

It is recommended that each laboratory establish its own normal range representing its patient population.

PRESENTATION

REF	1102210025	1102210075	1102212150	1102211000
Pack Size	25 ml	75 ml	2x 150 ml	1000 ml

L1 Enzyme Reagent 2	20 ml	60 ml	2x120 ml	4x200 ml
L2 Starter Reagent	5 ml	15 ml	2x30 ml	4x50 ml

COMPOSITION

Tris Buffer 100mM; LAlanine 500mM; LDH 2KU; Ketoglutarate 15mM; NADH 0.18mM; Goods Buffer 100mM; Non Reactive Stabilizers, Preservatives.

Storage/Stability

Contents are stable at 2-8°C till the expiry mentioned on the label.

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 (ALAT) is reported to be stable in serum for 3 days at 2-8°C.

SAMPLE WASTE AND DISPOSAL

Do not reuse the reagent containers, bottles, caps or plugs due to the risks of contamination and the potential to comprormise reagent performance.

This product requires the handling of human specimens. It is recommended that all human sourced material are considered potentially hazardous and are handled in accordance with the OSHA standard on blood borne pathogens.

Appropriate biosafety practices should be used for materials that contain or are suspected of containing infectious agents.

Handle specimens, solid and liquid waste and test conponents in accordance with local regulations and NCCLS guidelines M29, or other published biohazard safety guidelines.

MATERIALS REQUIRED BUT NOT PROVIDED

Photometer analyzer with standard thermostatic cuvette holder micropipette and appropriate laboratory equipment.

PROCEDURE

Wavelength/filter : 340nm

Temperature : 37°C/30°C/ 25°C

Light path : 1cm

Substrate Start Assay :

Pipette into a clean dry test tube labelled as Test (T)

Addition Sequence	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 min. and add

Starter Reagent (L2)	0.2 ml	0.2 ml

Mix well and read the initial absorbance A, after 1 min. and repeal the absorbance reading after every 1, 2, & 3 mins. Calculale the mean absorbance change per min. (ΔA /min).

Sample Start Assay:

Pipette into a clean dry test tube labelled 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 min. and add

Sample	0.2 ml	0.1 ml

Mix well and read the initial absorbance A, after 1 min. and repeat the absorbance reading after every 1, 2, & 3 mins. Calculate the mean absorbance change per min. (ΔA /min).

CALCULATIONS

Substrate/Sample start

SGPT (ALAT) Activity in U/L 25 C/30° C =ΔA /min. x 952

SGPT (ALAT) Activity in U/L 37°C = ΔA/min. x 1746

QUALITY CONTROL

The following process is recommended for QC during the assay of SGPT (ALAT). Define and establish acceptable range for your laboratory.

1. Two levels of control (Normal and Abnormal) are to be run on a daily basis.

2. If QC results fall ouiside acceptance criteria, recalibration may be necessary.

3. Review QC results and run acceptance criteria following a change

of reagent lot.

SPECIFIC PERFORMANCE CHARACTERISTICS

Linearity:

The procedure is linear upto 500 U/L at 37°C. If the absorbance change (ΔA/min.) exceeds 0.250, use only the value of the first 2 mins. to calculate the result or dilute the sample 1+ 9 with normal saline (NaCl 0.9%) and repeat the assay (Results x10).

Limit of detection:

The limit of detection for SGPT (ALAT) is 3.5 U/L.

Interferences:

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

N.S. BIO-TEC

ALKALINE PHOSPHATASE

Colorimetric determination of serum alkaline phosphatase

INTENDED USE

NS Biotec ALP reagent is intended for the in vitro quantitative determination of alkaline phosphatase in serum on manual systems.

CLINICAL SIGNIFICANCE

Alkaline phosphatase refers to a group of phosphatases (pH optimum approximately 10) found in almost every tissue in the body. Most alkaline phosphatase in normal adult serum is from the liver or biliary tract. Normal alkaline phosphatase levels are age dependant with young children and adolescents having much higher levels than adults. Adult males tend to have higher levels than females, but pregnant females have increased levels due to placental secretion of alkaline phosphatase.[2] Alkaline phosphatase in serum consists of four structural genotypes: the liver-bone-kidney type, the intestinal type, the placental type and the variant from germ cells. It occurs in osteoblasts, hepatocytes the kidneys, spleen, placenta, prostate, leukocytes and the small intestine. The liver-bone-kidney type is particularly important.[2] Elevation of alkaline phosphatase levels occurs in diseases such as hepatitis, cirrhosis, malignancy, chemical toxicity, and in bone diseases such as metastatic carcinoma, rickets, Paget's disease, and osteomalacia.[3] Moderate increase in serum alkaline phosphatase levels have been observed in Hodgkin's disease, congestive heart failure, ulcerative colitis, regional enteritis, and intra-abdominal bacterial infections. Alkaline phosphatase levels are normally elevated during periods of active bone growth, for example, in young children and adolescents[4].

ASSAY PRINCIPLE

Alkaline phosphatases catalyze the hydrolysis of a wide variety of physiologic and non-physiologic phosphoric acid esters in alkaline medium. The natural substrates for these enzymes have not yet been identified. Thus, a variety of synthetic substrates have been used in assay methods for ALP, the selection of which has been largely a matter of convenience. Kay demonstrated the presence of ALP in blood using β-glycerophosphate as the substrate.[5] This method requires measuring the rate of phosphate liberation against the background level of endogenous phosphate. Phenyl phosphate was used as a substrate by King and Armstrong, in this method, the liberated phenol has been measured in a variety of ways including the use of Folin-Ciocalteau reagent[6], and 4-aminoantipyrine.[1]

The series of reactions involved in the assay system is as follows:

1. Alkaline phosphatase (ALP) hydrolyzes the phenylphosphate to phenol and phosphate in the at pH 10.
2. The phenol librated formed color complex in the presence of 4-aminoantipyrine and potassium ferricyanide.
3. The presence of sodium arsenate in the reagent 3 stops the enzymatic reaction.

phenyl phosphate $\xleftarrow[\text{pH 10}]{\text{ALP}}$ **phenol +phosphate**

The intensity of the color produced is directly proportional to the catalytic ALP activity. It is determined by measuring the increase in absorbance at 510 nm.

EXPECTED VALUES

Serum[1]	
Children	10 – 20 Kind & King U/dl 71 - 142 IU/l
Adults	3 – 13 Kind & King U/dl 21 - 92 IU/l

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference range. For diagnostic purposes, the ALP results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

REAGENTS

R_1	Standard (phenol) equal to	142 IU/l 20 KK/dl
R_2	Carbonate-bicarbonate buffer, pH 10.0 Disodium phenylphosphate	50 mmol/l 5.0 mmol/l
R_3	4-aminoantipyrine Sodium arsenate **Toxic reagent** **R 23/25 : Toxic by inhalation and if swallowed.** **S 28 : after contact with skin, wash immediately with plenty of water.** **S 45 : In case of accident or if you feel unwell, seek medical advice immediately (Show label where possible)**	60 mmol/l 240 mmol/l
R_4	Potassium ferricyanide	150 mmol/l

- **Reagent Preparation & Stability**

All reagents are ready for use and stable up to the expiry date given on label when stored at 2–8^{0}C.

Specimen

Serum is the only accepted specimen. Avoid hemolysis

Specimen Preparation & Stability

Freshly collected unhemolyzed serum specimen is the preferred specimen. Heparinized plasma may also be used. Complexing anticoagulants such as citrate, oxalate, and EDTA inhibit alkaline phosphatase so are unsuitable as anticoagulant. Specimens should be kept cold and assayed as soon as possible after collection. Alkaline phosphatase levels in serum, plasma rise significantly when stored at 2-8^{0}C, or room temperature.

PROCEDURE

- **Manual Procedure**

Wavelength	510 nm
Cuvette	1 cm light path
Temperature	37 ^{0}C
Zero adjustment	against reagent blank
Specimen	Serum

	Blank	Standard	Specimen Blank	Specimen
R_2	2.0 ml	2.0 ml	2.0 ml	2.0 ml
Incubate for 5 minutes at 37^{0}C				
Specimen	.....	.....	.....	50 µl
Standard	.....	50 µl	.....	.....
Incubate for exactly 15 minutes at 37^{0}C				
R_3	500 µl	500 µl	500 µl	500 µl
Mix well or preferably vortex				
R_4	500 µl	500 µl	500 µl	500 µl
Specimen	.....	.....	50 µl	.....
H_2O	50 µl	.....	.....	.....

Mix well, incubate in dark for 10 minutes, read the absorbance of specimen ($A_{specimen}$), specimen blank ($A_{specimen\ blank}$), and standard ($A_{standard}$) against blank.

The color is stable for 45 minutes.

CALCULATION

Calculate the absorbance of specimen by subtracting the absorbance of specimen blank from absorbance of specimen.

Then calculate the ALP activity by using the following formulae:

$$\text{ALP activity} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Standard value*}$$

Standard Value*:

Kind and king unit/100 ml = 20

U/l = 142

Unit conversion

Kind & King U/dl x 7.1 = IU/l

Kind and King unit is that amount of enzyme which in the given conditions liberates 1 mg of phenol in 15 minutes at 37^{0}C.

LINEARITY

When run as recommended, the assay is linear up to 40 Kind & King U/dl or 285 IU/l.

If result exceeds 40 Kind & King U/dl or 285 IU/l, reassay using smaller volume of specimen, such as 10 µl instead of 50 µl. Multiply the result by 5.

SENSTIVITY

The sensitivity is defined as the lower detection limit represents the lowest measurable ALP activity that can be distinguished from zero.

When run as recommended the sensitivity of this assay is 1.0 Kind & King U/dl or 7.1 IU/l.

QUALITY

It is recommended that controls (normal and abnormal) be included in:

- Each set of assays, or
- At least once a shift, or
- When a new bottle of reagent is used, or
- After preventive maintenance is performed or a clinical component is replaced.

Commercially available control material with established ALP/AP values may be routinely used for quality control.

Failure to obtain the proper range of values in the assay of control material may indicate:

- Reagent deterioration,
- Instrument malfunction, or
- Procedure errors.

The following corrective actions are recommended in such situations:

- Repeat the same controls.
- If repeated control results are outside the limits, prepare fresh control serum and repeat the test.
- If results on fresh control material still remain outside the limits, then repeat the test with fresh reagent.
- If results are still out of control, contact NS Biotec Technical Services.

INTERFERING SUBSTANCES

- **Anticoagulants:**
  Complexing anticoagulants such as citrate, oxalate, and EDTA must be avoided. The only acceptable anticoagulant is heparin.
- **Bilirubin:**

No significant interference from free or conjugated bilirubin up to a level of 60 mg/dl.

- **Drugs:**
  Young[7] in 1990 has published a comprehensive list of drugs and substances which may interfere with this assay.
- **Haemolysis:**
  Haemoglobin levels higher than 250 mg/dl decrease the apparent ALP activity significantly.
- **Lipemia:**
  No significant interference.
- **Others:**
  Pathological high levels of albumin (7.0 g/dl) increase the apparent ALP activity significantly.

WARNING & PRECAUTIONS

- NS Biotec ALP reagent is for in vitro diagnostic use only. Normal precautions exercised in handling laboratory reagents should be followed.
- Warm up working solution to the corresponding temperature before use.
- The reagent and sample volumes may be altered proportionally to accommodate different spectrophotometer requirements.
- Valid results depend on an accurately calibrated instrument, timing, and temperature control.
- Don't use the reagent if it is turbid.

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Cromatest LINEAR

ACID PHOSPHATASE CE

REF 1100005
4 x 10 mL
CONTENTS
R1. Reagent 1 x 40 mL
R2. Reagent 1 x 20 mL
R3. Reagent 4 x 10 mL
R4. Reagent 1 x 3 mL

For *in vitro* diagnostic use only

ACID PHOSPHATASE
TOTAL AND PROSTATIC
Colorimetric method
KINETIC
OPTIMIZED

PRINCIPLE

The method[1,2] is based on the hydrolysis of α-naphtyl phosphate at pH 5.0 by acid phosphatase (ACP) to produce α-naphtol and inorganic phosphate. The pentanediol acts as a phosphate acceptor increasing the reaction sensitivity.
The α-naphtol reacts with Fast Red TR*, to produce a coloured complex directly proportional to the activity of the ACP in the sample.

$$\alpha\text{-Naphtyl phosphate} + H_2O \xrightarrow{ACP} \alpha\text{-Naphtol} + P_i$$

$$\alpha\text{-Naphtol} + \text{Fast Red TR} \xrightarrow[30\text{-}37^{\circ}C]{pH\ 5,0} \text{Azo dye}$$

* *Diazotized 2- Amino-5-chlorotoluene*

The sample tested in the presence of L-tartrate inhibits the prostatic acid phosphatase of the total ACP activity.

REAGENT COMPOSITION

R1 **Citrate buffer**. Sodium citrate 110 mmol/L, 1,5-pentanediol 220 mmol/L, pH 5.2.

R2 **Citrate/Tartrate buffer.** Sodium citrate 110 mmol/L, 1,5-pentanediol 220 mmol/L, L-tartrate 110 mmol/L, pH 5.2.

R3 **ACP sustrate**. Powder. α-Naphtyl phosphate 12.5 mmol/L, Fast Red TR 1.25 mmol/L, after reconstitution.

R4 **Stabilizer.** Acetate buffer 5 M/L, pH 5.2.

STORAGE AND STABILITY

Store at 2-8ºC.
All the kit compounds are stable until the expiry date stated on the label. Do not use reagents over the expiration date.
Store the vials tightly closed, protected from light and prevented contaminations during the use.

Discard if appear signs of deterioration:
- Presence of particles and turbidity.
- Blank absorbance (A) at 405 nm > 0.400 in 1cm cuvette.

REAGENT PREPARATION

Working reagent. Add 10 mL of **R1** (total ACP) or 10 mL of **R2** (non-prostatic ACP) into a vial of **R3**. Cap and swirl gently until complete solution. Do not shake. The reagent is stable for 10 days at 2-8ºC.

SAMPLES

Clear, unhemolyzed serum, separated from the clot, immediately. Do not use plasma. Oxalates and sodium fluoride inhibit ACP while heparin and EDTA cause turbidity in the sample.
To stabilize the enzyme after separation of the serum from the clot, add 50 µL of **R4** to 1 mL of sample.

Specimens not preserved in this manner are unsuitable for analysis. ACP activity in preserved serum is stable for 4-5 days at 2-8ºC.

INTERFERENCES

- Lipemia (intralipid >1,25 g/L) may affect the results.
- Bilirubin (>1,25 g/L) may affect the results.
- Hemoglobin may affect the results.
- Other drugs and substances may interfere[3-6].

MATERIALS REQUIRED

- Photometer or spectrophotometer with a thermostatted cell compartment set at 30/37ºC, capable of reading at 405 nm.
- Stopwatch, strip-chart recorder or printer.
- Cuvettes with 1-cm pathlength.
- Pipettes to measure reagent and samples.

PROCEDURE

1. Preincubate working reagent, samples and controls to reaction temperature.
2. Set the photometer to 0 absorbance with distilled water.
3. For Total acid and/or Non-Prostatic acid tests pipette into labelled cuvettes:

TUBES	Total	Non-Prostatic
Working reagent **R1**	1.0 mL	-
Working reagent R2	-	**1.0 mL**
Sample or control	100 µL	100 µL

4. Mix gently by inversion. Insert cuvette into the cell holder and start stopwatch.
5. Incubate for 5 minutes and record initial absorbance reading.
6. Repeat the absorbance readings exactly after 1, 2 and 3 minutes.
7. Calculate the difference between absorbances.
8. Calculate the mean of the results to obtain the average change in absorbance per minute (ΔA/min).

CALCULATIONS

A. ***Total Acid Phosphatase***

U/L = ΔA/ min x 853

B. ***Non-Prostatic Acid Phosphatase***

U/L = ΔA/ min x 853

C. ***Prostatic Acid Phosphatase***

A (U/L) – B (U/L) = Prostatic Acid Phosphatase

Samples with ΔA/min exceeding 0.170 at 450 nm should be diluted 1:3 with saline and assayed again. Multiply the results by 3.

If results are to be expressed as SI units apply:
U/L x 16.67 = µkat/L

QUALITY SYSTEM CERTIFIED ISO 9001 ISO 13485

LINEAR CHEMICALS, S.L.U. Joaquim Costa 18 2ª planta. 08390 Montgat (Barcelona) SPAIN
Telf. (+34) 934 694 990; E-mail: info@linear.es ; website: www.linear.es NIF-VAT:B60485687

cromatest

LINEAR

REFERENCE VALUES [4]

Serum

Reaction temperature	37ºC	30ºC
Total ACP, up to	6.6 U/L (110 nkat/L)	7.0 U/L (278 nkat/L)
Prostatic ACP, up to	3.5 U/L (108 nkat/L)	2.6 U/L (43 nkat/L)

It is recommended that each laboratory establishes its own reference range.

QUALITY CONTROL

To ensure adequate quality control (QC), each run should include a set of controls (normal and abnormal) with assayed values handled as unknowns.

REF **1980005** HUMAN MULTISERA NORMAL
Borderline level of ACP. Assayed.

REF **1985005** HUMAN MULTISERA ABNORMAL
Elevated level of ACP. Assayed.

If the values are found outside of the defined range, check the instrument, reagents and procedure.
Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

CLINICAL SIGNIFICANCE

Determination of acid phosphatase activity in serum is directed toward the prostatic enzyme with the intent of detecting carcinoma of the prostate.

Elevations of the activity are found in the sera of about 60% of men with prostatic cancer with metastases.
Slight elevations in total enzyme are observed in cases of thromboembolic phenomena, multiple myeloma, thrombocytopenia and liver disease.

Moderate elevations in total acid phosphatase activity often occur in Paget´s disease, in hyperparathyroidism with skeletal involvement, and in the presence of malignant invasion of the bones by cancers. The serum activity in these cases is not inhibited by tartrate. The only non-bone condition in which elevated activities of tartrate-resistant osteoclast-type acid phosphatase are found in serum is Gaucher´s disease.

ANALYTICAL PERFORMANCE

- ***Detection Limit :*** 2.74 U/L
- ***Linearity :*** Up to 150 U/L
- ***Precision:***

U/L	Within-run		Between-run	
Mean	28.2	63.6	28.2	63.6
SD	0.40	0.89	1.41	2.85
CV%	1.43	1.41	4.98	4.48
N	10	10	10	10

- ***Sensitivity :*** 1,3 mA/min/U/L Acid phosphatase.
- ***Correlation:*** This assay (y) was compared with a similar commercial method (x). The results were:
  N = 50 r = 0.987 y = 1.078 x – 2.166

The analytical performances have been generated using on automatic instrument. Results may vary depending on the instrument.

NOTES

1. This method may be used with different instruments. Any application to an instrument should be validated to demonstrate that results meets the performance characteristics of the method. It is recommended to validate periodically the instrument. Contact to the distributor for any question on the application method.
2. Clinical diagnosis should not be made on findings of a single test result, but should integrate both clinical and laboratory data.

REFERENCES

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LINEAR CHEMICALS, S.L.U. Joaquim Costa 18 2ª planta. 08390 Montgat (Barcelona) SPAIN
Telf. (+34) 934 694 990; E-mail: info@linear.es ; website: www.linear.es NIF-VAT:B60485687

Enzymatic Method for Determining Amylase Activity (Amylase Activity Assay)

This assay protocol is suitable for the colorimetric detection of Amylase activity in cell and tissue culture supernatants, urine, plasma, serum, and other biological samples using the Amylase Activity Assay Kit (MAK009). Amylase activity is determined using a coupled enzymatic assay, which results in a colorimetric (405 nm) product, proportional to the amount of substrate, ethylidene-pNP-G7, cleaved by the amylase. One unit is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 µmole of p-nitrophenol per minute at 25 °C.

PRECAUTIONS AND DISCLAIMER :

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

REAGENTS

Amylase Assay Buffer 25 mL

Catalog Number MAK009A

Amylase Substrate Mix 5 mL

Catalog Number MAK009B

Amylase Positive Control 1 vL

Catalog Number MAK009C

Nitrophenol Standard 150 µL

Catalog Number MAK009D

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED :

96 well flat-bottom plate - It is recommended to use clear plates (Catalog Number M4436 or equivalent) for colorimetric assays.

Spectrophotometric multiwell plate reader.

PREPARATION INSTRUCTIONS

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Amylase Assay Buffer – Allow buffer to come to room temperature before use.

Amylase Positive Control – Reconstitute with 50 µL of Amylase Assay Buffer. Mix well by pipetting, then aliquot and store at –20 °C. Use within 2 months of reconstitution.

STORAGE/STABILITY

The kit is shipped on wet ice. Storage at –20 °C, protected from light, is recommended.

PROCEDURE

All samples and standards should be run in duplicate.

Nitrophenol Standards for Colorimetric Detection

Add 0, 2, 4, 6, 8, 10 µL of the 2 mM Nitrophenol Standard into a 96 well plate, generating 0 (blank), 4, 8, 12, 16, and 20 nmole/well standards. Add water to each well to bring the volume to 50 µL.

Sample Preparation

Tissue (100 mg) or cells (4 x106) can be homogenized in 0.5 mL of the Amylase Assay Buffer. Centrifuge the samples at 13,000 x g for 10 minutes to remove insoluble material.

Serum and urine samples can be directly added to wells.

Add 1–50 µL of sample into wells of a 96 well plate. Bring samples to a final volume of 50 µL with Amylase Assay Buffer.

Note: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

For the positive control (optional), add 5 µL of the Amylase Positive Control solution to wells and adjust to 50 µL with the Amylase Assay Buffer.

ASSAY REACTION

1. Prepare the Master Reaction Mix according to Table 1. 100 µL of the Master Reaction Mix is required for each reaction (well).

Table 1.

Reagent	Volume
Amylase Assay Buffer	50 µL
Amylase Substrate Mix	50 µL

2. Add 100 µL of the Master Reaction Mix to each of the sample, standard, and positive control wells. Mix well using a horizontal shaker or by pipetting.

3. After 2–3 minutes (Tinitial), measure the absorbance at 405 nm (A405)initial

Note: It is essential that (A405)initial is in the linear range of the standard curve.

4. Incubate the plate at 25 °C, measuring the absorbance (A405) every 5 minutes. Protect the plate from light during the incubation.

5. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (20 nmole/well). At this time, the most active sample is near or exceeds the end of the linear range of the standard curve.

6. The final absorbance measurement [(A405)final] for calculating the enzyme activity would be the penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve (see step 5). The time of the penultimate reading is Tfinal.

Note: It is essential the final measurement falls within the linear range of the standard curve.

RESULTS

Calculations

Correct for the background by subtracting the final measurement (A405)final obtained for the 0 (blank) nitrophenol standard from the (A405) final measurement of the standards and samples.

Note: A new standard curve must be set up each time the assay is run.

Calculate the change in absorbance from Tinitial to Tfinal for the samples.

$\Delta A405 = (A405)final - (A405)initial$

Compare the ΔA405 of each sample to the standard curve to determine the amount of nitrophenol (B) generated by the amylase between Tinitial to Tfinal

The amylase activity of a sample may be determined by the following equation:

Amylase Activity = (B x Sample Dilution Factor)/(Reaction Time) x V

B = Amount (nmole) of nitrophenol generated between Tinitial and Tfinal

Reaction Time = Tfinal – Tinitial (minutes)

V = sample volume (mL) added to well

Amylase activity reported as nmole/min/mL. One unit of amylase is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 µmole of p-nitrophenol per minute at 25 °C.

FOR RESEARCH USE ONLY!

Creatine Kinase Activity Colorimetric Assay Kit

(Catalog # K777-100; 100 assays; Store at -20°C) *rev. 2/15*

I. Introduction:

Creatine Kinase (CK) also known as creatine phosphokinase (CPK) and ATP: creatine N-phosphotransferase is a common cellular enzyme (EC 2.7.3.2). It catalyzes the reversible conversion of creatine and ATP into ADP and phosphocreatine. CK is widely expressed in various tissues and cell types, with highest activity in striated muscles, heart tissue and brain. CK consists of two subunits: M (muscle) and B (brain), and has three isoenzymes: CK-MM (skeleton muscle), CK-MB (cardiac muscle), and CK-BB (brain). Increased CK level is associated with many diseases such as myocardial infarction, muscular dystrophy, pulmonary infarction and brain tumors. Accurate measurement of CK is crucial for early diagnosis, prediction and therapeutic strategy. In BioVision's Creatine Kinase Activity Colorimetric Assay kit, creatine kinase converts creatine into phosphocreatine and ADP. The generated phoshocreatine and ADP reacts with CK Enzyme Mix to form an intermediate, which reduces a colorless Probe to a colored product with strong absorbance at 450 nm. The CK Activity Assay is high-throughput adaptable, simple and sensitive. This assay kit can detect Creatine Kinase activity less than 1 mU.

$$\text{Creatine} + \text{ATP} \xrightarrow{\text{Creatine Kinase}} \text{Phosphocreatine} + \text{ADP}$$

$$\text{ADP} \xrightarrow{\text{CK Enzyme Mix}} \text{Intermediate} \xrightarrow{\text{CK Developer}} \text{Color detection } (\lambda = 450 \text{ nm})$$

II. Application:

- Measurement of Creatine Kinase activity in various samples.
- Diagnostic marker for many diseases.
- Screening new therapeutic drugs.

III. Sample Type:

- Serum & plasma.
- Animal tissues: muscle, brain, heart etc.
- Cell culture: Adherent or suspension cells.

IV. Kit Contents:

Components	K777-100	Cap Code	Part Number
CK Assay Buffer	25 ml	WM	K777-100-1
CK Substrate	1 ml	Blue	K777-100-2
ATP (Lyophilized)	1 vial	Orange	K777-100-3
CK Enzyme Mix (Lyophilized)	1 vial	Green	K777-100-4
CK Developer (Lyophilized)	1 vial	Red	K777-100-5
NADH Standard (Lyophilized)	1 vial	Yellow	K777-100-6
Positive Control (Lyophilized)	1 vial	Purple	K777-100-7

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom.
- Multi-well spectrophotometer (ELISA reader).

VI. Storage and Handling:

Store kit at –20°C, protected from light. Warm Assay Buffer to room temperature before use. Briefly centrifuge small vials prior to opening.

VII. Reagent Preparation and Storage Conditions:

- **ATP:** Reconstitute with 220 µl dH_2O. Pipette up and down to dissolve completely. Aliquot & store at -20°C. Use within two months.
- **CK Enzyme Mix:** Reconstitute with 220 µl CK Assay Buffer. Pipette up and down to dissolve completely. Aliquot and store at -20°C. Avoid repeated freeze thaw. Use within two months. Keep on ice while in use.
- **CK Developer:** Reconstitute with 220 µl dH_2O. Pipette up and down to dissolve completely. Store at -20°C. Use within two months.
- **NADH Standard:** Reconstitute with 50 µl CK Assay Buffer to generate 10 mM (10 nmol/µl) NADH Standard solution. Store at –20°C. Use within two months. Keep on ice while in use.
- **Positive Control:** Reconstitute with 200 µl CK Assay Buffer to generate 10 mU/µl stock and mix thoroughly. Aliquot and store at –20°C. Use within two months.

VIII. Creatine Kinase Activity Assay Protocol:

1. **Sample Preparation:** Rapidly homogenize tissue (10 mg) or cells (1 x 10^6) with 100 µl ice cold CK Assay Buffer for 10 minutes on ice. Centrifuge at 12000 rpm for 5 min. Collect the supernatant. Add 1-50 µl sample (100 µg) per well. Adjust final volume to 50 µl with CK Assay Buffer. For Positive Control, add 2-10 µl of Positive Control into desired well(s). Adjust final volume to 50 µl with CK Assay Buffer.
   Notes:
   a. Small molecules such as ADP, NADH etc. in some tissue samples such as liver may generate background. To remove small molecules, we suggest using 10K spin column (Biovision Cat# 1997-25).
   b. For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.

155 S. Milpitas Blvd., Milpitas, CA 95035 USA | T: (408)493-1800 F: (408)493-1801 | www.biovision.com | tech@biovision.com

2. **NADH Standard Curve:** Dilute NADH Standard to 1 mM by adding 10 µl of 10 mM NADH Standard to 90 µl CK Assay Buffer. Add 0, 2, 4, 6, 8 and 10 µl of 1 mM NADH Standard into a series of wells in 96 well plate to generate 0, 2, 4, 6, 8 and 10 nmol/well of NADH Standard. Adjust volume to 50 µl/well with CK Assay Buffer.

3. **Reaction Mix:** Mix enough reagents for the number of assays to be performed. For each well, prepare 50 µl Mix containing:

	Reaction Mix
CK Assay Buffer	34 µl
CK Enzyme Mix	2 µl
CK Developer	2 µl
ATP	2 µl
CK Substrate	10 µl

Add 50 µl of the Reaction Mix to each well containing Standard, Positive Control and samples, mix well.

4. **Measurement:** Incubate for 20-40 min at 37^{0}C and measure OD_{450nm}.
Note: Incubation time depends on the Creatine Kinase activity in the samples. We recommend measuring the OD in a kinetic mode and choose two time points (T_1 & T_2) in the linear range to calculate the CK activity of the samples. The NADH Standard curve can read in Endpoint mode (i.e., at the end of incubation time).

5. **Calculation:** Subtract 0 Standard reading from all readings. Plot the NADH Standard Curve. Calculate the Creatine Kinase activity of the test sample: $\Delta OD = A_2 - A_1$. Apply the ΔOD to the NADH Standard Curve to get B nmol of NADH generated by Creatine Kinase during the reaction time ($\Delta T = T_2 - T_1$).

Sample Creatine Kinase Activity = B/(ΔT X V) x Dilution Factor = nmol/min/ml = mU/ml

Where: **B** is the NADH amount from standard curve (nmol).
ΔT is the reaction time (min).
V is the sample volume added into the reaction well (ml).

Unit Definition: One unit of Creatine Kinase is the amount of enzyme that will generate 1.0 µmol of NADH per min at pH 9.0 at 37^{0}C.

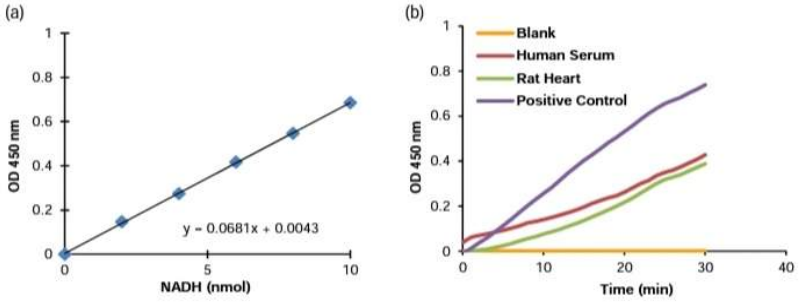

Figure 1: NADH Standard curve (a). Creatine Kinase activity in human serum (5 µl) & rat heart lysate (192 ng) (b). Assays were performed following kit protocol.

IX. RELATED PRODUCTS:

Creatine Assay Kit
Glucose Assay kit
Glucose-1-Phosphate Assay Kit
PicoProbe™ Glucose-6-Phosphate Assay Kit
Glucose Dehydrogenase Activity Assay Kit
Glucose-6-Phosphate Dehydrogenase Assay Kit
Glucose and Sucrose Assay Kit
Glucose Uptake Colorimetric Assay Kit
Glucose Uptake Fluorometric Assay Kit
Hexokinase Assay Kit
Maltose and Glucose Assay Kit
NAD/NADH Quantification Kit
NADP/NADPH Quantification Kit
Phosphoglucomutase Assay Kit

FOR RESEARCH USE ONLY! Not to be used on humans.

Lactate Dehydrogenase (LDH) Assay Kit (Colorimetric)

LS-K306-100 (100 Tests) • **Store at -20°C**

LSBio
LifeSpan BioSciences, Inc.

Introduction

LACTATE DEHYDROGENASE (LDH) is an oxidoreductase which catalyzes the interconversion of lactate and pyruvate. When disease or injury affects tissues containing LDH, the cells release LDH into the bloodstream, where it is identified in higher than normal levels. Therefore, LDH is most often measured to evaluate the presence of tissue or cell damage. The non-radioactive colorimetric LDH assay is based on the reduction of the tetrazolium salt MTT in a NADH-coupled enzymatic reaction to a reduced form of MTT which exhibits an absorption maximum at 565 nm. The intensity of the purple color formed is directly proportional to the enzyme activity.

Key Features

- High sensitivity and wide linear range. Use 3 µL serum or plasma sample. The detection limit is 2 U/L, linear up to 200 U/L.
- Homogeneous and simple procedure. Simple "mix-and-measure" procedure allows reliable quantitation of LDH activity within 30 minutes.
- Robust and amenable to HTS. All reagents are compatible with high-throughput liquid handling instruments.

Applications

- Direct Assays: LDH activity in serum, plasma and other sources.
- Characterization and Quality Control for LDH production.
- Drug Discovery: screen and evaluation of LDH modulators.

Components

Component	K306-100
	100 Tests
Substrate Buffer	20 mL
NAD Solution	1 mL
MTT Solution	1.5 mL
Diaphorase	120 µL
Calibrator	1.5 mL

Materials Not Supplied

Pipetting devices and accessories (e.g. multi-channel pipettor), clear flat-bottom 96-well plates (e.g. VWR cat# 82050-760) or cuvettes and plate reader or spectrophotometer capable of measuring OD 565 nm.

Storage

The kit is shipped at ambient temperature. Store all components at -20°C upon receiving. Shelf life: 6 months after receipt.

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www.LSBio.com • (206) 464-1554 • TechnicalSupport@LSBio.com

Lactate Dehydrogenase (LDH) Assay Kit (Colorimetric)

LS-K306-100 (100 Tests) • **Store at -20°C**

LSBio
LifeSpan BioSciences, Inc.

Assay Procedure

This assay is based on a kinetic reaction. To ensure identical incubation time, addition of Working Reagent to samples should be quick and mixing should be brief but thorough. Use of a multi-channel pipettor is recommended. Assays can be executed at room temperature or 30°C.

Sample Preparation

Serum and plasma are assayed directly.

Tissue: prior to dissection, rinse tissue in phosphate buffered saline (pH 7.4) to remove blood. Homogenize tissue in 5 mL buffer containing 100 mM potassium phosphate (pH 7.0) and 2 mM EDTA, per gram tissue. Centrifuge at 10,000 x g for 15 min at 4°C. Remove supernatant for assay.

Cell Lysate: collect cells by centrifugation at 2,000 x g for 5 min at 4°C. For adherent cells, do not harvest cells using proteolytic enzymes; rather use a rubber policeman. Homogenize or sonicate cells in an appropriate volume of cold buffer containing 100 mM potassium phosphate (pH 7.0) and 2 mM EDTA. Centrifuge at 10,000 x g for 15 min at 4°C. Remove supernatant for assay.

All samples can be stored at –20 to –80°C for at least one month.

Reagent Preparation

Equilibrate reagents to desired reaction temperature (e.g. 25°C or 37°C). Briefly centrifuge reagent tubes before use.

Prepare sufficient Working Reagent (WR) for all sample wells by mixing, for each well: 14 µL MTT Solution, 8 µL NAD Solution, 1 µL Diaphorase and 175 µL Substrate Buffer. Fresh reconstitution is recommended.

Procedure

1. Transfer 200 µL H_2O (OD_{H2O}) and 200 µL Calibrator (OD_{CAL}) solution into separate wells of a clear flat bottom 96-well plate.
2. Transfer 10 µL of each sample into separate wells and then add 190 µL WR to each sample well. Tap plate briefly to mix.
3. Read OD_{565nm} immediately (OD_{S0}), and again after 25 min (OD_{S25}) on a plate reader.

Calculations

LDH activity can then be calculated as follows:

$$\text{LDH Activity} = \frac{OD_{S25} - OD_{S0}}{\varepsilon_{mtt} \cdot l} \times \frac{\text{Reaction Vol (µL)}}{\text{Time} \cdot \text{Sample Vol (µL)}} \times n$$

$$= 43.68 \times \frac{OD_{S25} - OD_{S0}}{OD_{CAL} - OD_{H20}} \times n \quad \text{(IU/L)}$$

where OD_{S25} and OD_{S0} are OD_{565nm} values of sample at 25 min and 0 min. ε_{mtt} is the molar absorption coefficient of reduced MTT. l is the light path length which is calculated from the calibrator. OD_{CAL} and OD_{H20} are OD_{565nm} values of the Calibrator and water. Reaction Vol and Sample Vol are 200 µL and 10 µL, respectively. n is the dilution factor.

Note: if sample LDH activity exceeds 200 IU/L, dilute samples in water and repeat the assay.

Unit definition: 1 Unit (IU) of LDH will catalyze the conversion of 1 µmole of lactate to pyruvate per min at pH 8.2.

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Lactate Dehydrogenase (LDH) Assay Kit (Colorimetric)

LS-K306-100 (100 Tests) • **Store at -20°C**

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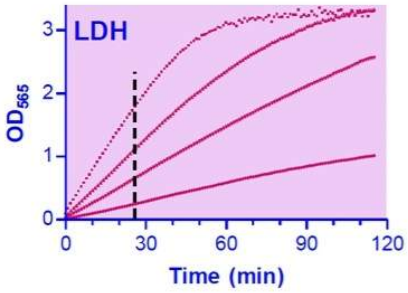
Sample Data

Samples were assayed using the 96-well plate protocol. The LDH activity (IU/L) was 41 for a human serum, 220 for rat serum and 88 for fetal bovine serum, respectively.



Kinetics of LDH reaction in 96-well plate assay with increasing serum concentration

Version: V.08.09.2018

Atlas Medical CE

Calcium Arsenazo III Colorimetric Method

IVD **For in vitro-diagnostic use only.**

2°C 8°C **Store at 2-8°C**

INTENDED USE

For the measurement of calcium concentration in human serum, plasma or urine.

INTRODUCTION

Calcium the most abundant mineral in the body, is found in some foods, added to others, available as a dietary supplement, and is present in some medicines (such as antacids). Calcium is required for vascular contraction and vasodilation, muscle function, nerve transmission, intracellular signaling and hormonal secretion, though less than 1% of total body calcium is needed to support these critical metabolic functions. The remaining 99% of the body's calcium supply is stored in the bones and teeth where it supports their structure and function.

A decrease in albumin level causes a decrease in serum calcium. Low levels of calcium are found in hypoparathyroidism, pseudohypoparathyroidism, vitamin D deficiency, malnutrition and intestinal malabsortion.

Among causes of hypercalcemia are cancers, large intake of vitamin D, enhanced renal retention, osteoporosis, sarcosidosis, thyrotoxicosis, hyperparathyroidism.

Clinical diagnosis should not be based on a single test result; it should integrate clinical and other laboratory data.

PRINCIPLE

Calcium with Arsenazo III (1, 8-Dihydroxy-3, 6-disulpho-2, 7-naphthalene-bis(azo)-dibenzenearsonic acid), at neutral pH, yields a blue colored complex.

The intensity of the color formed is proportional to the calcium concentration in the sample.

MATERIAL

MATERIAL PROVIDED

R (Arsenazo III)	Imidazol buffer ph 6.5 Arsenazo III	100mmol/L 120mmol/L
Calcium STD	Calcium aqueous primary standard 10mg/dl	

MATERIALS REQUIRED BUT NOT PROVIDED

- Spectrophotometer or colorimeter measuring at 650 nm.
- Matched cuvette 1.0 cm light path.
- General laboratory equipment.

PRECAUTIONS

R: May damage fertility or the unborn child.

STD: May be corrosive to metals.

STORAGE AND STABILITY

- All components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C protected from light and contaminations prevented during their use.
- Do not use reagents over the expiration date.
- **Signs of reagent deterioration:**
  - Presence of particles and turbidity.
  - Blank absorbance (A) at 650 nm $\geq$ 0.50.

PREPARATION

Regents are ready to use.

SAMPLES

Serum or plasma: separated from cells as rapidly as possible .Blood anticoagulants with oxalate or EDTA are not acceptable since these chemicals will strongly chelate calcium.

Urine: collect 24 hour urine specimen in calcium free containers. The collecting bottles should contain 10 ml of diluted nitric acid (50% v/v).Record the volume.

Dilute a sample 1/2 in distilled water. Mix. Multiply result by 2 (dilution factor).

Stability of the samples: calcium is stable for 10 days at 2-8°C

PROCEDURE

1. Assay conditions:
   Wavelength..... 650 nm
   Cuvette..... 1 cm. light path

mg/dl.
If the results obtained were greater than linearity limit, dilute the sample 1/2 with NaCl 9 g/l and multiply the result by 2.

2. Precision

	INTRA -ASSAY (N=20)		INTER -ASSAY (N=20)	
Mean (mg/dl)	8.35	14.28	8.58	14.57
SD	0.08	0.08	0.19	0.34
CV (%)	0.96	0.56	2.21	2.33

3. *Sensitivity.*

1mg/dl=0.0316 A.

4. *Accuracy:*

Results obtained using ATLAS reagents (y) did not show systematic differences when compared with other commercial reagents(x).
The results obtained using 50 samples as follows:
Correlation coefficient(r):0.9506.
Regression equation=0.8944x+1.3421.
The results of the performance characteristics depend on the analyzer used.

INTERFERENCES

No interference was observed with triglyceride up to 1.25g/l.
A list of drugs and other interfering substances with calcium determination has been reported by Young.

NOTES

1. Calibration with the aqueous standard may cause a systematic error in automatic procedures. In these cases, it is recommended to use a serum Calibrator.
2. Use clean disposable pipette tips for its dispensation.
3. CALCIUM CAL: proceed carefully with this product as, due its nature, it can get contaminated easily.
4. Most of the detergents and water softening products used in the laboratories contain chelating agents. Defective rinsing will invalidate the procedure.

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Atlas Medical
Ludwig-Erhard Ring 3
15827 Blankenfelde-Mahlow
Germany
Tel: +49 - 33708 – 3550 30
Email: Info@atlas-medical.com

PPI1459A01
Rev A (02.09.2019)

REF	Catalogue Number		Temperature limit
IVD	*In Vitro* diagnostic medical device		Caution
	Contains sufficient for <n> tests and Relative size		Consult instructions for use (IFU)
LOT	Batch code		Manufacturer
	Fragile, handle with care		Use-by date
	Manufacturer fax number		Do not use if package is damaged
	Manufacturer telephone number		Date of Manufacture
	Keep away from sunlight		Keep dry

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email: techservice@sial.com sigma-aldrich.com

Product Information

Phosphate Colorimetric Assay Kit

Catalog Number **MAK030**
Store at Room Temperature

TECHNICAL BULLETIN

Product Description
Phosphate is an essential component in living organisms and contributes to a variety of biological functions, including structural roles within nucleic acids, cellular membranes, and bone. Phosphate is also important in the transport of cellular energy, nucleic acid metabolism, and signal transduction. Hyperphosphatemia, a condition of excess phosphate levels in the blood, can lead to calcification of organs and interference with usage of other inorganic ions, such as iron, calcium, magnesium, and zinc.

The Phosphate Colorimetric Assay Kit provides a simple and direct procedure for measuring phosphate levels (ranging from 1–5 nmole/well) in a variety of samples. Phosphate reacts with a chromogenic complex, which results in a colorimetric (650 nm) product proportional to the amount of phosphate present.

Components
The kit is sufficient for 500 assays in 96 well plates, or 100 assays in 1 mL cuvettes.

Phosphate Reagent Catalog Number MAK030A	15 mL
Phosphate Standard, 10 mM Catalog Number MAK030B	0.5 mL

Reagents and Equipment Required but Not Provided.
- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Reagents are supplied ready to use. Briefly centrifuge vials before opening. Use ultrapure water for dilutions in the assay.

Notes: Many laboratory detergents contain high levels of phosphates, which can adhere to cleaned glassware. It is highly recommended to use disposable plastic labware for all samples, standards, and reagents to avoid contamination.

The Phosphate Reagent may contain a precipitate that does not harm the performance of the reagent. Avoid pipetting the precipitate into the assay wells.

Storage/Stability
The kit is shipped at room temperature. Storage at room temperature, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

Phosphate Standards for Colorimetric Detection
Dilute 10 µL of the 10 mM Phosphate Standard with 990 µL of water to prepare a 0.1 mM Phosphate Standard Solution. Add 0, 10, 20, 30, 40, and 50 µL of the 0.1 mM Phosphate Standard Solution into a 96 well plate, generating 0 (blank), 1, 2, 3, 4, and 5 nmole/well standards. Add water to each well to bring the volume to 200 µL.

Sample Preparation
Samples can be measured directly. Add 1–200 µL of sample to wells. Bring samples to a final volume of 200 µL with water.

Note: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Assay Reaction
1. Add 30 µL of the Phosphate Reagent to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 30 minutes at room temperature. Cover the plate and protect from light during the incubation.

2. Measure the absorbance at 650 nm (A_{650}).

Note: When using 1.0 mL cuvettes, increase the volume of all reaction components 5-fold. The 1 mL total reaction volume will contain 1–500 µL of sample, 150 µL of Phosphate Reagent, and bring to a final volume of 1 mL with water. Incubate at room temperature for 30 minutes. Measure the absorbance at 650 nm (A_{650}).

Results

Calculations

The background for the assay is the value obtained for the 0 (blank) Phosphate Standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings.

Use the values obtained from the appropriate Phosphate Standards to plot a standard curve.
Note: A new standard curve must be set up each time the assay is run.

Using the corrected measurement, the amount of phosphate present in the samples may be determined from the standard curve.

Concentration of Phosphate

$S_a/S_v = C$

where:
S_a = Amount of Phosphate in unknown sample well (nmole) from standard curve
S_v = Sample volume (µL) added to reaction well
C = Concentration of Phosphate in sample

Sample Calculation

Amount of Phosphate (S_a) = 2.84 nmole (from standard curve)

Sample volume (S_v) = 50 µL

Concentration of Phosphate in sample:
2.84 nmole/50 µL = 0.0568 nmole/µL

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Master Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Master Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

KVG,MF,MAM 03/15-1

BIOLABO
www.biolabo.fr
MANUFACTURER :
BIOLABO SAS,
Les Hautes Rives
02160, Maizy, France

CHLORIDE Colorimetric method

Reagent for quantitative determination of chloride ions in human serum and plasma, urines or cerebrospinal fluid (CSF).

REF K1005	R1	8 x 20 mL
REF K2005	R1	8 x 50 mL

TECHNICAL SUPPORT AND ORDERS
Tel: (33) 03 23 25 15 50
Fax : (33) 03 23 256 256
support@biolabo.fr
Latest revision : www.biolabo.fr

CE

IVD

Made In France

I: corresponds to significant modifications

INTENDED USE

I This reagent is designated for professional use in laboratory (automated method).
It allows the quantification of chloride ions in human serum and plasma, urines or cerebrospinal fluid (CSF).

GENERALITIES (1)

Chloride is the major extracellular anion. Together with sodium, chloride is significantly involved in maintenance of water distribution, osmotic pressure and anion-cation balance in extracellular fluids.

PRINCIPLE (1) (4) (5)

$Hg(SCN)_2 + 2Cl^- \longrightarrow HgCl_2 + 2SCN^-$

$3(SCN)^- + Fe^{3+} \longrightarrow Fe(SCN)_3$

Chloride ions react with undissociated mercuric thiocyanate to form undissociated mercuric chloride and free thiocyanate ions. Thiocyanate ions react with ferric ions to form a highly colored reddish complex of ferric thiocyanate which absorbance, proportional to the amount of chloride in the specimen, is measured at 500 nm (450-500).

REAGENTS

R1 CL Thiocyanate Reagent

Ferric nitrate	22.2	mmol/L
Chloride mercuric	0.55	mmol/L
Mercuric Thiocyanate	1.33	mmol/L
Nitric acid	30	mmol/L
Surfactant	1	mL/L

According to 1272/2008/EC regulation, this reagent is not classified as dangerous

SAFETY CAUTIONS

- Refer to current Material Safety Data Sheet available on request or on www.biolabo.fr
- Verify the integrity of the contents before use.
- Waste disposal: Respect legislation in force in the country.
- All specimens or reagents of biological origin should be handled as potentially infectious. Respect legislation in force in the country.

I Any serious incident that has occurred in connection with the device is notified to the manufacturer and the competent authority of the Member State in which the user and/or patient is based.

REAGENTS PREPARATION

Ready for use.

STABILITY AND STORAGE

Stored away from light, well cap in the original vial at 18-25°C, reagent is stable when stored and used as described in the insert:

Unopened,

- Until the expiry date stated on the label of the Kit.

Once opened,

- Reagent is stable at least 2 years.

Discard any reagent if cloudy or if absorbance at 505 nm is > 0.100.

SPECIMEN COLLECTION AND HANDLING (2) (6)

Unhemolysed serum or heparinized plasma.

Urines or CSF.

Chloride is stable in the specimen for:
✓1 week at room temperature or 2-8°C.

LIMITS (3)

For a more comprehensive review of factors affecting this assay refer to the publication of Young D.S.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Basic medical analysis laboratory equipment.
2. Biochemistry Clinical Analyzer Kenza One, Kenza 240TX/ISE or Kenza 450TX/ISE

Manufacturer	Expiry date	In vitro diagnostic	Storage temperature	Dematerialized water	Biological risk
Product Reference	See Insert	Batch number	Store away from light	Sufficient for	Dilute with

REFERENCE INTERVALS (2)

In serum or plasma	mEq/L	[mmol/L]
In cord	96-104	[96-104]
Premature	95-110	[95-110]
0 to 30 days	98-113	[98-113]
up to 90 years	98-107(108)	[98-107(108)]
> 90 years	98-111	[98-111]
In 24 h Urines	**mEq/L**	**[mmol/L]**
Newborn	2-10	[2-10]
Child < 6 years	15-40	[15-40]
6-10 years, M	36-110	[36-110]
6-10 years, F	18-74	[18-74]
10-14 years, M	64-176	[64-176]]
10-14 years, F	36-173	[36-173]
Adult	110-250	[110-250]
> 60 years	95-195	[95-195]
In CSF	**mEq/L**	**[mmol/L]**
Child	110-130	[110-130]
Adult	118-132	[118-132]

Each laboratory should establish its own normal ranges for the population that it serves.

PERFORMANCES

On Kenza ONE, 505 nm, 37°C

Detection limit: approx. 1.8 mEq/L

Linearity Range: between 70 and 140 mEq/L

Precision:

Within-run N = 20	*Low level*	*Normal level*	*High level*
Mean (mEq/L)	80,7	103,1	123,1
S.D. mEq/L	0,9	0,8	0,9
C.V. %	1,1	0,8	0,7

Between run N = 20	*Low level*	*Normal level*	*High level*
Mean (mEq/L)	82,9	106,1	122,1
S.D. mEq/L	1,3	1,4	1,9
C.V. %	1,5	1,3	1,5

Analytical Sensitivity: approx. 0.042 abs for 10 mEq/L

Comparison studies with commercially available reagent:
Realized on automated analyzer with specimens (n=69) between 69 and 129 mEq/L

y =1.0391 x – 2.9153 r = 0.9944

Interferences:

Turbidity	Positive interference from 0.067 OD
Total bilirubin	Positive interference from 558 µmol/L
Direct bilirubin	No interference up to 24 µmol/L
Ascorbic acid	No interference up to 2500 mg/dL
Glucose	No interference up to 1044 mg/dL
Hemoglobin	Positive interference from 76 µmol/L

Other substances may interfere (see § Limits)

On the board stability: 2 months

Calibration Stability: 14 days

Make a new calibration when changing reagent batch, if quality control results are found out of the established range and after maintenance operations

Performances and stability data on Kenza 240TX/ISE and Kenza 450TX/ISE are available on request.

CALIBRATION (7)

- REF 95015 Multicalibrator *traceable to SRM 909*

The calibration frequency depends on proper instrument functions and on the preservation of the reagent.

QUALITY CONTROL

- REF 95010 EXATROL-N level I
- REF 95011 EXATROL-P level II
- REF 95012 Urinary controls
- External quality control program.

It is recommended to control in the following cases:
- At least once a run.
- At least once within 24 hours.
- When changing vial of reagent.
- After maintenance operations on the instrument.

If control is out of range, apply following actions:
1.Prepare a fresh control serum and repeat the test
2.If control is still out of range, use a new vial of fresh calibrator
3.If control is still out of range, use a new vial of reagent and reassay
If control is still out of range, please contact BIOLABO technical support or your local Agent.

PROCEDURE

Refer to validated application of the Kenza Analyzer used

CALCULATION

The analyzer provides directly final result.
Refer to the instruction of use of Kenza analyzer.

REFERENCES

(1) *TIETZ N.W. Textbook of clinical chemistry, 3rd Ed. C.A. Burtis, E.R. Ashwood, W.B. Saunders (1999) p. 1063-1064, 1104.*
(2) *Clinical Guide to Laboratory Test, 4th Ed., N.W. TIETZ (2006) p. 234-239*
(3) *YOUNG D.S., Effect of Drugs on Clinical laboratory Tests, 4th Ed. (1995) p. 3-137 à 3-141*
(4) *Zall D.M., Fisher D., Garner D.O., Anal. Chem. 28, 1665 (1956).*
(5) *Florence T.M. and Y.J. FARRAR: Spectrophotometric determination of Chloride at the parts per-billion level by the mercury (II) thiocyanate method, Anal. Chim. Acta., 54: 373-377 (1971).*
(6) *HENRY R. J. (Ed), Clinical chemistry: Principles and technics (2nd ed.), Harper and Row, p.718-719 (1974)*
(7) *SRM: Standard Reference Material ®*

CHLORIDE KIT

(Thiocyanate Method)

ELYTE 2 KIT

(Na^+& K^+Colorimetric)

ELYTE 3 KIT

(Na^+/K^+ & Cl^-Colorimetric)

For the determination of Na^+/ K^+ & Cl^-in serum.
(For Invitro Diagnostic Use Only)

Summary

Sodium and Potassium are the major cations of extracellular and intra cellular fluids respectively. Sodium maintains the normal distribution of water and the osmotic pressure in the various fluid compartments. Potassium influences the acid base balance and osmotic pressure including water retention. Increased sodium levels are found in severe dehydration and excessive treatment with sodium salts. Decreased levels are found in severe polyurea, metabolic acidosis, diarrhoea and renal insufficiency. Increased potassium levels are found in renal failure, dehydration, shock and adrenal insufficiency. Decreased levels are found in malnutrition, gastro-intestinal fluid loss, and hyperactivity of the adrenal cortex.Chloride is a major extracellular anion and maintains the cation/ anion balance between intra and extra cellular fluids, mostly as a salt with sodium. Increased levels are usually found in dehydration, kidney dysfunction, and anaemia. Decreased levels are found in extensive burns, vomiting, diarrhoea, intestinal obstructions, & salt losing nephritis

Principle

1. Sodium is precipitated as a triple salt with magnesium and Uranyl acetate. The excess of uranyl ions are reacted with ferrocyanide in an acidic medium to develop a brownish colour. The intensity of the colour produced is inversely proportional to the concentration of sodium in the sample.

Uranyl ions + Mg ions + Na^+ ⟶ **UranylMgNa precipitate**
Free Uranyl ions + $K_4Fe(CN)_6$ ⟶ **Brown coloured complex**

2. Potassium reacts with sodium tetraphenyl boron in a specially prepared buffer to form a colloidal suspension. The amount of the turbidity produced is directly proportional to the concentration of potassium in the sample.

Tetraphenyl Boron + K^+ ⟶ **White turbidity**

3. Chloride ions combine with free mercuric 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 colour formed is directly proportional to the amount of chloride present in the sample.

$2Cl^- + Hg(SCN)_2$ ⟶ **$HgCl_2 + 2(SCN)$**
$3(SCN) + Fe^{3+}$ ⟶ **$Fe(SCN)_3$**

Normal reference values

Sodium	:	135 - 155 mmol/l
Potassium	:	3.5 - 5.5 mmol/l
Serum / Plasma Chloride	:	98 - 106 mmol/l
Urine Chloride	:	170 - 250 mmol/24hours
CSF Chloride	:	120 - 135 mmol/l

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents

	CHLORIDE KIT 75 ml 3x75 ml	ELYTE 2 15 Tests	ELYTE3 15 Tests
Sodium Kit			
L1 : Precipitating Reagent		35 ml	35 ml
L2 : Acid reagent		45 ml	45 ml
L3 : Colour Reagent		5 ml	5 ml
Potassium Kit			
L1 : Potassium Reagent		45 ml	45 ml
S : Na^+ / K^+ Standard (150/5 mmol/l)		5 ml	5 ml
Chloride Kit			
L1 : Chloride Reagent	75 ml 3 x 75 ml	-	45 ml
S : Chloride Standard (100 mmol/l)	5 ml 5 ml	-	5 ml

Storage / stability
All reagents are stable at R.T. till the expiry mentioned.

Reagent Preparation

Reagents are ready to use.

Sample material

For Sodium & Potassium : Serum. Free from hemolysis. Serum should be separated from the clot immediately / as soon as possible.
For Chloride : Serum, plasma, urine, & 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 **Sodium**	:	530 nm (Hg 546) / Green
Wavelength / filter **Potassium**	:	630 nm (Hg 623) / Red
Wavelength / filter **Chloride**	:	505 nm (Hg 546) / Green
Temperature	:	R.T.
Light path	:	1 cm.

Sodium Assay :

1. Precipitation :

Pipette into a clean dry test tubes labelled as Standard (S) and Test (T)

Addition Sequence	S (ml)	T (ml)
Precipitating Reagent (L1)	1.0	1.0
Na^+/K^+ Standard (S)	0.02	-
Sample	-	0.02

Mix well and let stand at R.T. for 5 min. with shaking well intermittently. Centrifuge at 2500 to 3000 RPM to obtain a clear supernatant.

2. Colour Development :

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 (L2)	1.0	1.0	1.0
Supernatant from Step 1.	-	0.02	0.02
Precipitating Reagent (L1)	0.02	-	-
Colour Reagent (L3)	0.1	0.1	0.1

Mix well and incubate at R.T. for 5 min. Measure the absorbance of the Blank (Abs.B), Standard (Abs.S), and Test Sample (Abs.T) against distilled water within 15 min.

Potassium Assay :

Pipette into clean dry test tubes labeled as Blank (B), Standard (S), and Test (T)

Addition Sequence	B (ml)	S (ml)	T (ml)
Potassium Reagent (L1)	1.0	1.0	1.0
Deionised water	0.02	-	-
Na^+/K^+ Standard (S)	-	0.02	-
Sample	-	-	0.02

Mix well and incubate at R.T. for 5 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against Blank, within 15 min.

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 R.T. for 2 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against Blank, within 60 min.

Calculations

Sodium Assay :

$$\text{Sodium in mmol/l} = \frac{\text{Abs.B} - \text{Abs.T}}{\text{Abs.B} - \text{Abs.S}} \times 150$$

Potassium Assay :

$$\text{Potassium in mmol/l} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 5$$

Chloride Assay :

$$\text{Chloride in mmol/l} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 100$$

Linearity

The Sodium assay is linear upto 200 mmol/l. The Potassium assay is linear upto 8 mmol/l. The Chloride assay is linear between 70-140 mmol/l. If values exceed this limit, dilute the sample with deionised water (free from Na^+ / K^+ / Cl^- ions) and repeat the assay. Calculate the value using the proper dilution factor.

Notes

Bring all reagents to R.T. before use.
The sodium reaction is an inverse reaction and hence the blank has higher absorbance than the standard or test. During precipitation, inadequate shaking or centrifugation will result in lower values.
Separate serum from the clot as soon as possible as potassium may leach from the RBC's which have a very high potassium level.
Turbid or icteric samples may produce falsely elevated results
The procedure for chloride measures total halides such as bromides, iodides, & 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 & reading.
This common package insert is supplied with the Chloride Kit, Elyte 2 Kit (Na^+/K^+) and the Elyte 3 Kit (Na^+/K^+/Cl^-)

References

IMaruna, R.F.L., (1958) Clin. Chem. Acta. 2 : 581
Trinder, P., (1951) Analyst 76 : 596
Terri, A.E., et. al. (1958) J. Clin. Path. 29 : 86
Sunderman, F.W., et. al. (1959) Am. J. Clin. Path. 29 : 95
Schales, O., Schales, S.S., (1941) J. Biol. Chem. 140 : 879
Schoenfeld, R.G., Lewellen, C.J., (1964) Clin. Chem. 10 : 553

System Parameters, Na^+

Reaction	: End Point	**Interval**	: ---
Wavelength	: 530 nm	**Sample Vol.**	: 0.02 ml
Zero Setting	: Reagent Blank	**Reagent Vol.**	: 1.10 ml
Incub. Temp.	: R.T.	**Standard**	: 150 mmol/l
Incub. Time	: 5 min.	**Factor**	: ---
Delay Time	: ---	**React. Slope**	: Decreasing
Read Time	: ---	**Linearity**	: 200 mmol/l
No. of read.	: ---	**Units**	: mmol/l

System Parameters, K^+

Reaction	: End Point	**Interval**	: ---
Wavelength	: 630 nm	**Sample Vol.**	: 0.02 ml
Zero Setting	: Reagent Blank	**Reagent Vol.**	: 1.00 ml
Incub. Temp.	: R.T.	**Standard**	: 5.0 mmol/l
Incub. Time	: 5 min.	**Factor**	: ---
Delay Time	: ---	**React. Slope**	: Increasing
Read Time	: ---	**Linearity**	: 8.0 mmol/l
No. of read.	: ---	**Units**	: mmol/l

System Parameters, Cl^-

Reaction	: End Point	**Interval**	: ---
Wavelength	: 505 nm	**Sample Vol.**	: 0.01 ml
Zero Setting	: Reagent Blank	**Reagent Vol.**	: 1.00 ml
Incub. Temp.	: R.T.	**Standard**	: 100 mmol/l
Incub. Time	: 2 min.	**Factor**	: ---
Delay Time	: ---	**React. Slope**	: Increasing
Read Time	: ---	**Linearity**	: 70 - 140 mmol/l
No. of read.	: ---	**Units**	: mmol/l

Serological and immunological test

Experiment : Study of precipitation, agglutination and coagulation test.

Study of Immunoprecipitation: Immunoprecipitation: Quantitative precipitin assay

AIM: To analyze quantitative analysis of precipitation reaction between antigen and antibody in solution.

INTRODUCTION: Immunoprecipitation (IP) involves the interaction between a protein and its specific antibody. This technique provides a rapid and simple means to separate a specific protein from whole cell lysate or culture supernatants. We can use immunoprecipitation to confirm the identity or study biochemical characteristics, post-translational modifications, and expression levels of a protein of interest. This assay can be used to determine the point of equivalence in antigen-antibody interaction.

PRINCIPLE: Antigen-antibody interacts in certain proportion to form precipitate. Increasing amounts of antigen are added to a constant amount of antibody and the rate of precipitation formed in each tube is determined spectrophotometrically. Optimal ratio of antibody to antigen allows the maximal precipitation.

MATERIALS: Antigen, specific antibody to the antigen, PBS (pre chilled), 0.1M sodium hydroxide, microfuge tubes, micropipette and tips, glasswares, centrifuge, spectrophotometer.

PROCEDURE:

1. Add antigen, phosphate buffered saline, antibody to a series of numbered centrifuge tubes provided as given below:

Microfuge tubes Nos.	1	2	3	4	5	6
Antigen (µl)	0	10	30	50	100	250
PBS (µl)	250	240	220	200	150	0
Antibody (µl)	50	50	50	50	50	50

2. Mix the reagents thoroughly and incubate at 37°C for one hour and then incubate at 4°C for overnight.

3. Centrifuge at 10000 rpm for 10 minutes, discard the supernatant. An angle head rotor may be used as the precipitate to form at the side of the tube, thus facilitate to remove the supernatant.

4. Wash the precipitate by re-suspending the pellet with 1 ml PBS and centrifugation at 10000 rpm for 10 minutes.

5. Aspirate the supernatant carefully and dissolve the pellet/ precipitate in 1 ml of 0.1 M NaOH.

6. Measure the absorbance at 280 nm by using 1 ml of 0.1 M Sodium Hydroxide as blank.

RESULT & CALCULATION:

Calculate the protein content of al tubes from the following formula:

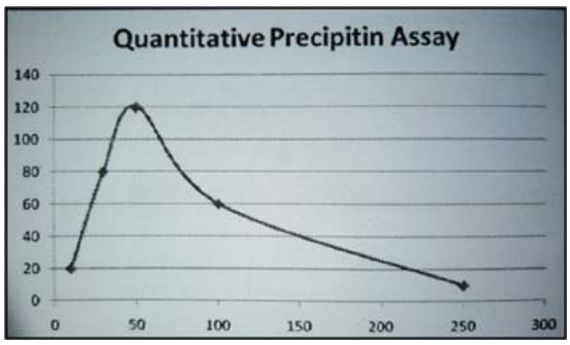
Protein content in the precipitate = (A280 X M)/1.4 mg

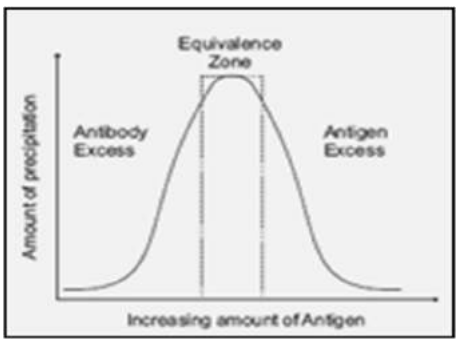
Where M is the amount of NaOH added in "ml" and 1.4 is the Extinction Coefficient.

Record the absorbance values and protein content as follows:

Tube No.	Absorbance at 280nm	Protein Concentration (µg)
1.		
2.		
3.		
4.		
5.		

Plot the amount of protein (in the precipitate) in µg/tube on Y axis and corresponding antigen added (in µg) on X axis as follows:





From the graph, get the A280 values and the corresponding amount of antigen at the point of equivalence which is the point where the maximum amount of precipitate forms.

Amount of active antibody in antiserum = (Pm – Ag)/V

Where; Pm = Amount of maximum precipitate in µg

Ag = Amount of antigen in the tube

V = Volume of antibody (antiserum) taken in µl

INTERPRETATION:

The Equivalence point, when the entire antigen – antibody complex in the precipitate occurs just before the point of maximum precipitation. If the supernatant from each tube is examined for the presence of excess antigen or antibody, there will be one point at which no free antigen or antibody can be detected. This is the point of equivalence which occurs just before maximum precipitation. The amount of precipitation increases after the equivalence point because of continued incorporation of antigen into complex. Eventually, soluble complexes are formed in excess of antigen and the amount of precipitate decreases.

Agglutination reactions: Determination of IgM titer by hemagglutination reaction.

AIM: To quantify the antibody-mediated hemagglutination reaction by determining the hemagglutination titer.

INTRODUCTION:

Agglutination is defined as the formation of clumps of cells or inert particles by specific antibodies to surface antigenic components (direct agglutination) or to antigenic components adsorbed or chemically coupled to red cells or inert particles (passive hemagglutination and passive agglutination, respectively).

PRINCIPLE: IgM antibodies agglutinate foreign cells such as RBC bearing appropriate antigenic determinants on their surface. The concentration of such antibodies is measured indirectly by determining strength of their agglutination reactions upon two-fold serial dilution. In this agglutination assay, the highest dilution of the antiserum that causes complete agglutination of RBC represents the hemagglutination titer for the sample. The titer values are directly proportional to the strength of the activity or concentration of the antibodies in the given sample.

MATERIALS: Test tubes (5ml), test tube stand, pipettes (1 ml & 5 ml),conical flask, human A or B RBC, antiserum (commercially available anti-A or anti-B serum depending on the type of RBC to be used).

REAGENTS:

(1) 0.9% Saline: Dissolve 900 mg NaCl in 100 ml of glass distilled water (0.9% saline) and to this add 20 mg sodium azide (0.02%) as preservative.

(2) Alsever's solution: Dissolve 10.25 gm dextrose, 4 gm sodium citrate, 0.28 gm citric acid and 2.10 gm sodium chloride successively in 500 ml distilled water. Autoclave this solution for 15min at 15 pounds per square inch (psi).

(3) Citrate-saline solution: dissolve 4 gm sodium citrate and 850 mg sodium chloride in 100 ml distilled water and autoclave this solution at 15 psi for 15 min. This solution has to be freshly prepared.

(4) RBC suspension: collect about 10 ml of human blood sample in a conical flask containing 25 ml of Alsever's solution or citrate-saline solution. The blood sample thus collected can be stored for up to 2 (with citrate saline solution) or 7 days (with Alsever's solution). Prior to use add about 1 ml of this RBC suspension to 9 ml of saline and mix well. Centrifuge the RBC suspension at 1500 rpm for 5 min and decant the supernatant. Again add 9 ml of saline and repeat the procedure. Remove the supernatant carefully without disturbing the pellet, take 150µl of RBC pellet and resuspend it in 10 ml of saline to get 1.5% RBC suspension.

PROCEDURE:

1. Pipette out 25 µl of saline to each test tube in a row 6 test tubes

2. Add 25 µl of antiserum to the first test tube and mix it well.

3. Take 25 µl from the first tube and add it to the second tube and then mix it well.

4. Then take 25 µl from the second tube repeat the above procedure up to tube 5

5. Finally take 25 µl from test tube no 5 and discard it.

6. Add 50 µl of 1.5% human RBC suspension to each tube including test tube no. 6 (serves as control). Mix all tubes well.

7. Close all the tubes with aluminium foil, hold them vertically in the test tube stand and leave it at room temperature for 1 hr.

8. Note down the final dilution of anti-serum in each test tube.

9. Examine all the tubes for the occurrence of hemagglutination of RBC.

10. Determine the hemagglutination titer based on the reciprocal of the highest dilution of the antiserum which causes complete agglutination of human RBC.

RESULTS:

Record the hemagglutination titers obtained with a neat diagram indicating hemagglutination reaction at each dilution step.

INFERENCE:

Relate the titer values to the concentration of antibodies in the test sample.

1C. Coagulation test: Detection of hemophilia by coagulation test

INTRODUCTION: Clotting prevents excessive bleeding when there is cut. But the blood moving through our vessels shouldn't clot. If such clots form, they can travel through your bloodstream to your heart, lungs, or brain. This can cause a heart attack, stroke, or even death. Coagulation tests measure our blood's ability to clot, and how long it takes to clot. Testing can help your doctor assess your risk of excessive bleeding or developing clots (thrombosis) somewhere in your blood vessels. Hemophilia, an inherited bleeding disorder, occurs when blood clotting factors are faulty or missing. It almost always affects males. Bleeding can happen both internally and externally.

Blood tests are then performed to determine how much factor VIII or factor IX is present. These tests will show which type of hemophilia you have and whether it is mild, moderate, or severe, depending on the level of clotting factor in the blood:

• People who have 5%-30% of the normal amount of clotting factors in their blood have mild hemophilia.

• People with 1%-5% of the normal level of clotting factors have moderate hemophilia.

• People with less than 1% of the normal clotting factors have severe hemophilia.

PRINCIPLE: Whole blood clotting time and is a measure of the plasma clotting factors. It is a screening test for coagulation disorders. Various other tests for coagulation disorders include: prothrombin time (PT), partial thromboplastin time with kaolin (PTTK) or activated partial thromboplastin time with kaolin (APTTK), and measurement of fibrinogen.

PROCEDURE:

Lee-white method:

1. Obtain at least 3ml of blood in a plastic syringe by careful vein puncture (start a stop watch).

2. Place 1ml of blood into each of the three tubes.

3. Place the test tube in a water bath at 37°C.

4. After 2 minutes one of the three test tubes is tipped gently at one-minute interval.

5. Test the third test tube in the same manner.

6. The time elapsed between the first appearance of the blood in the syringe and clot formation in the third tube is clotting time.

Capillary tube method:

REQUIREMENT: Capillary tube, filter paper, clock watch.

PROCEDURE:

1. Clean the tip of a finger with spirit.

2. Puncture it upto 3 mm deep with a disposable needle.

3. Start the stopwatch.

4. Fill two capillary tubes with free flowing blood from the

5. puncture after wiping the first drop of blood.

6. Keep these tubes at body temperature.

7. After 2 minutes, start breaking the capillary tube at 1 cm distance to see whether a thin fibrin strand is formed between the two broken ends.

8. Stop the watch and calculate the time from average of the two capillary tubes.

INTERPRETATION:

If blood coagulation found prolonged or not coagulated, the patient may suffering from hemophilia.

Normal value

• Lee- white method in glass tube --- 3-12 minutes

• Capillary tube method---3-15 minute in horse and cattle---1-5 minutes other animals

Prolonged

• Deficiency in coagulation factors.

• Vitamin K deficiency. • Thrombocytopenia

• The presence of circulating anticoagulants. • Afibrinogenaemia.

• Administration of heparin. • Disseminated intravascular coagulation (DIC).

• Administration of drugs such as anticoagulants.

Antigen-Antibody reaction testing by Ouchterlony test :

AIM: To analyze the precipitation reaction between soluble antigen and IgG antibody by double immune-diffusion test.

PRINCIPLE:

When soluble antigen and an appropriate type of antibody (IgG) are allowed to diffuse towards each other in an agar medium, antigen-antibody complexes are formed at the optimal concentrations (=zone of equivalence). These complexes spontaneously aggregate to form visible line or band of precipitation in the agar. The precipitin bands are very useful in detecting the reactions between IgG and the antigens and to identify the cross reactivity of specific antibodies with other antigens. This method of analyzing precipitation reaction between antigen and antibody is called double immuno-diffusion test or Ouchterlony double diffusion test (Garvey et al., 1979).

MATERIALS: Agar (agarose with no or low electro-endo-osmosis), antigen and antiserum (sheep serum and anti-sheep serum; commercially available), amido black, methanol, acetic acid, microscope glass slides, well cutter, Pasteur pipettes, petri dish, staining jar and filter paper.

REAGENTS: (1) Borate- buffered saline (BBS: pH 8.5): Dissolve 0.618 gm boric acid, 0.953gm borax (disodium tetra borate) and 0.438 gm NaCl in 100 ml of distilled water. Adjust pH of this solution to 8.5 using 2 N HCl or 0.1 N NaOH solution. Prepare BBS by mixing 5 parts of buffer with 95 parts of 0.9% saline. Dissolve 5 mg Thimerasal or merthiolate (a microbial agent) in 10 ml BBS. (2) 0.9% saline: Dissolve 900 mg NaCl in 100 ml distilled water to obtain 0.9% saline and to this add 20 mg (0.02%) sodium azide as preservative. (3) Staining solution: Mix methanol, acetic acid and water in the ratio of 4.5:1:4.5.

PROCEDURE:

1. Dissolve 10 mg agar in 10 ml of distilled water (0.1% agar) in a glass test tube and ensure complete dissolution by heating in a boiling water bath.

2. Pre-coat the glass sides by evenly spreading about 2 ml of this solution on each slide and allowing it to dry in a closed chamber for 2 to 3 hrs at room temperature.

3. Add 500 mg agar to 5 ml BBS in a test tube and soak it for few minutes.

4. Dissolve the agar completely by heating in a boiling water bath and this solution contains 1% agar.

5. Place the pre-coated glass slides on a flat surface and distribute 3 or 4 ml of hot agar solution on each slide to obtain an even gel coat. First pour the agar solution on the slides and then on the top of the slides.

6. Cool these slides for 30 min in a closed chamber at room temperature and then for 5 min at 10□C to solidify the agar.

7. Cut the wells in the agar using a well-cutter (Template) according to the pattern given provided.

8. Remove agar from the wells by using a pasteur pipette attached to a rubber teat or by suction. This is easily achieved by inserting the pipette vertically into the center of each precut well and completely aspiring the gel to produce wells of uniform dimension.

9. Fill well 2 in slide No. 1 and well 3 in slide No.2 each with 10 µl of antiserum using a pipetteman or a capillary tube.

10. Fill wells 1 and 3 in slide No. 1 and wells 1 and 2 in slide no 2 with 10 µl of antigens as described for the antiserum.

11. Allow the diffusion of antigens and antiserum for 24 hrs in a humid chamber at room temperature.

12. Observe the slides for the development of precipitation line against a dark background and record your observations.

13. Wash away the unreacted proteins by placing the slides in a petri dish containing 0.9% saline containing 0.0% sodium azide over a period of 48 hrs. Change the medium with fresh saline once in 12 hrs.

14. Wash the slides with distilled water containing 0.02% sodium azide for 48hrs to remove the salts from the gels. Change the medium with fresh distilled water once in 24hrs.

15. Cover the slides by rolling with filter paper and allow it to dry for 1 hr at 60° C.

16. Moisten the filter paper by spraying water, remove paper carefully and recover the dried gels firmly attached on the glass slides.

17. Stain the precipitation line by immersing the slides for 3 min in a staining jar containing 0.6% amido black solution.

18. Remove access stain by transferring the slides to a staining jar containing destaining solution.

19. Remove the slides from the destaining solutions and air-dry them in a vertical position.

RESULTS: Using the stained preparation of slides, draw the precipitin lines formed between antigen and antibody wells.

INFERENCE: 1. Comment on the antigen – antibody reactions based on the number of precipitin lines formed. 2. Discuss the merits and limitations of this test.

Experiment : Quantitative assay of Immunoglobins in plasma (IgG, IgM)

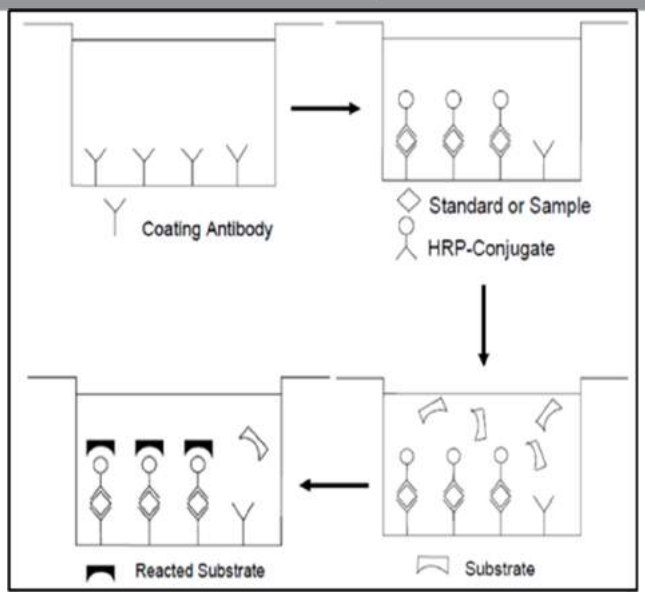
AIM: Enzyme-linked Immunosorbent Assay for quantitative detection of human total IgG in human serum or plasma.

INTRODUCTION: IgG is the major immunoglobulin in blood, lymph fluid, cerebrospinal fluid, and peritoneal fluid and a key player in the humoral immune response. Serum IgG in healthy humans presents approximately 15% of total protein beside albumins, enzymes, other globulins and many more. The Fc portion of IgG, but not F(ab´)2 or Fab fragments, can cross the placenta of a mother to enter the fetal circulation providing the fetus with postpartum protection. IgG molecules are able to react with Fc γ receptors that are present on the surfaces of macrophages, neutrophils, natural killer cells, and can activate the complement system. The

binding of the Fc portion of IgG to the receptor present on a phagocyte is a critical step in the opsonizing property IgG provides to the immune response. Phagocytosis of particles coated with IgG antibodies is a vital mechanism to cope with microorganisms. IgG is produced in a delayed response to an infection and can be retained in the body for a long time. The longevity in serum makes IgG most useful for passive immunization by transfer of this antibody. Detection of IgG usually indicates a prior infection or vaccination.

PRINCIPLE: The Human IgG solid-phase sandwich ELISA (enzyme-linked immunosorbent assay) is designed to measure the amount of the target bound between a matched antibody pair. A target-specific antibody has been pre-coated in the wells of the supplied microplate. Samples, standards, or controls are then added into these wells and bind to the immobilized (capture) antibody. The sandwich is formed by the addition of the second (detector) antibody, a substrate solution is added that reacts with the enzyme-antibody-target complex to produce measurable signal. The intensity of this signal is directly proportional to the concentration of target present in the original specimen.

An anti-human total IgG coating antibody is adsorbed onto microwells. Human total IgG present in the sample or standard binds to antibodies adsorbed to the microwells and a HRP-conjugated antihuman total IgG antibody is added and binds to human total IgG captured by the first antibody. Following incubation unbound HRP-conjugated anti-human total IgG antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of human total IgG present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human total IgG standard dilutions and human total IgG sample concentration determined.



MATERIALS:

Reagents Supplied in Kit:

• 1aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human total IgG.

• 1vial (70 µL) HRP-Conjugate anti-human total IgG monoclonal antibody.

• 2 vials human total IgG Standard lyophilized, 0.2 µg/mL upon reconstitution.

• 3 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

• 1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

• 1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

• 1 vial (15 mL) Stop Solution (1M Phosphoric acid)

• 2 Adhesive Films

Storage instructions: Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C to 8°C). Expiry of the kit and reagents is stated on labels do not use the kit after expiry date. Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

Sample collection and storage instructions:

Serum and plasma (citrate, heparin, EDTA) were tested with this assay. Other biological samples might be suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic samples. Samples should be aliquoted and must be stored frozen at –20°C to avoid loss of bioactive human total IgG. Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required (Apart from Kit):

• 5 mL and 10 mL graduated pipettes.

• 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips.

• 50 µL to 300 µL adjustable multichannel micropipette with disposable tips.

• Multichannel micropipette reservoir.

• Beakers, flasks, cylinders necessary for preparation of reagents.

• Device for delivery of wash solution (multichannel wash bottle or automatic wash system).

• Microwell strip reader capable of reading at 450 nm (620 nm as reference wave length).

• Glass-distilled or deionized water.

• Statistical calculator with program to perform regression analysis.

Preparation of reagents:

1. Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.

2. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

Wash buffer (1x)

1. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water. Mix gently to avoid foaming.

2. Transfer to a clean wash bottle and store at 2°C to 25°C. Wash Buffer (1x) is stable for 30 days.

3. Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer (20x) (mL)	Distilled Water (mL)
1-6	25	475
1-12	50	950

Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer Concentrate (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.

2. Store at 2°C to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

3. Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer (20x) (mL)	Distilled Water (mL)
1-6	5	95
1-12	10	190

HRP-Conjugate

The HRP-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated HRP-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (1x) (mL)
1-6	0.03	2.97
1-12	0.06	5.94

Human total IgG standard

1. Reconstitute human total IgG standard by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard =0.2 µg/mL).

2. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.

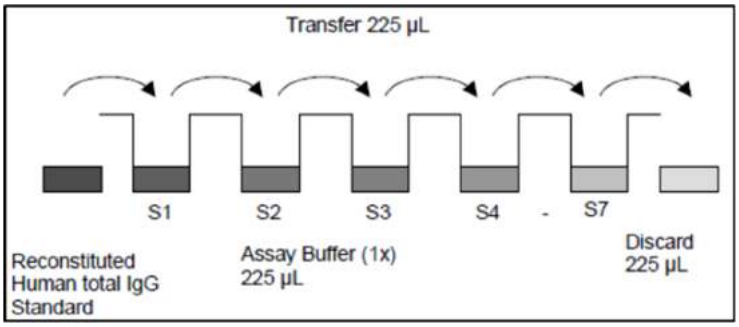
2. Prepare 2-fold serial dilutions for the standard curve as follows: Pipette 225 µL of Assay Buffer (1x) into each tube.

3. Pipette 225 µL of reconstituted standard (concentration =0.2 µg/mL) into the first tube, labeled S1, and mix (concentration of S1 = 0.1 µg/mL.

4. Pipette 225 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.

5. Repeat serial dilutions 5 more times thus creating the points of the standard curve.

6. Assay Buffer (1x) serves as blank.



PROCEDURE:

1. Pre-dilute samples before starting with the test procedure. Dilute serum and plasma samples 1:500,000 with Assay Buffer (1x) according to the following scheme:

10 µL sample + 990 µL Assay Buffer (1x) = Pre-dilution A

10 µL Predilution A + 990 µL Assay Buffer (1x) = Pre-dilution B

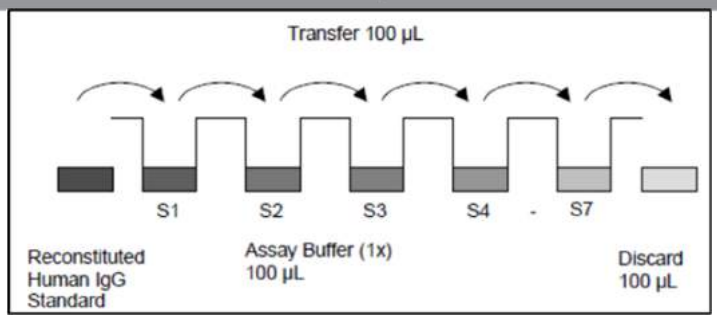
10 µL Predilution B + 490 µL Assay Buffer (1x) = Final Pre-dilution

2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°C to 8°C sealed tightly.

3. Prepare HRP-conjugated antibody.

4. Wash the microwell strips twice with approximately 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10–15 seconds before aspiration. Do not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

5. Standard dilution on the microwell plate. Add 100 µL of Assay Buffer (1x) in duplicate to all standard wells. Pipette 100 µL of prepared standard (concentration = 0.2 µg/mL), in duplicate, into well A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 0.1 µg/mL), and transfer 100 µL to wells B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human total IgG standard dilutions, ranging from 0.1 µg/mL to 0.002 µg/mL. Discard 100 µL of the contents from the last microwells (S7) used.



In case of an external standard dilution, pipette 100 µL of these standard dilutions (S1–S7) in the standard wells according to Table 1.

6. Add 100 µL of Assay Buffer (1x) in duplicate to the blank wells.

7. Add 100 µL of each final prediluted sample in duplicate to the sample wells.

8. Add 50 µL of diluted HRP-conjugated antibody to all wells, including the blank wells.

Table 1 Example of the arrangement of blanks, standards, and samples in the microwell strips.

	1	**2**	**3**	**4**
A	Standard 1 0.1 µg/mL	Standard 1 0.1 µg/mL	Sample 1	Sample 1
B	Standard 2 0.05 µg/mL	Standard 2 0.05 µg/mL	Sample 2	Sample 2
C	Standard 3 0.025 µg/mL	Standard 3 0.025 µg/mL	Sample 3	Sample 3
D	Standard 4 0.013 µg/mL	Standard 4 0.013 µg/mL	Sample 4	Sample 4

E	Standard 5 0.006 µg/mL	Standard 5 0.006 µg/mL	Sample 5	Sample 5
F	Standard 6 0.003 µg/mL	Standard 6 0.003 µg/mL	Sample 6	Sample 6
G	Standard 7 0.002 µg/mL	Standard 7 0.002 µg/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

9. Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 1 hour on a microplate shaker.

10. Remove adhesive film and empty wells. Wash microwell strips 4 times according to point 3. of the test protocol. Proceed immediately to the next step.

11. Pipette 100 µL of TMB Substrate Solution to all wells.

12. Incubate the microwell strips at room temperature (18°C to 25°C) for 30 minutes. Avoid direct exposure to intense light. The color development on the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively, the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9–0.95.

13. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2°C to 8°C in the dark.

14. Read absorbance of each microwell on a ELISA reader using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank

the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

CALCULATION:

• Calculate the average absorbance values for each set of duplicate standards and samples.

• Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human total IgG concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).

• To determine the concentration of circulating human total IgG for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human total IgG concentration.

• If samples have been diluted 1:500,000 and the concentration read from the standard curve must be multiplied by the dilution factor (x 500,000).

• It is suggested that each testing facility establishes a control sample of known human total IgG concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

Table 2 Sample data of the human total IgG ELISA:

Standard	human total IgG Concentration (µg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm
1	0.100	2.213 2.169	2.191
2	0.050	1.182 1.248	1.215
3	0.025	0.613 0.671	0.642
4	0.013	0.358 0.376	0.367
5	0.006	0.213	0.219

		0.224	
6	0.003	0.128 0.136	0.132
7	0.002	0.092 0.099	0.095
Blank	0.000	0.048 0.052	0.050

Quantitative assay of Immunoglobins in plasma (IgM)

AIM: Enzyme-linked Immunosorbent Assay for quantitative detection of human IgM in human serum or plasma.

INTRODUCTION:

Immunoglobulin M is the third most common serum Ig and can exist as pentamer where all heavy chains are identical and all light chains are identical or as a monomer (e.g., found on B lymphocytes as B – cell receptors). The large pentameric structure allows for building of bridges between encountered epitopes on molecules that are too distant as to be connected by smaller IgG antibodies. IgM is built as the first antibody during an immune response and is responsible for agglutination and cytolytic reactions since its pentameric structure gives it 10 free antigen binding sites in theory and it posseses a high avidity. Due to conformational constraints among the 10 Fab portions, IgM only has a valence of 5. Additionally, IgM is not as versatile as IgG, but it is of vital importance in complement activation and agglutination. IgM is predominantly found in the lymph fluid and the blood and is a very effective neutralizing agent in the early stages of disease. Elevated levels can be a sign of recent infection or exposure to antigen.

PRINCIPLE:

An anti-human IgM coating antibody is adsorbed onto microwells. Human IgM present in the sample or standard binds to antibodies adsorbed to the microwells and a HRP-conjugated anti-human IgM antibody is added and binds to human IgM captured by the first antibody. Following incubation unbound HRP-conjugated anti-human IgM antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of human IgM present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IgM standard dilutions and human IgM sample concentration determined.

Reagents provided

•1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human IgM.

•1 vial (70 µL) HRP-Conjugate anti-human IgM monoclonal antibody

•2 vials human IgM Standard lyophilized, 2000 ng/mL upon reconstitution

•2 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

•1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

•1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

•1 vial (15 mL) Stop Solution (1M Phosphoric acid)

•2 Adhesive Films

Remaining Protocol, Calculation same as IgG.

RPR test

AIM:

Rapid Plasma Reagin (RPR) Test for the diagnosis of Syphilis.

INTRODUCTION:

Rapid plasma reagin (RPR) is macroscopic, non treponemal, flocculation card test used to screen for syphilis caused by *Treponema pallidum.* RPR is simple test can be done within few minutes. This test is less sensitive than treponemal test in early syphilis infection. Two types of antibodies are produced in syphilis i.e. autoantibody (cardiolipin) response and treponemal antibody response. Autoantibodies are produced in 2-3 weeks of treponemal infection due to tissue damage. These auto antibodies are often referred to as cardiolipin antibodies because they can be detected in serological test using cardiolipin antigen. This test doesn't look for antibodies against actual bacterium but rather for antibodies against substances released by cells they are damaged by *Treponema pallidum.* The anti-lipodial antibodies are antibodies that are not produce only in syphilis infection but also in other non treponemal disease of an acute and chronic nature in which tissues are damaged. RPR measures IgM and IgG antibodies to lipodial materials released from damaged host cells as well as lipoprotein like material and possibly cardiolipin released from treponems. Antigen used in RPR test contain cardiolipin lecithin, cholesterol, 10% choline chloride, EDTA, charcoal in buffer. This test cannot be performed on CSF. Serum or plasma can be used for testing, serum not heated. This test tends to give negative results during late syphilis.

PRINCIPLE:

RPR is 18 mm circle card test is a macroscopic flocculation test for syphilis. The antigen is prepared from modified VDRL (Venereal Disease Research Laboratory), antigen suspension containing choline chloride and EDTA (ethylenediamine tetra acetic acid) to enhance stability of suspension, finely divided charcoal particles as visualizing agents. In this test antigen is mixed with unheated serum on plastic –coated card. This test measures IgM & IgG antibodies to lipodial material released from damaged host cells as well as possibly cardiolipin released from treponems. If antibodies are present, they combine with lipid particles of the antigen, causing them to agglutinate. The charcoal particles co-agglutinate with antibodies and shows black clumps on white cards. If antibodies are not present, the test mixture is uniformly gray.

REAGENTS:

1. RPR antigen suspension: RPR antigen suspension is a stabilized combination of 0.003% cardiolipin, 0.020-0.022% lecithin, 0.09% cholesterol, 10% choline chloride, 0.0125M EDTA, 0.01875% charcoal, 0.01M Na2HP04, 0.01M KH2P04, 0.1% thimerosal in distilled water.

2. Control serum samples: Control serum samples are lyophilized reactive (R), minimally reactive (Rm), and nonreactive (N) control serum specimens on a card, or liquid or lyophilized serum samples of graded reactivity. If quantitative tests are to be performed, a control serum that can be titered to at least a 1:4 dilutions should be used.

3. 0.9% Saline: Add 0.9 gm of dry sodium chloride (ACS) to 100 ml of distilled water.

4. Diluent: Prepare a 2% solution of human serum in 0.9% saline, by diluting a human serum nonreactive for syphilis 1:50 in 0.9% saline.

PROCEDURE:

Qualitative Test

1. Place 50µl of serum or plasma on 18mm circle of RPR test using a disposable dispenstirs or a safety pipetting device.

2. Spread serum or plasma to fill the entire circle. Don’t spread the specimen beyond the confines of the circle.

3. Gently shake the antigen dispensing bottles to re-suspend the particle.

4. Dispense several drops of antigen (17µl of ag) suspension to each circle containing serum or plasma.

5. Mix the suspension well in one direction.

6. Rotate card for 4-8 mins and observed for flocculation.

Quantitative Test

1. Dilute the endpoint titre all serum specimen with rough non-reactive results in qualitative test. Test each specimen undiluted (1:1) and in 1:2, 1:4, 1:8, 1:16 dilution.

2. Place 50µl of 0.9% saline in circles. Don't spread saline.

3. Using safety pipette device, place 50µl of serum in circle labeled 1 and 50µl of serum in circle 2. Mix the saline and serum in circles.

4. Transfer 50µl from circle 2 (1:2) to circle3, & mix

5. Transfer 50µl from circle 3 (1:4) to circle 4 &Mix

6. Same way transfer 50µl from circle (1:8) to circle (1:16), mix and discard the last 50µl.

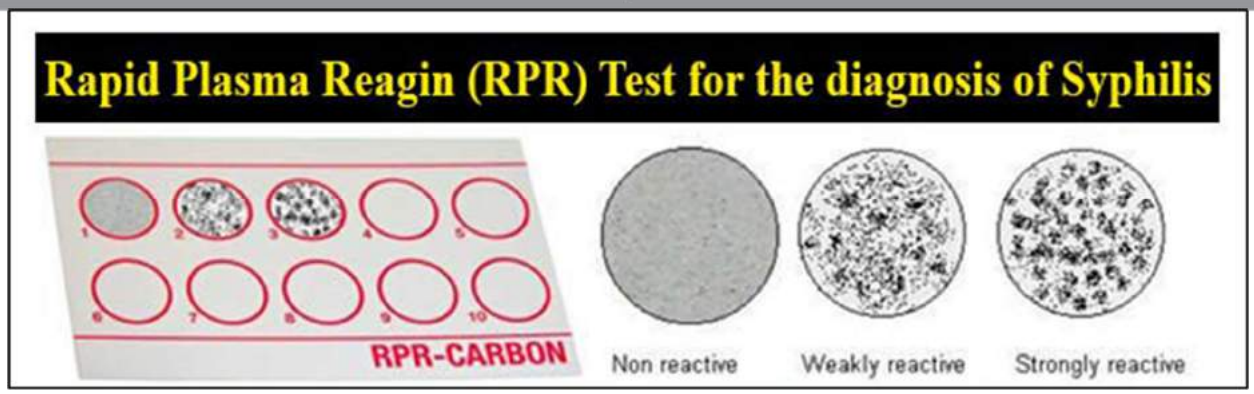
7. Spread the serum dilution using clean dispenstirs to fill entire circle.

8. Gently shake the dispensing bottles to re-suspend the antigen particles.

9. Add (17µl of ag) antigen suspension in each circle.

10. Place the card in rotator for 8 min at 100v 2rpm under humidifying cover.

11. Remove card from rotator and tilt the card by hand (three or four to and fro motions) to aid in differentiating non-reactive from minimally reactive results.



RESULTS AND INTERPRETATION:

Positive Result (Reactive): Clumping (Characteristic clumping ranging from marked and intense (reactive) to Reactive (R) slight but definite (minimally to moderately) reactive).

Negative Result (Non-Reactive): No Clumping or slight roughness.

WIDAL test

AIM:

To rapid detect the typhoid from serum sample.

INTRODUCTION:

Widal Test is an agglutination test which detects the presence of serum agglutinins (H and O) in patients serum with typhoid and paratyphoid fever. When facilities for culturing are not available, the Widal test is the reliable and can be of value in the diagnosis of typhoid fevers in endemic areas. It was developed by Georges Ferdinand Widal in 1896. The patient's serum is tested for O and H antibodies (agglutinins) against the following antigen suspensions (usually stained suspensions):

S. typhi 0 antigen suspension, 9, 12

S. typhi H antigen suspension, d

S. paratyphi A 0 antigen suspension, 1, 2, 12

S. paratyphi A H antigen suspension, a

S. paratyphi B 0 antigen suspension, 1, 4, 5, 12

S. paratyphi B H antigen suspension, b, phase 1

S. paratyphi C 0 antigen suspension, 6, 7

S. paratyphi C H antigen suspension, c, phase 1

Salmonella antibody starts appearing in serum at the end of first week and rise sharply during the 3rd week of endemic fever. In acute typhoid fever, O agglutinins can usually be detected 6–8 days after the onset of fever and H agglutinins after 10–12 days. It is preferable to test two specimens of sera at an interval of 7 to 10 days to demonstrate a rising antibody titre. Salmonella antigen suspensions can be used as slide and tube techniques.

PRINCIPLE:

Bacterial suspension which carry antigen will agglutinate on exposure to antibodies to Salmonella organisms. Patients' suffering from enteric fever would possess antibodies in their sera which can react and agglutinate serial doubling dilutions of killed, coloured Salmonella antigens in a agglutination test. The main principle of widal test is that if homologous antibody is present in patients serum, it will react with respective antigen in the reagent and gives visible clumping on the test card and agglutination in the tube. The antigens used in the test are "H" and "O" antigens of *Salmonella typhi* and "H" antigen of *S. paratyphi*. The paratyphoid "O" antigen is not employed as they cross react with typhoid "O" antigen due to the sharing of factor 12. "O" antigen is a somatic antigen and "H" antigen is flagellar antigen.

Preparation of Widal Antigens

•H suspension of bacteria is prepared by adding 0.1 per cent formalin to a 24 h broth culture or saline suspension of an agar culture.

•For preparation of O suspensions of bacteria, the organisms are cultured on phenol agar (1:800) to inhibit flagella.

•Standard smooth strains of the organism are used; *S Typhi* 901, O and H strains are employed for this purpose.

•The growth is then emulsified in small volume of saline, mixed with 20 times its volume of alcohol, heated at 40° C to 50° C for 30 minutes and centrifuged.

•The antigens are treated with chloroform (preservative) and appropriate dyes are added for easy identification of antigens.

•The growth is then emulsified in small volume of saline, mixed with 20 times its volume of alcohol, heated at 40° C to 50° C for 30 minutes and centrifuged.

•The antigens are treated with chloroform (preservative) and appropriate dyes are added for easy identification of antigens.

ASO test

AIM:

For the qualitative and semi-quantitative measurement of antibodies to streptococcal exoenzymes in human serum.

INTRODUCTION:

The group A β-hemolytic streptococci produces various toxins that can act as antigens. One of these exotoxins Streptolysin O was discovered by Todd in 1932. A person infected with group A β -hemolytic streptococci produces specific antibodies against these exotoxins, one of which is anti-streptolysin O (ASO). The quantity of this antibody in a patient's serum will establish the degree of infection due to the β-hemolytic streptococcal. The usual procedure for the determination of the antistreptolysin titer is based on the inhibitory effect that the patient's serum produces on the hemolytic power of a pretitrated and reduced Streptolysin O. However, the antigen-antibody reaction occurs independently of the hemolytic activity of Streptolysin O. This property enables the establishment of a qualitative and quantitative test for the determination of the ASO by agglutination of latex particles on slide.

PRINCIPLE:

ASO test method is based on an immunological reaction between streptococcal exoenzymes bound to biologically inert latex particles and streptococcal antibodies in the test sample. The reagent has been adjusted in the way that presence of an ASO titer of 200 IU/mL or higher in the serum gives a visible agglutination of the latex particles without previous sample dilution.

REAGENTS & SPECIMEN/SAMPLE:

1. ASO Latex Reagent: A suspension of polystyrene particles coated with streptococcal exoenzymes. MIX WELL BEFORE USING.

2. ASO Positive Control: A stabilized human serum containing at least 200 IU/mL of ASO reactive with the test reagent. Ready for use; do not dilute.

3. ASO Negative Control: A stabilized human serum containing less than 200 IU/mL of ASO non- reactive with the test reagent. Ready for use; do not dilute.

4. Glycine-Saline Buffer (20x) pH = 8.2 ±0.1. A diluent containing 0.1 M glycine and 0.15 M NaCl. Dilute buffer according to instructions on the label. All reagents contain 0.1% (w/v) sodium azide as a preservative. Store all reagents at 2 - 8°C.

REAGENT STORAGE AND STABILITY

1. Reagents are stable until stated expiration date on bottle label when stored refrigerated (2 - 8°C).

2. The ASO Latex Reagent, once shaken, must be uniform without visible clumping. When stored refrigerated, a slight sedimentation may occur and should be considered normal.

3. Do not use the latex reagent or controls if they become contaminated.

SPECIMEN COLLECTION AND STORAGE

1. Use fresh serum collected by centrifuging clotted blood. Patient may be instructed not to eat (fast) six hours before the test.

2. If the test cannot be carried out on the same day, the serum may be stored between 2 - 8°C for no longer than 48 hours after collection. For longer periods the sample must be frozen.

3. As in all serological tests, hemolytic or contaminated serum must not be used.

4. DO NOT USE PLASMA.

PRECAUTIONS

1. Reagents containing sodium azide may combine with copper and lead plumbing to form highly explosive metal azides. Dispose of reagents by flushing with large amounts of water to prevent azide build-up.

2. For in vitro diagnostic use.

3. Positive and negative controls predated using human sera found negative for hepatitis B surface antigen (HBsAg) and HIV, however, handle controls as if potentially infectious.

MATERIALS AND REAGENTS PROVIDED

1. ASO Latex Reagent.

2. ASO Positive Control.

3. ASO Negative Control.

4. Glycine - Saline Buffer.

5. Reaction Slide.

6. Pipette/Stir Sticks.

OTHER MATERIALS REQUIRED:

1. Timer

2. Test Tubes, Rack

3. Serological Pipettes

PROCEDURE:

Qualitative Test:

1. Bring reagents and specimens to room temperature before use.

2. Place one drop (50 µl) of ASO Positive Control on field #1 of the reaction slide. Place one drop (50 µl) of the ASO Negative Control on field #2 of the reaction slide. Use pipette/stir stick to deliver 1 drop (50µl) of undiluted test serum sample to field #3. Continue likewise with additional unknowns. Retain pipette/stir sticks for mixing step.

3. Gently resuspend the ASO Latex Reagent and add one drop to each test field.

4. Mix well with the flat end of the pipette. Gently rock the slide for two (2) minutes and read immediately under direct light.

Semi-quantitative Test:

1. Set up at least five test tubes: 1:2, 1:4, 1:8, 1:16, 1:32, etc. and dilute samples according to dilution factors on each test tube with diluted saline solution NOTE: Saline solution has to be diluted with distilled water before use.

2. Place one drop each of positive and negative controls onto the slide rings. Place one drop of each dilution on successive fields of the reaction slides.

3. Gently resuspend the ASO Latex Reagent and add one drop to each test field.

4. Mix well with the flat end of the pipette. Gently rock the slide for two (2) minutes and read immediately under direct light.

Note:

1. Positive and negative controls should be included in each test batch.

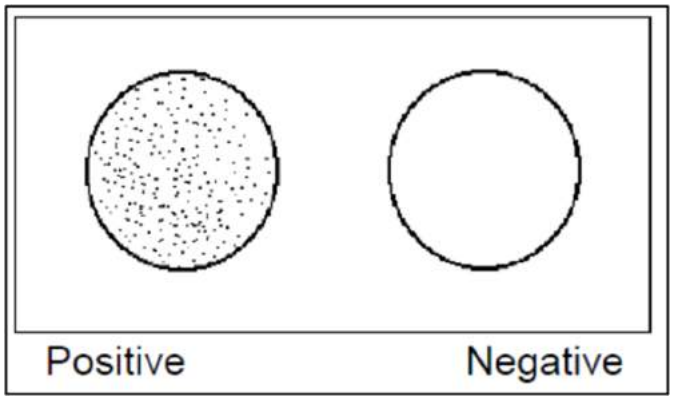
2. Acceptable performance is indicated when a uniform milky suspension with no agglutination is observed with the ASO Negative Control and agglutination with large aggregates is observed with the ASO Positive Control.

3. Results should be read two (2) minutes after the mixing of the reagent on the slide. A reading obtained after this period of time may be incorrect.

RESULTS:

Qualitative Test: 1. Negative reaction: Uniform milky suspension with no agglutination as observed with the ASO Negative Control.

2. **Positive reaction**: Any observable agglutination in the reaction mixture. A positive reaction indicates that the concentration of ASO in the specimen is equal or greater than 200 IU/mL. The specimen reaction should be compared to the ASO Negative Control.



Semi-quantitative Test:

A positive reaction is indicated by any observable agglutination in the reaction mixture. Record the last dilution showing a positive reaction. Concentration of ASO can be determined by multiplying the last positive dilution factor of the sample with the concentration of the positive control (200 IU/ml).

The titer of the serum is the reciprocal of the highest dilution, which exhibits a positive reaction.
IU/ml of sample = Conc. of positive control x reciprocal

DILUTION	RECIPROCAL	IU/ml
1/2	2	400

1/4	4	800
1/8	8	1600

EXPECTED VALUES

1. Normal values can vary with age, season of the year and geographical area, the "upper limit of normal" ASO titers for preschool children is less than 100 IU/ml and in school age children or young adults is usually between 166 and 250 IU/ml. In any case, the average can be established at less than 200 IU/ml.

2. Following acute streptococcal infection, the ASO titer will usually rise after one week, increasing to a maximum level within 3 to 5 weeks and usually returning to the pre-infection levels in approximately 6 to 12 months.

INFERENCE:

The ASO titer normal range is one below 200 in case of adults while an ASO test value below 100 is considered normal for kids below age 5. However, results may slightly vary from lab to lab and your doctor will consider this before suggesting aso titre treatment for any complications. The test may have to be repeated after two weeks in order to confirm the diagnosis since ASO antibodies are produced with a week of the infection. As a thumb rule, increasing antibodies indicates that the infection is recent while decreasing antibodies show that the infection is reducing.

RA (Rheumatoid Arthritis) test:

AIM:

To rapid test the qualitative detection of all subtypes of rheumatoid factor (RF) in human serum, plasma or whole blood at a sensitivity of 8 IU/mL. by a lateral flow immunoassay.

INTRODUCTION:

Rheumatoid factors are human auto-antibodies that bind to the Fc moieties of immunoglobulins leading to tissue damage. Elevated levels of RF are found in 70-90% of rheumatoid arthritis, 75-95% of Sjögren’s syndrome, 95% of Felty’s syndrome cases, as well as a number of connective tissue and inflammatory diseases including infectious mononucleosis, SLE, scleroderma and

hepatitis. The majority of RF are of the IgM subtype, but it can also be of IgG, IgA, IgE or IgD subtypes. IgM RF, IgA RF and IgG RF are seen in 92%, 65% and 66% of RA patients respectively.

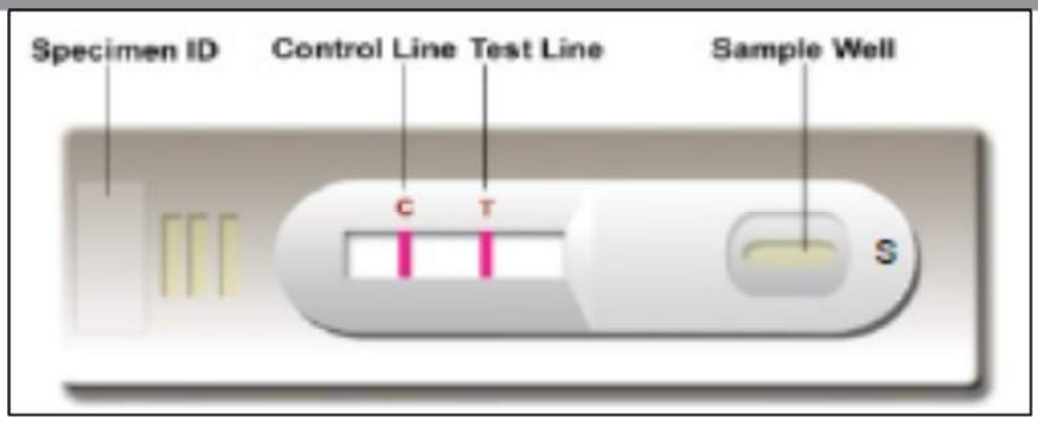
Therefore, detection of all the isotypes of RF is necessary. Pathological values of RF vary with subtypes, test methods and laboratories. Levels of IgM RF are reported to be ≥ 3-15 IU/mL, IgA RF are ≥ 4-15 IU/mL and IgG RF ≥ 6-60 IU/mL. RF is traditionally detected by agglutination assays. In recent years, many laboratories have begun to use nephelometry and enzyme-linked immunosorbent assays (ELISA) for the quantitative detection of RF.

The RF Rapid Test is a lateral flow immunoassay for the qualitative detection of all subtypes of RF in serum, plasma or whole blood. The RF Rapid Test can be performed within 10 minutes by minimally skilled personnel and without the use of laboratory equipment**.**

PRINCIPLE:

The RF Rapid Test is a lateral flow chromatographic immunoassay. The test cassette consists of: 1) a burgundy colored conjugate pad containing immunoglobulins conjugated with colloidal gold (Ig conjugates) and a control antibody conjugated with colloidal gold, 2) a nitrocellulose membrane strip containing a test line (T line) and a control line (C line). The T line is pre-coated with another immunoglobulin for the detection of rheumatoid factor, and the C line is pre-coated with a control line antibody.

When an adequate volume of test specimen is dispensed into the sample well of the test cassette, the specimen migrates by capillary action across the cassette. RF, if present in the specimen, will bind to the Ig conjugates. The immunocomplex is then captured on the membrane by the pre-coated immunoglobulin forming a burgundy colored T line, indicating a level of RF greater than or equal to 8 IU/mL. Absence of the T line suggests that the RF level in the specimen is lower than 8 IU/mL. The test contains an internal control (C line) which should exhibit a burgundy colored line of the immunocomplex of the control antibodies regardless of any color development on the T line. If the C line does not develop, the test result is invalid, and the specimen must be re-tested with another device.



MATERIALS:

REAGENTS PROVIDED

1. Individually sealed foil pouches containing: a. One cassette device; b. One desiccant

2. 5 µL capillary tubes

3. Sample diluent (5 mL/bottle)

4. One package insert (instruction for use)

5. Clock or timer

6. Lancing device for whole blood test

7. RF Positive Control

8. RF Negative Control

All reagents are ready to use as supplied. Store unused test devices unopened at 2-30°C. If stored at 2-8°C, ensure that the test device is brought to room temperature before opening. The test device is stable through the expiration date printed on the sealed pouch. Do not freeze the kit or expose the kit to temperatures above 30°C.

SAMPLE COLLECTION AND HANDLING:

Consider any materials of human origin as infectious and handle them using standard bio-safety procedures.

Plasma/Serum:

Step 1: Collect blood specimen into collection tube containing EDTA, citrate or heparin for plasma or collection tube containing no anticoagulants for serum by venipuncture.

Step 2: To make plasma specimen, centrifuge collected specimens and carefully withdraw the plasma into a new pre-labeled tube.

Step 3: To make serum specimen, allow blood to clot, then centrifuge collected specimens and carefully withdraw the serum into a new pre-labeled tube.

Test specimens as soon as possible after collecting. Store specimens at 2-8°C, if not tested immediately. The specimens can be stored at 2-8°C for up to 5 days. The specimens should be frozen at -20°C for longer storage. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen specimens to room temperature slowly and mix gently. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

Whole Blood:

Drops of whole blood can be obtained by either fingertip puncture or venipuncture. Collect blood specimen into a collection tube containing EDTA, citrate or heparin. Do not use hemolyzed blood for testing. Whole blood specimens should be stored in refrigeration (2-8°C), if not tested immediately. The specimens must be tested within 24 hours of collection.

PROCEDURE:

1: Bring the specimen and test components to room temperature if refrigerated or frozen. Once thawed, mix the specimen well prior to assay.

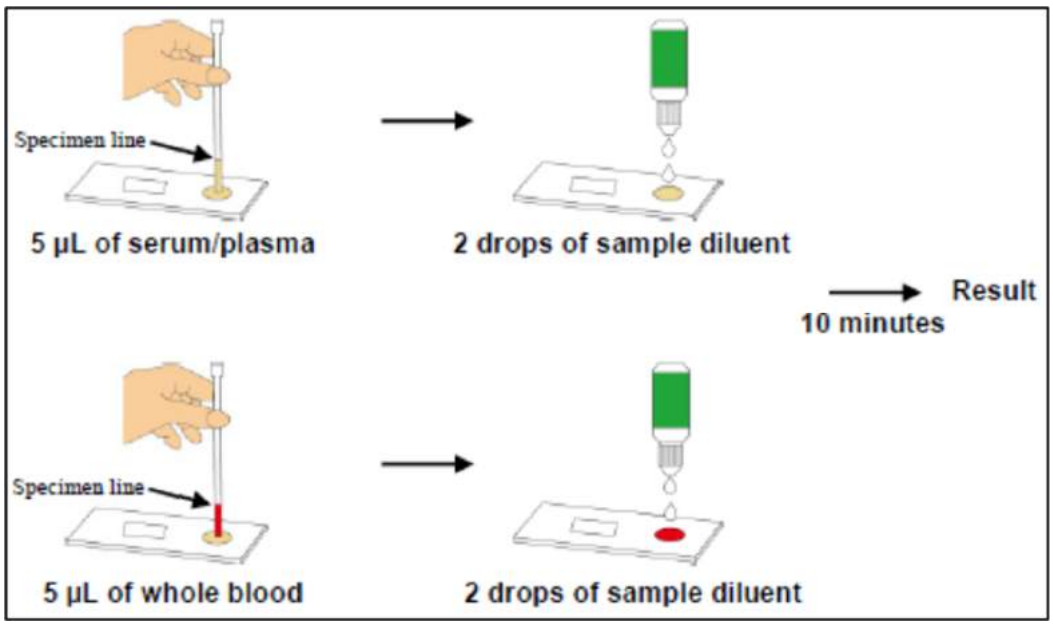
2: When ready to test, open the pouch at the notch and remove the device. Place the test device on a clean, flat surface.

3: Be sure to label the device with the specimen ID number.

4: Using a squeezing motion, fill the capillary tube with specimen (about 5 µL) not to exceed the specimen line. Holding the capillary tube vertically, dispense the entire amount of specimen into the center of the sample well making sure that there are no air bubbles. Immediately add 2 drops (about 60-80 µL) of sample diluent to the sample well with the bottle positioned vertically.

Step 5: Set up timer.

Step 6: Results should be read at 10 minutes. Positive results may be visible in as short as 1 minute. Negative results must be confirmed at the end of the 15 minutes only.



RESULT & INTERPRETATION:

2. NEGATIVE RESULT: If only the C line develops, the test indicates that the level of rheumatoid factor is less than 8 IU/mL. The result is negative or non-reactive.

3. POSITIVE RESULT: If both the C and T lines develop, the test indicates that the level of rheumatoid factor is greater than or equal to 8 IU/mL. The result is positive or reactive.

4. INVALID: If no C line develops, the assay is invalid regardless of color development on the T line as indicated below. Repeat the assay with a new device.

Defined as the 95% detection level, the limit of detection or sensitivity for the RF Rapid Test is 8 IU/mL. Serum RF levels greater than or equal to 8 IU/mL routinely test positive. Samples containing RF less than 8 IU/mL may also produce a very faint positive line.

Detection of C-reactive protein (CRP) in serum/plasma sample by latex test.

AIM:

Rapid detection of CRP in patient sample.

INTRODUCTION:

C-reactive protein (CRP) is an acute-phase protein found in concentrations of up to 5µg/ml in the serum of healthy persons. However, during an increase by as much as one thousand fold. This increase in CRP levels in serum can be used to monitor certain diseases. The changes in concentration of CRP usually be demonstrated in cases of acute myocardial infarction, rheumatoid arthritis, bacterial and viral infections.

PRINCIPLE:

This test is based on the immunologic reaction between CRP as an antigen and latex particles have been coated with mono specific anti- human CRP and sensitized to detect levels greater than 6µg/ ml CRP. The latex slide test has the advantage of rapid performance in comparison to other tests for detection of CRP.

PROPCEDURE:

1. Test serum has to be used undiluted.

2. Using the disposable plastic dropper, place one drop of test serum within the circled area on the special slide provided in the kit.

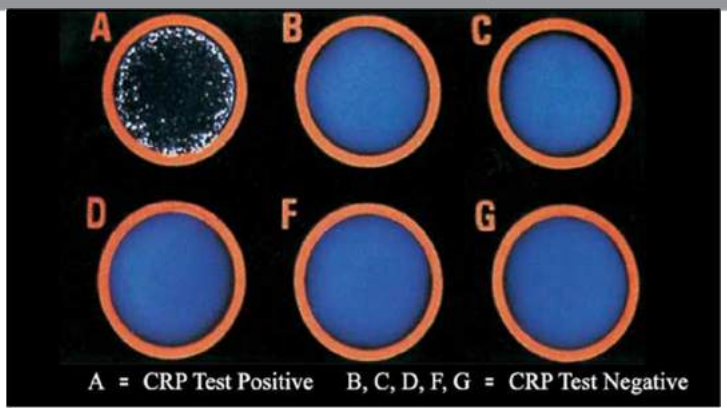
3. Add one drop of latex CRP Reagent (shake well the vial gently immediately before use) above the test serum drop and mix well with a disposable applicator stick & spread out in the test area.

4. Rock the slide gently to and for 2 minutes and examine for macroscopic agglutination under direct light source. Do not examine beyond 2 minutes.

OBSERVATION AND RESULT:

Formation of coarse agglutination shows strong positive, whereas finer agglutination shows weakly positive and negative result indicates smooth suspension without any noticeable change.

Dilution	CRP (ug/ml) in undiluted sample
1:2	14
1:4	28
1:8	56
1:16	112
1:32	224
1:64	448



Positive: Agglutination of latex particles, indicating the presence of C – reactive protein at a significant and detectable level.

Negative: No Agglutination.

For Semi-Quantitative Test Results, the last dilution of serum with visible agglutination is the CRP titre of the serum.

CALCULATION OF TITRE:

CRP ug/ml = 7 x D, where D is the highest dilution of serum showing agglutination and 7 is the sensitivity in ug/ml.

AIDS test

AIM:

To determine HIV-1/2 Ag/Ab Combo is not intended for newborn screening or for use with cord blood specimens or specimens from individuals less than 12 years of age.

INTRODUCTION:

HIV–1/2 Ag/Ab Combo is an in vitro, visually read, qualitative immunoassay for the simultaneous detection of Human Immunodeficiency Virus Type 1 (HIV-1) p24 antigen (Ag) and antibodies (Ab) to HIV Type 1 and Type 2 (HIV-1 and HIV-2) in human serum, plasma, capillary (fingerstick) whole blood or venipuncture (venous) whole blood. It is intended for use as a point-of-care test to aid in the diagnosis of infection with HIV-1 and HIV-2, including an acute HIV-1 infection, and may distinguish acute HIV-1 infection from established HIV-1 infection when the specimen is positive for HIV-1 p24 antigen and negative for anti-HIV-1 and

anti-HIV-2 antibodies. The test is suitable for use in multi-test algorithms designed for the statistical validation of rapid HIV test results. When multiple rapid HIV test are available, this test can be used in appropriate multi-test algorithms.

PRINCIPLE:

HIV–1/2 Ag/Ab Combo is an immunochromatographic test for the simultaneous and separate qualitative detection of free HIV-1 p24 antigen and antibodies to HIV-1 and HIV-2. The test device is a laminated strip that consists of a Sample Pad containing monoclonal biotinylated anti-HIV-1 p24 antibody, a Conjugate Pad containing monoclonal anti-HIV-1 p24 antibody-colloidal selenium and HIV-1 and HIV-2 recombinant antigen-colloidal selenium, and a nitrocellulose membrane with an immobilized mixture of recombinant and synthetic peptide HIV-1 and HIV-2 antigens in the Lower Test Area, immobilized streptavidin in the Upper Test Area, and an immobilized mixture of anti-HIV-1 antibodies, HIV-1/2 antigens, and HIV-1 p24 recombinant antigen and anti-HIV-1 p24 monoclonal antibody in the Control Area.

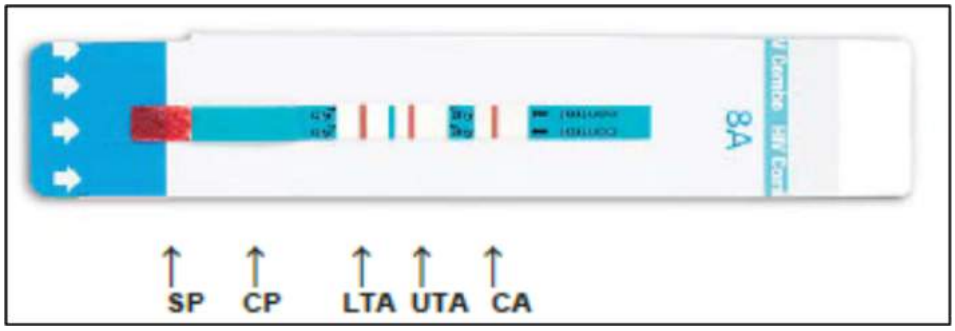
A specimen (venipuncture or capillary whole blood, serum, or plasma) is applied to the Sample Pad (followed by Chase Buffer for venipuncture or fingerstick whole blood specimens) and migrates by capillary action through the Conjugate Pad and then through the nitrocellulose membrane.

If HIV-1 p24 antigen is present in the specimen; it binds with the monoclonal biotinylated anti-HIV-1 p24 antibody from the Sample Pad and then with monoclonal anti-HIV-1 p24 antibody-colloidal selenium from the Conjugate Pad to form a complex (biotinylated antibody-antigen-colloidal selenium-antibody). This complex migrates through the solid phase by capillary action until it is captured by immobilized streptavidin at the Upper Test Area (labeled “Ag”) where it forms a single pink/red “Ag” line. If HIV-1 p24 antigen is not present in the specimen or is below the limit of detection of the test, no pink/red Ag line is formed. NOTE: The monoclonal biotinylated anti-HIV-1 p24 antibody used in this assay does not cross react with HIV-2 p26 antigen.

If antibodies to HIV-1 and/or HIV-2 are present in the specimen, the antibodies bind to recombinant gp41 (HIV-1) and gp36 (HIV-2) antigen-colloidal selenium conjugates from the Conjugate Pad. The complex migrates through the solid phase by capillary action until it is captured by immobilized HIV-1 and HIV-2 synthetic peptide antigens and recombinant gp41 antigen at the Lower Test Area (labeled “Ab”) and forms a single pink/red “Ab” line. If

antibodies to HIV-1 and/or HIV-2 are absent or are below the detection limit of detection of the test, no pink/red Ab line is formed.

To ensure assay validity, a procedural "Control" line containing a mixture of anti-HIV-1 antibody, HIV-1/2 antigens, and HIV-1 p24 recombinant antigen and anti-HIV-1 p24 monoclonal antibody is incorporated in the nitrocellulose membrane. For a test result to be valid there must be a visible pink/red Control line. During the testing procedure the colloidal selenium conjugates released from the Conjugate Pad will be captured by the antibodies and antigens immobilized in the Control Area and form a pink/red Control line for samples that are either positive or negative.



SP = Sample Pad; CP = Conjugate Pad; LTA = Lower Test Area; UTA = Upper Test Area; CA = Control Area

REAGENTS:

Materials:

1. HIV-1/2 Ag/Ab Combo Cards. Each Card consists of 5 or 10 Test Units which can be separated from each other by tearing along the perforated lines. Each Test Unit has a cover that is to be removed for sample application and visualization of test results.

2. Desiccant Package

3. Chase Buffer: Containing sodium chloride, disodium hydrogen phosphate, and Nipasept as a preservative.

4. Quick Reference Guide

5. Package Insert

6. Subject Information Notices: 25 in the 25 Test Units kit, and 100 in the 100 Test Units kit.

7. Customer Letter

8. Disposable Capillary Tubes: For collection and transfer of fingerstick samples.

9. Disposable Workstations: 25 in the 25 Test Units kit, and 100 in the 100 Test Units kit.

Accessory:

• Fingerstick Sample Collection Kit

• HIV–1/2 Ag/Ab Combo Controls:

HIV-1 p24 Antigen Control: 1.5mL, HIV-1 viral lysate in defibrinated pooled normal human plasma; negative for antibodies to HIV-1, HIV-2 and HCV; negative for HBsAg.

HIV-1 Reactive Control: 1.5mL, human plasma positive for anti-HIV-1 antibodies, diluted in defibrinated pooled normal human plasma; negative for antibodies to HIV-2 and HCV; negative for HBsAg.

HIV-2 Reactive Control: 1.5mL, human plasma positive for anti-HIV-2 antibodies, diluted in defibrinated pooled normal human plasma; negative for antibodies to HIV-1 and HCV; negative for HBsAg and HIV-1 p24.

Nonreactive Control: 1.5mL, defibrinated normal human plasma; negative for antibodies to HIV-1, HIV-2, and HCV; negative for HBsAg and HIV-1 p24.

Materials General

• Clock, watch, or other timing device.

• Precision pipette capable of delivering 50µL of sample with disposable tips.

• Disposable gloves

• Sterile gauze (for fingerstick whole blood specimens)

• Antiseptic wipes

• Biohazard disposal container

• Collection devices for specimens (other than fingerstick whole blood specimens)

PROCEDURE:

HIV-1/2 Ag/Ab Combo Controls should be tested prior to testing patient specimens when a new operator performs testing, a new test kit lot is to be used, a new shipment of test kits is received, and at periodic intervals indicated by the testing facility. Controls should be tested in the same manner as serum or plasma samples in the following Test Procedure.

Kit Component Preparation

1. Remove the desired numbers of test units from the 5- or 10-Test Unit Card by bending and tearing at the perforation.

2. Lay the Test Unit flat in the workstation and remove the protective foil cover from each Test

Unit. The test should be initiated within 2 hours after removing the protective foil cover from each Test Unit.

For serum or plasma samples:

1. Apply 50 µL of sample (precision pipette) to the Sample Pad (marked by the arrow symbol). Do not add Chase Buffer when using serum or plasma specimens.

2. Read the test result between 20 and 30 min after the addition of the Sample. Do not read test results after 30 min.

For whole blood (venipuncture) samples:

1. Using a precision pipette with a disposable tip, apply 50 µL of sample to the Sample

Pad (marked by the arrow symbol).

2. Wait for one minute, then apply one drop of Chase Buffer to the Sample Pad.

3. Read the test result between 20 and 30 minutes after the addition of the Chase Buffer.

For whole blood (fingerstick) samples using the Disposable Capillary Tube:

1. Align the tip of the Capillary Tube containing the blood sample with the Sample Pad (marked by the arrow symbol) and gently squeeze the bulb. Avoid air bubbles. Wait until all the blood is transferred from the Capillary Tube to the Sample Pad.

2. Do not lift the Capillary Tube from the Sample Pad before all the blood has been transferred – a bubble may form which will prevent the complete transfer of sample.

3. Wait for one minute, then apply one drop of Chase Buffer to the Sample Pad.

4. Read the test result between 20 and 30 minutes after the addition of the Chase Buffer. Do not read Test Results after 30 minutes.

5. Discard the used pipette tips, Capillary Tube, Test Units and any other test materials into a biohazard waste container.

RESULTS & INTERPRETATION:

1. ANTIBODY REACTIVE (Two Lines - Control Line and Ab Line) A pink/red Control line appears in the Control Area and a pink/red Ab line appears in the Lower Test Area of the Test Unit. The intensity of the Ab and Control lines may vary. Any visible pink/red color in both the Control and Lower Test Areas, regardless of intensity, is considered REACTIVE. A Reactive test result means that HIV-1 and/or HIV-2 antibodies have been detected in the specimen. The test result is interpreted as PRELIMINARY POSITIVE for HIV-1 and/or HIV-2 antibodies.

2. ANTIGEN (HIV-1p24) REACTIVE (Two Lines - Control Line and Ag Line) A pink/red Control line appears in the Control Area and a pink/red Ag line appears in the Upper Test Area of the Test Unit. The intensity of the Ag and Control lines may vary. Any visible pink/red color in both the Control and Upper Test Areas, regardless of intensity, is considered REACTIVE. A Reactive test result means that HIV-1 p24 antigen has been detected in the specimen. The test result is interpreted as PRELIMINARY POSITIVE for HIV-1 p24 antigen.

3. ANTIBODY REACTIVE AND ANTIGEN (HIV-1 p24) REACTIVE (Three Lines - Control, Ab and Ag Lines) A pink/red Control line appears in the Control Area and a pink/red Ab line appears in the Lower Test Area and a pink/red Ag line appears in the Upper Test Area of the Test Unit. The intensity of the Ab, Ag and Control lines may vary. Any visible pink/red color in the Control Area, the Lower Test Area and the Upper Test Area, regardless of intensity, is

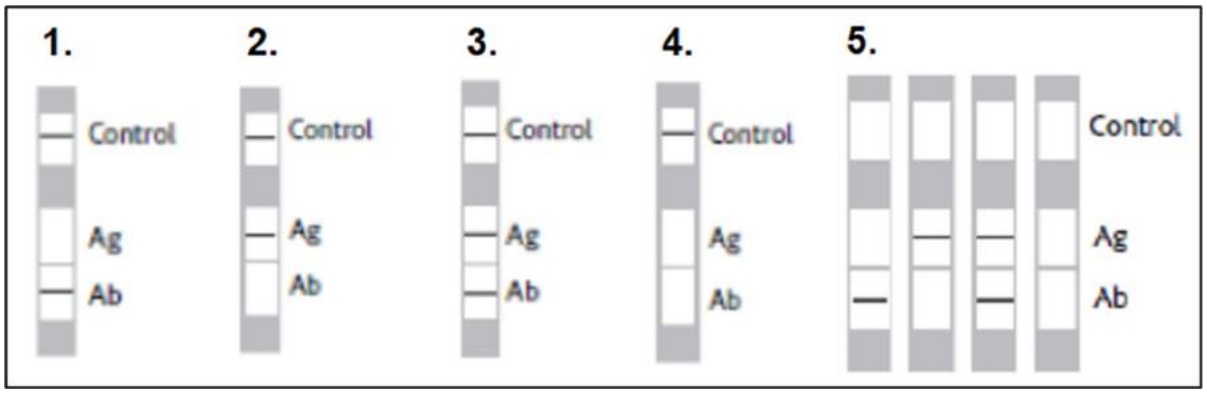
considered REACTIVE. The test result is interpreted as PRELIMINARY POSITIVE for HIV-1 and/or HIV-2 antibodies and HIV-1 p24 antigen.

4. NONREACTIVE (One Line – Control Line)

A pink/red Control line appears in the Control Area of the Test Unit, and no pink/red Ab or Ag line appears in the Lower Test Area and the Upper Test Area of the Test Unit, respectively. A NONREACTIVE test result means that HIV-1 or HIV-2 antibodies and HIV-1 p24 antigen were not detected in the specimen.

5. INVALID (No Control Line)

If there is no pink/red Control line in the Control Area of the Test Unit, even if a pink/red line appears in the Lower Test Area or the Upper Test Area of the Test Unit, the result is INVALID and the test should be repeated. If the problem persists, contact Alere Technical Support.



Dengue test

cromatest LINEAR Chemicals, S.L.

Dengue IgG/IgM cassette CE

CONTENTS			
REF	4273240	Dengue IgG/IgM	40 Tests
For professional *in vitro* diagnostic use only			

Dengue IgG/IgM

A rapid one test for simultaneous detection and differentiation of IgG and IgM anti–dengue virus (DEN 1, 2, 3, and 4) in human serum, plasma or whole blood

ONE STEP

PRINCIPLE

LINEAR Dengue IgG/IgM cassette is intended to be used as a screening test and provides a preliminary test result to aid in the diagnosis of infection with dengue viruses.
Any interpretation of this test result must rely on other clinical findings and on the professional judgment of health care providers. Alternative test method(s) should be considered to confirm the test result obtained.
The test consists of: 1) a burgundy colored conjugate pad containing dengue recombinant envelope antigens conjugated with colloidal gold (dengue Ag conjugates) and a control antibody conjugated with colloidal gold, 2) a nitrocellulose membrane strip containing two test lines (G and M lines) and a control line (C line). The G line is pre-coated with antibodies for the detection of IgG anti-dengue virus, the M line is pre-coated with antibodies for the detection of IgM anti-dengue virus, and the C line is pre-coated with a control line antibody.
When an adequate volume of specimen is dispensed into the sample well of the cassette, the specimen migrates by capillary action along the cassette. IgG anti-dengue virus, if present in the specimen, will bind to the dengue Ag conjugates. The immunocomplex is then captured by the pre-coated anti-human IgG, forming a burgundy colored G line, indicating an IgG anti-dengue virus positive test result and suggesting a secondary or past infection with dengue virus.
IgM anti-dengue virus, if present in the specimen, will bind to the dengue Ag conjugates. The immunocomplex is then captured by the pre-coated anti-human IgM, forming a burgundy colored M line, indicating an IgM anti-dengue virus positive result and suggesting either an acute primary or secondary dengue infection. An IgM and IgG positive result indicates a late primary or early secondary infection.
Absence of any G, M or T lines suggests a negative result. Each test contains an internal control (C line) which should exhibit a burgundy colored line of the control antibodies regardless of color development on any of the test lines. If the C line does not develop, the test result is invalid and the specimen must be retested with another device.

PACKAGING CONTENTS

REF 4273240 40 Dengue IgG/IgM test device
40 Capillary tubes (5 µL)
1 Sample diluent (5 mL)

STORAGE AND STABILITY

Store at 2-30°C. The test device is stable through the expiration date printed on the sealed pouch. The test device must remain in the sealed pouch until use. **Do not freeze the kit or expose the kit over 30°C**. Do not use beyond the expiration date.

SPECIMEN COLLECTION AND PREPARATION

Serum, (EDTA, citrate or heparin) or plasma unhemolyzed.
Test specimens as soon as possible after collecting. Store specimens at 2°C-8°C if not tested immediately. Stable up to 5 days at 2-8°C or frozen at -20°C for longer storage. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen specimens to room temperature slowly and mix gently. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Do not use samples demonstrating gross lipemia, gross hemolysis or turbidity in order to avoid interference on result interpretation.

Whole Blood
Drops of whole blood can be obtained by either finger tip puncture or veinpuncture. Do not use any hemolized blood for testing.
Store at 2-8°C if not tested immediately. The specimens must be tested within 24 hours of collection.
Consider any materials of human origin as infectious and handle them using standard biosafety procedures.

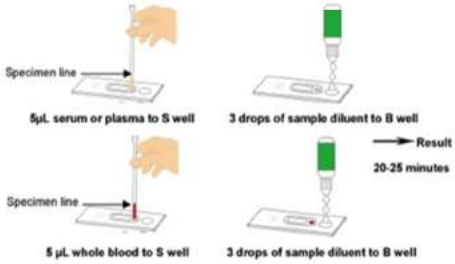
MATERIAL REQUIRED

- Timer or Clock

PROCEDURE

Allow test device, specimen, and/or controls to equilibrate to room temperature (15-30°C) prior to testing.

1. Remove the test device from the sealed pouch and use it as soon as possible.
2. Be sure to label the device with specimen's ID number.
3. Fill the capillary tube with the serum, plasma or whole blood specimen not exceeding the specimen line as shown in the image below. The volume of the specimen is around 5µL. ***For better precision, transfer the specimen by a pipette capable of delivering 5µL of volume.***
   Holding the capillary tube vertically, dispense the entire specimen (5 µL) into the center of the sample well **(S well)** making sure that there are no air bubbles.
   Immediately add 3 drops (about 90-120 µL) of Sample Diluent into the buffer well **(B well)** with the bottle positioned vertically.



4. Set up a timer.
5. Read the result at 20-25 minutes. Positive results may be visible in as short as 1 minute. Negative results must be confirmed at the end of the 25 minutes only. **Any results interpreted outside of the 20-25 minute window should be considered invalid and must be repeated. Discard used devices after interpreting the result following local laws governing the disposal of devices.**

NEGATIVE: If only the C band is present, the absence of any burgundy color in the both test bands (G and M) indicates that no anti- dengue virus antibodies are detected. The result is negative or non-reactive.

POSITIVE:
1. Two distinct red lines appear. C band and if only G band is developed, indicates the presence of IgG anti- dengue virus; the result suggests past infection or re-infection of dengue virus.

QUALITY SYSTEM CERTIFIED ISO 9001 ISO 13485

LINEAR CHEMICALS S.L. Joaquim Costa 18 2ª planta. 08390 Montgat, Barcelona, SPAIN
Telf. (+34) 934 694 990 Fax. (+34) 934 693 435. website www.linear.es

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cromatest LINEAR Chemicals, S.L.

Dengue IgG/IgM cassette CE

CONTENTS			
REF	4273240	Dengue IgG/IgM	40 Tests
For professional *in vitro* diagnostic use only			

Dengue IgG/IgM

A rapid one test for simultaneous detection and differentiation of IgG and IgM anti–dengue virus (DEN 1, 2, 3, and 4) in human serum, plasma or whole blood

ONE STEP

PRINCIPLE

LINEAR Dengue IgG/IgM cassette is intended to be used as a screening test and provides a preliminary test result to aid in the diagnosis of infection with dengue viruses.
Any interpretation of this test result must rely on other clinical findings and on the professional judgment of health care providers. Alternative test method(s) should be considered to confirm the test result obtained.
The test consists of: 1) a burgundy colored conjugate pad containing dengue recombinant envelope antigens conjugated with colloidal gold (dengue Ag conjugates) and a control antibody conjugated with colloidal gold, 2) a nitrocellulose membrane strip containing two test lines (G and M lines) and a control line (C line). The G line is pre-coated with antibodies for the detection of IgG anti-dengue virus, the M line is pre-coated with antibodies for the detection of IgM anti-dengue virus, and the C line is pre-coated with a control line antibody.
When an adequate volume of specimen is dispensed into the sample well of the cassette, the specimen migrates by capillary action along the cassette. IgG anti-dengue virus, if present in the specimen, will bind to the dengue Ag conjugates. The immunocomplex is then captured by the pre-coated anti-human IgG, forming a burgundy colored G line, indicating an IgG anti-dengue virus positive test result and suggesting a secondary or past infection with dengue virus.
IgM anti-dengue virus, if present in the specimen, will bind to the dengue Ag conjugates. The immunocomplex is then captured by the pre-coated anti-human IgM, forming a burgundy colored M line, indicating an IgM anti-dengue virus positive result and suggesting either an acute primary or secondary dengue infection. An IgM and IgG positive result indicates a late primary or early secondary infection.
Absence of any G, M or T lines suggests a negative result. Each test contains an internal control (C line) which should exhibit a burgundy colored line of the control antibodies regardless of color development on any of the test lines. If the C line does not develop, the test result is invalid and the specimen must be retested with another device.

PACKAGING CONTENTS

REF 4273240 40 Dengue IgG/IgM test device
40 Capillary tubes (5 µL)
1 Sample diluent (5 mL)

STORAGE AND STABILITY

Store at 2-30ºC. The test device is stable through the expiration date printed on the sealed pouch. The test device must remain in the sealed pouch until use. **Do not freeze the kit or expose the kit over 30°C**. Do not use beyond the expiration date.

SPECIMEN COLLECTION AND PREPARATION

Serum, (EDTA, citrate or heparin) or plasma unhemolyzed.
Test specimens as soon as possible after collecting. Store specimens at 2°C-8°C if not tested immediately. Stable up to 5 days at 2-8°C or frozen at -20°C for longer storage. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen specimens to room temperature slowly and mix gently. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Do not use samples demonstrating gross lipemia, gross hemolysis or turbidity in order to avoid interference on result interpretation.

Whole Blood
Drops of whole blood can be obtained by either finger tip puncture or veinpuncture. Do not use any hemolized blood for testing.
Store at 2-8°C if not tested immediately. The specimens must be tested within 24 hours of collection.
Consider any materials of human origin as infectious and handle them using standard biosafety procedures.

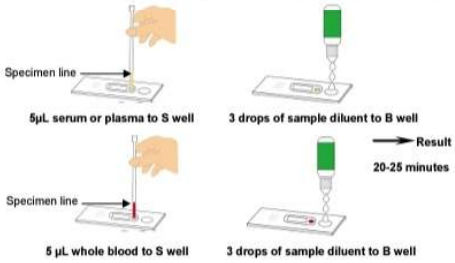
MATERIAL REQUIRED

- Timer or Clock

PROCEDURE

Allow test device, specimen, and/or controls to equilibrate to room temperature (15-30°C) prior to testing.

1. Remove the test device from the sealed pouch and use it as soon as possible.
2. Be sure to label the device with specimen's ID number.
3. Fill the capillary tube with the serum, plasma or whole blood specimen not exceeding the specimen line as shown in the image below. The volume of the specimen is around 5µL. ***For better precision, transfer the specimen by a pipette capable of delivering 5µL of volume.***
   Holding the capillary tube vertically, dispense the entire specimen (5 µL) into the center of the sample well **(S well)** making sure that there are no air bubbles.
   Immediately add 3 drops (about 90-120 µL) of Sample Diluent into the buffer well **(B well)** with the bottle positioned vertically.



4. Set up a timer.
5. Read the result at 20-25 minutes. Positive results may be visible in as short as 1 minute. Negative results must be confirmed at the end of the 25 minutes only. **Any results interpreted outside of the 20-25 minute window should be considered invalid and must be repeated. Discard used devices after interpreting the result following local laws governing the disposal of devices.**

NEGATIVE: If only the C band is present, the absence of any burgundy color in the both test bands (G and M) indicates that no anti- dengue virus antibodies are detected. The result is negative or non-reactive.

POSITIVE:
1. Two distinct red lines appear. C band and if only G band is developed, indicates the presence of IgG anti- dengue virus; the result suggests past infection or re-infection of dengue virus.

QUALITY SYSTEM CERTIFIED ISO 9001 ISO 13485

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2. In addition to the presence of C band, if only M band is developed, the test indicates for the presence of IgM anti-dengue virus. The result suggests fresh infection of dengue virus.

3. In addition to the presence of C band, both G and M bands are developed, indicates for the presence of IgG and IgM anti-dengue virus. The result suggests current infection or secondary infection of dengue virus.

Samples with positive results should be confirmed with alternative testing method(s) and clinical findings before a diagnosis is made.

INVALID:
If no C band is developed, the assay is invalid regardless of any burgundy color in the test bands (G and M) as indicated below. Repeat the assay with a new device.

QUALITY CONTROL

An internal procedural control is included in the test. A colored line appearing in the control line region (C) is considered an internal procedural control. It confirms sufficient specimen volume, adequate membrane wicking and correct procedural technique.
External controls are not supplied with this kit; however, it is recommended that positive and negative controls be tested as good laboratory testing practice to confirm the test procedure and to verify proper test performance. particularly under the following circumstances:

a. A new operator uses the kit, prior to performing testing of specimens.
b. A new lot of test kit is used.
c. A new shipment of kits is used.
d. The temperature during storage of the kit falls outside of 2-30°C.
e. The temperature of the test area falls outside of 15-30°C.
f. To verify a higher than expected frequency of positive or negative results.
g. To investigate the cause of repeated invalid results.

CLINICAL SIGNIFICANCE

Dengue virus is an enveloped, single-stranded, positive-sense RNA virus that comprises four related but distinct serotypes (DEN1, 2, 3, and 4). The virus is transmitted by mosquitoes of the daytime-biting *Stegemyia* family, principally *Aedes aegypti* and *Aedes albopictus*. Today, more than 2.5 billion people living in areas of tropical Asia, Africa, Australia, and the Americas are at risk for dengue infection. An estimated 100 million cases of dengue fever and 250,000 cases of life-threatening dengue hemorrhagic fever occur annually on a worldwide basis[1-3]. Serological detection is a common method for the diagnosis of infection with dengue virus. IgM anti-dengue virus starts to appear 3 days after initial exposure and remains in circulation for about 30-60 days. IgG anti-dengue virus levels rise around 7 days, peak at 2-3 weeks and persist for the duration of life[4-6].

ANALYTICAL PERFORMANCE

Clinical Performance For IgM Test
A total of 314 specimens were collected from susceptible subjects and tested with the Linear Dengue IgG/IgM cassette and by a commercial EIA. Comparison for all subjects is shown in the following table:

	Linear Dengue IgG/IgM Cassette		
IgM EIA	**Positive**	**Negative**	**Total**
Positive	31	1	32
Negative	3	279	282
Total	34	280	314

Relative Sensitivity: 96.9%, Relative Specificity: 98.9%, Overall Agreement: 98.7%

Clinical Performance For IgG Test
A total of 326 specimens were collected from susceptible subjects and tested with the Linear Dengue IgG/IgM cassette and by a commercial EIA. Comparison for all subjects is shown in the following table:

	Linear Dengue IgG/IgM Cassette		
IgG EIA	Positive	Negative	Total
Positive	36	1	37
Negative	2	287	289
Total	38	288	326

Relative Sensitivity: 97.3%, Relative Specificity: 99.3%, Overall Agreement: 99.1%.

Cross Reactivity
No false positive IgG and IgM anti-dengue virus test results were observed on 1-13 specimens from the following disease states or specific conditions, respectively:

HAV	HBV	HCV	HEV	HIV	*H. pylori*
CMV	Chagas	Chikungunya	hCG	Rubella	*T. gondii*
Typhi	*T. pallidum*	ANA	HAMA	RF (up to 8,400 IU/mL)	

LIMITATIONS OF TEST

1. This package insert must be followed closely when testing the presence of antibodies to dengue virus from individual subjects. Failure to follow the procedure may lead to inaccurate results.
2. The Linear Dengue IgG/IgM cassette is limited to the qualitative detection of IgG and IgM anti–dengue virus. The intensity of the test band does not have linear correlation with the antibody titer in the specimen.
3. Information about the dengue virus serotype(s) present in a specimen cannot be provided from this test.
4. The Linear Dengue IgG/IgM cassette cannot differentiate primary or secondary infection.
5. Serological cross-reactivity with other flaviviruses is common (e.g., Japanese encephalitis, West Nile virus, yellow fever, etc.). Therefore, it is possible that patients who were exposed to these viruses may show some level of reactivity with this test.
6. A negative or non-reactive result for an individual subject indicates absence of detectable dengue virus antibodies. However, a negative result does not preclude the possibility of exposure to or infection with dengue virus.
7. A negative result can occur if the quantity of antibodies to dengue virus present in the specimen is below the limits of detection, or if the antibodies that are detected are not present during the stage of disease in which a sample is collected.
8. Some specimens containing unusually high titer of heterophile antibodies or rheumatoid factor may affect expected results.
9. Infection may progress rapidly. If the symptoms persist, while the result is negative or non-reactive, it is recommended to test with an alternative test method.
10. The results obtained with this test should be interpreted in conjunction with other diagnostic procedures and clinical findings.

PRECAUTIONS

1. Do not open the sealed pouch, unless ready to conduct the assay.
2. Do not use expired devices.
3. Do not use the components of any other type of test kit as a substitute for the components in this kit.
4. Do not use hemolyzed blood specimen for testing.
5. Wear protective clothing and disposable gloves while handling the kit reagents and clinical specimens. Wash hands thoroughly after performing the test.
6. Users of this test should follow the US CDC Universal Precautions for prevention of transmission of HIV, HBV and other blood-borne pathogens.
7. Dispose of all specimens and materials used to perform the test as bio-hazardous waste.

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3. Monath TP. Dengue: The risk to developed and developing countries. Proc Natl Acad Sci U S A 1994;91:2395–2400.
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5. Innis BL, and Nisalak A, et al: An enzyme-linked immunosorbent assay to characterize dengue infections where denude and Japanese encephalitis co-circulate. Am. J. Trap. Med. Hygiene. 1989: 40: 418-427.
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LINEAR CHEMICALS S.L. Joaquim Costa 18 2ª planta. 08390 Montgat, Barcelona, SPAIN
Telf. (+34) 934 694 990 Fax. (+34) 934 693 435. website www.linear.es

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Montoux test

AIMS:

To rapid detect of tuberculosis.

INTRODUCTION:

The Mantoux test or Mendel–Mantoux test (also known as the Mantoux screening test, tuberculin sensitivity test, Pirquet test, or PPD test for purified protein derivative) is a tool for screening for tuberculosis (TB) and for tuberculosis diagnosis. It is one of the major tuberculin skin tests used around the world. The Mantoux test is endorsed by the American Thoracic Society and Centers for Disease Control and Prevention. It was also used in the USSR and is now prevalent in most of the post-Soviet states.

PRINCIPLE:

Tuberculin is a glycerol extract of the tubercle bacillus. Purified protein derivative (PPD) tuberculin is a precipitate of species-nonspecific molecules obtained from filtrates of sterilized, concentrated cultures. This active agent is tuberculin, a protein. A person who has been exposed to the bacteria is expected to mount an immune response in the skin containing the bacterial proteins. The response is a classical example of delayed-type hypersensitivity reaction (DTH), a type IV of hypersensitivities. T cells and myeloid cells are attracted to the site of reaction in the timeframe of 1-3 days and generate local inflammation. The reaction is read by measuring the diameter of induration (palpable raised, hardened area) across the forearm (perpendicular to the long axis) in millimeters. If there is no induration, the result should be recorded as "0 mm". Erythema (redness) should not be measured. In the Pirquet version of the test tuberculin is applied to the skin via scarification.

MATERIALS:

Tuberculin, Alcohol swab, Syringe, Scale.

PROCEDURE:

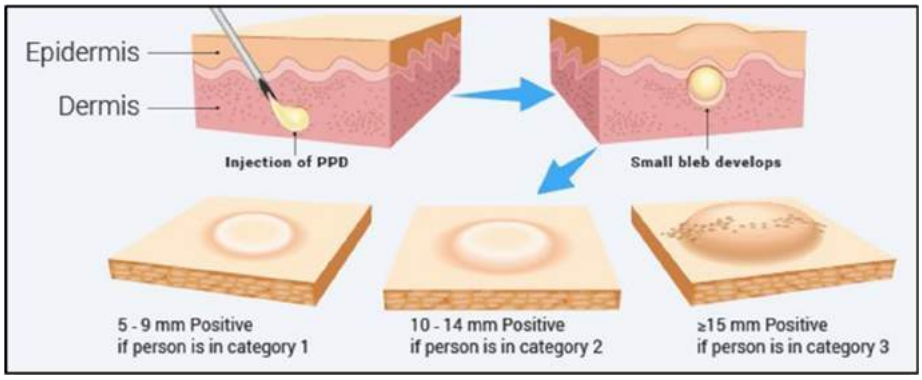
1. In the Mantoux test, a standard dose of 5 tuberculin units (TU - 0.1 ml), according to the CDC, or 2 TU of Statens Serum Institute (SSI) tuberculin RT23 in 0.1 ml solution, according to the NHS, is injected intra-dermally (between the layers of dermis) on the flexor surface of the left forearm, mid-way between elbow and wrist.

2. The injection should be made with a tuberculin syringe, with the needle bevel facing upward. Alternatively, the probe can be administered by a needle-free jet injector.

3. When placed correctly, injection should produce a pale wheal of the skin, 6 to 10 mm in diameter.

4. The result of the test is read after 48-96 hours but 72 hours (3rd day) is the ideal.

5. This intradermal injection is termed the Mantoux technique.



RESULTS & INTERPRETATION:

The person's medical risk factors determine at which increment (5 mm, 10 mm, or 15 mm) of induration the result is considered positive. A positive result indicates TB exposure.

According to the guidelines published by Centers for Disease Control and Prevention in 2005, the results are re-categorized into 3 parts based on their previous or baseline outcomes:

•Baseline test: ≥10 mm is positive (either first or second step); 0 to 9 mm is negative

•Serial testing without known exposure: Increase of ≥10 mm is positive.

Known exposure:

o≥5 mm is positive in patients with baseline of 0 mm

o≥10 mm is positive in patients with negative baseline or previous screening result of >0 mm

Diameter 5 mm or more is positive:

An HIV-positive person; Persons with recent contacts with a TB patient; Persons with nodular or fibrotic changes on chest X-ray consistent with old healed TB; Patients with organ transplants, and other immunosuppressed patients.

Diameter 10 mm or more is positive: Recent arrivals (less than five years) from high-prevalence countries; Injection drug users; Residents and employees of high-risk congregate settings (e.g., prisons, nursing homes, hospitals, homeless shelters, etc.); Mycobacteriology lab personnel; Persons with clinical conditions that place them at high risk (e.g., diabetes, prolonged corticosteroid therapy, leukemia, end-stage renal disease, chronic malabsorption syndromes, low body weight, etc.); Children less than four years of age, or children and adolescents exposed to adults in high-risk categories.

Diameter 15 mm or more is positive: Persons with no known risk factors for TB.

A tuberculin test conversion is defined as an increase of 10 mm or more within a two-year period, regardless of age. Alternative criteria include increases of 6, 12, 15 or 18 mm.