BACHELOR OF MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

3rd Semester

Prepared By

Paramedical & Allied Science Dept.

BMLT

MIDNAPORE CITY COLLEGE

Haematology: Paper-VII, Unit-14

Technique of blood collection

Blood is collected from the vein for various haematological investigations. In order to obtain accurate and precise results in the laboratory which will help the clinician to make a correct diagnosis of the patient's disease, it is of paramount importance to collect the blood sample in a correct manner. Each sample is sent to the laboratory accompanied by a laboratory requisition form filled in by the clinician. Brief clinical details and any other relevant information must be mentioned on the form. Prior to blood sample collection it is essential to check the patient's identity and make sure that it corresponds to the name and other details mentioned on the requisition form. Blood can be withdrawn from the vein, usually the antecubital vein on the forearm (Venous blood) or from the finger or heel (Capillary blood). Venous blood is preferred. It can be collected using a syringe and needle or a vacuum tube. Both these methods will be described separately.

Collection

Blood is usually withdrawn from the antecubital vein or any other vein which is well identified on the forearm. The vein selected should be large, readily accessible, and sufficiently close to the surface to be seen and palpated Preparation of venipuncture site. Clean the skin of the area around the identified vein with 70% isopropyl alcohol in a circular fashion beginning at the site and moving outward. Allow to dry spontaneously. Do not touch the venipuncture site after it has been cleaned. Apply a tourniquet 3-4 inches above the venipuncture site. Ask the patient to make a fist a few times. Veins suitable for puncture will then become more apparent. Veins can become distended and easier to enter by allowing the arm to hang down for 2 or 3 minutes or by gently slapping the site of puncture.

Collection of venous blood using a syringe

1. Clean hands thoroughly with soap and water.

2. Write the name and hospital number of the patient on the tube in which blood is to be collected. A printed label with these particulars can also be used for patient identification.

3. Place the needle into the syringe. Keep the cap over the needle capped till it is used. Check that the syringe works smoothly.

4. With the needle bevel up and parallel to the surface of the skin insert it into the vein. Appearance of blood in the hub of the needle indicates that the needle has successfully entered the vein. Release the tourniquet as soon as blood enters the syringe.

5. Withdraw the piston slowly to avoid frothing.

6. After obtaining the requisite amount of blood, place a sterile gauze pad over the point where the needle entered the skin and deftly withdraw the needle simultaneously while applying pressure over the site.

7. Deliver the blood gently into the specified receiver. Cap it firmly to prevent leakage.

8. Maintain light pressure on the gauze pad over venipuncture site till the bleeding stops and then cover the puncture site with a small adhesive dressing.

9. Destroy the needle in a special device (needle destroyer) immediately after use. DONOT break, bend or recap needles after use.

10. Place the used swab, syringe and any other contaminated material in a puncture resistant container for adequate disposal.

Collection of venous blood using a vacuum tube

The vacutainer system consists of a double-pointed needle, a plastic holder or adapter, and several vacuum tubes with rubber stoppers of various colors depending on the sample to be collected. The color of the vacuum tube indicates the anticoagulant which it contains. The blood is directly collected into the tube from the vein.

Procedure

1. Place the identification details of the patient on each vacuum tube and ensure that they tally with the form.

2. Identify the vein and clean the area as described before. Apply a tourniquet 3-4 inches above the identified vein. Do not touch the venipuncture site after cleaning.

3. Place the vacuum tube in a reusable plastic holder and attach a disposable needle to it. Insert the tube into the holder till the top of the stopper is level with the marked guideline.

4. Place the patient's arm in a downward position to reduce the risk of backflow of any anticoagulant into the patient's circulation.

5. Insert the needle into the vein. Push the tube into the needle, puncturing the stopper/vacuum seal.

6. Remove the tourniquet as soon as the blood appears in the tube. Do not allow contents of the tube to come into contact with the stopper.

7. Do not allow the contents of the tube to come into contact with the stopper during the procedure.

8. If more than one sample is required, successive tubes may be fitted into the holder after removing the previous tube and blood collected. The needle remains in the vein. While each

successive tube is filling, the previous tube may be inverted gently to mix the sample well. Do not shake vigorously as this may lead to hemolysis of the blood sample.

9. After completion of blood collection, remove the holder and cover the site with a sterile swab and apply pressure till bleeding stops completely.

10. Destroy the needle in the destroyer without recapping it.

Collection of capillary blood

This can be obtained by skin puncture with a needle or lancet and is specially used in small children or very obese adults in whom venepuncture fails. Samples can be used for making peripheral blood films, performing hematocrit/Hb and point of care testing. In adults capillary blood sample can be obtained from the lateral side of tip of the 3rd or 4th finger while in infants the sample can be obtained by a deep puncture of the plantar surface of the heel.

Procedure

1. Clean the area with 70% alcohol and allow it to dry spontaneously. Puncture the skin to a depth of 2-3 mm with a sterile disposable lancet/needle.

2. Wipe the first drop of blood and squeeze gently to allow free flow of blood and collect the sample. In an adequate puncture, large drops of blood should exude spontaneously. Do not squeeze firmly as this gives unreliable results.

Making and Staining a Blood Smear

1. A well-made blood smear is a beauty to behold, and likely to yield interesting and significant information for a research project. A poor slide is a torment. The extra time and care taken during the field season will be rewarded later when the smears must be scanned, and parasites identified and counted. Here, the methods for making and staining smears are given, as well as a list of sources for high quality slides, stain, and chemicals. Photographs showing well-made smears are shown on the website.

2. Dried blood samples for genetic studies should always be made at the same time as the smears. The method is very easy and modern research must combine studies of morphology under the microscope with molecular methods. The technique for making and storing dried blood samples is given in the section “Dried Blood Samples”.

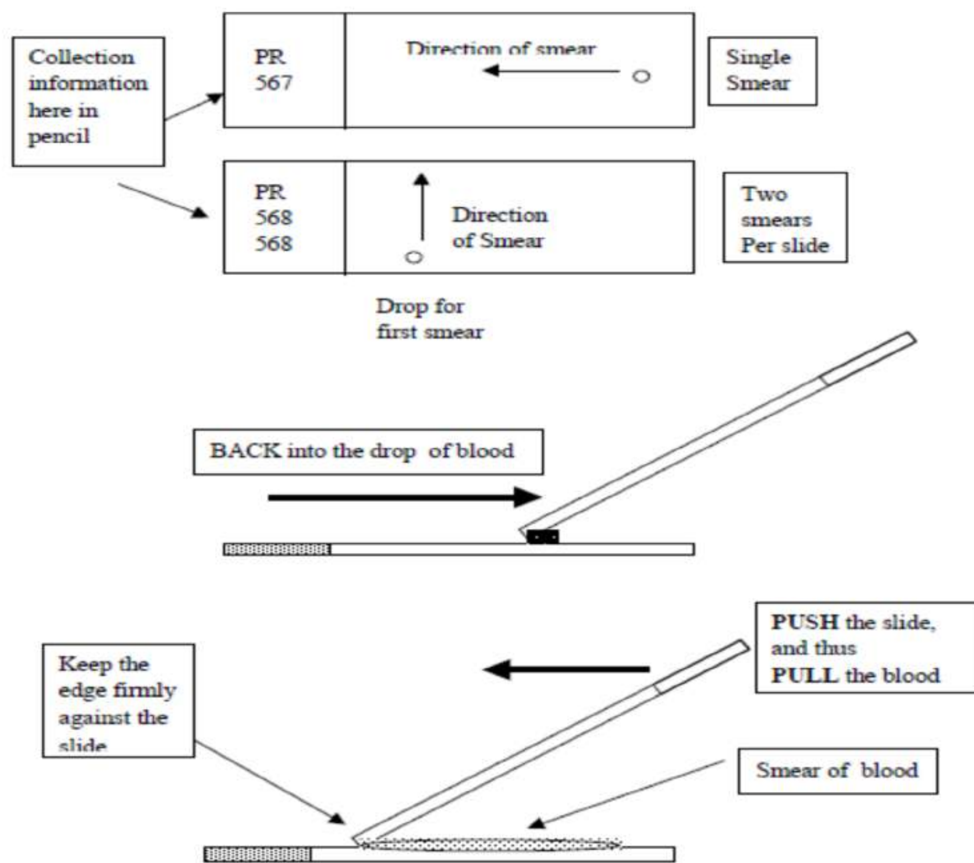
Making a smear

1. A single smear can be made per slide (smear running the length of the slide) or two (or even three) smears can share a slide, with the smears running the width of the slide. Putting two smears per slide saves on weight (glass is heavy) for field trips, and storage space. A picture showing both versions is included on the website.

2. It is easiest to use microscope slides with a frosted end, so that identifying information can be written there with pencil. Warning: Compare different pencils to find one that does not yield labels that rub off or wash off in the methanol dip.

3. Place a drop of blood approximately 4 mm in diameter on the slide (near the end if one smear is to be made, or at the proper location if two smears are to share a slide). See the drawing below.

4. Spread the drop by using another slide (called here the “spreader”), placing the spreader at a 45° angle and BACKING into the drop of blood. The spreader catches the drop and it spreads by capillary action along its edge. To make a short smear, hold the spreader at a steeper angle, and to make a longer smear, hold it closer to the drop. Now, push the spreader across the slide; this PULLS the blood across to make the smear. Do not push the blood by having it ahead of the smearing slide! It should take about one second to smear the drop. A smooth action is required, with the edge of the spreader held against the slide. This will yield a nice, even smear.



5. If doing one smear per slide, the spreader then becomes the next slide to receive a smear. Thus, each slide serves two duties, as a spreader, then as a slide to receive a smear. If two smears are made per slide, be sure to flip over the spreader to use the other edge for the second smear produced. The spreader then is used to receive the next two smears. Warning: If there is surplus blood on the spreader, wipe it off carefully before flipping it over to make the second smear on the slide.

6. Photographs are shown in the website.

7. For blood taken from mammals, a THICK blood film can also be made, but this is not possible with blood from birds or reptiles. Only mammals have erythrocytes that lack a nucleus. Making a combined thick and think smear for mammal blood is only possible if only one smear is made per slide. Make the thin smear starting about 1/3 from the nonfrosted end of the slide. Then, place another drop of blood at the clear end and use the edge of the smearing slide to spread the drop out to about a 1 cm circle. The thick smear will take longer to dry. Because the erythrocytes of mammals lack a nucleus, thousands of cells can be stacked, and parasites still seen (not for identification, but simply to detect an infected animal).

8. Smears should be air-dried, and then dipped into 100% methanol. A coplin jar with a screw top is best for this. We use a plastic version, which won’t break in the field, but has a poorly sealing top. Slides can be stored while drying in a small plastic slide box (holds 25 slides). Then, they are placed, two at a time, back-to-back, into the slots in the coplin jar. Thus, ten slides can be dipped at once. Be sure the alcohol does not reach the frosted end of the slide. After one minute, the slides are removed and placed on end to drain the alcohol. They can then be placed into a plastic slide box for complete drying.

9. In the field, we place the plastic slide box or boxes into a zip-lock bag with silica gel, and they are allowed to dry overnight.

10. To store slides during long field trips, and where many slides are to be made, they can be placed back into their original cardboard boxes, with a piece of index card or other clean paper between each slide.

Staining smears

1. First prepare the buffer. The stock buffer should be kept in the refrigerator, but if not possible, can be stored at room temperature for several weeks. Make working buffer which can be stored at room temperature for a few days. Buffer should be pH 7.0 to 7.2. Although this is a higher pH than normally used to stain blood cells, the parasites will stain darker and be more visible under the microscope.

2. A high-quality Giemsa should be used. Not all Giemsa stains are equal in quality. We place a layer of stain in the bottom of a glass coplin jar (about 3 mL), then add buffer to a level that will just cover the slides (except for frosted ends!) when they are in the jar. A little practice will tell the amount of buffer to add. Place the slides, back-to-back into the slots of the jar, and stain at room temperature for about 50 minutes.

3. Remove slides, rinse by dipping a few times into plain buffer, then stand on end to dry. Some workers prefer to run a thin stream of tap water over the slide to remove all the remaining stain; we have not found this necessary. Be sure to wash out the coplin jars after each use. If not properly washed, stain builds up inside the jar and reduces the quality of staining.

4. There is no need to cover-ship the slides. Immersion oil can be placed directly on the smear for observing under 1000x.

Preparing staining buffer

Stock buffers (two):

The alkaline stock is Sodium phosphate, dibasic anhydrous, N2HPO4 , Sigma Chemical S-0879. Mix 9.5 gm with distilled water to make 1000 mL.

The acid stock is Potassium phosphate monobasic anhydrous, KH2PO4 , Sigma P5379, mix 9.07 gm with distilled water to make 1000 mL

Working buffer:

Mix 39 mL of acid stock with 61 mL of the alkaline stock, and 900 mL of distilled water. Check pH, and adjust to ph 7 or 7.2 by adding the acid buffer stock to lower pH or alkaline to raise pH. Just a very few mL should be necessary to reach the required pH.

ESTIMATION OF TOTAL RED BLOOD CORPUSCLES (RBC) COUNT

Background:

Red blood cell count is an enumeration of red cells or is any erythrocyte count.

The red blood cells or erythrocytes are circular, biconcave, non nucleated cells containing haemoglobin and are embeded in blood plasma. After birth bone marrow is the main site of formation of red blood corpuscles. These are involved in acting as a carrier of oxygen and carbon dioxide. RBCs also maintain the ionic balance of human physiological system and maintain viscosity of blood. Various pigments like bilirubin and biliverdin are derived from RBC after their degradation. The basic principle is that the blood specimen is diluted (usually 200 times) with red cell diluting fluid which does not remove the white blood cells but allows the red cells to be counted under magnification in a known volume of fluid. Finally, the number of cells in undiluted blood is calculated and reported as the number of red cells/µl of whole blood. Blood cell counts can be performed using the hemacytometer.

Significance:

The red cell count is the number of red cells present in one cubic millimeter of blood. The normal values of the red blood cell count are: Woman : 4-5.5 million per cubic millimeter Men : 4.5-6.0 million per cubic millimeter Infants : 5- 6.5 million per cubic millimeter Variations in normal values is observed in pregnancy, severe burns, diseased conditions and it also depends upon altitude. It drops below normal values in anaemia and leukemia and rises above the normal values in polycythemia and dehydration conditions. Therefore, the red cell count is useful in diagnosis.

The aim of the experiment is to estimate red blood cell count of a blood specimen.

Requirements:

Neubaur chamber, RBC pipette, Cover slip, RBC diluting fluid, Needle, spirit, cotton.

Procedure:

1. Sterilise the finger tip with cotton plug soaked in spirit and let it dry.

2. Take a bold prick with needle to have free flow of blood and draw the blood in a RBC pipette upto 0.5 marks.

3. Dip the RBC pipette in red blood cell diluting fluid and suck up diluting fluid upto 101 marks.

4. Rotate the pipette equally in your hands to mix the solution well by swirling.

5. Take the haemocytometer and place it on the flat surface of the work bench. Place the cover slip on the counting chamber.

6. Allow a small drop of diluted blood, hanging from the pipette, to sweep into the counting chamber by capillary action. Make sure that there is no air bubble and the counting chamber must not be flooded.

7. Leave the counting chamber on the bench for 3 minutes to allow the cells to settle. Observe the cells by placing the counting chamber on the mechanical stage of the microscope.

8. Focus on the centre room of the chamber and start counting the cells from upper left corner of the room. It is advisable to complete all counts of the four squares and then move to the centre square, which is the fifth square to be counted.

Data Analysis:

$$\frac{\text{No. of cells X Dilution factor X Depth factor X Total ruled area}}{\text{Area count}}$$

Where; Dilution factor = 200; Depth factor = 10; Total ruled area = 25; Area count = 5

ESTIMATION OF WHITE BLOOD CELL COUNT (TLC/ Total Leucocyte Count)

Background:

White blood cell count is an enumeration of white corpuscles or leucocyte count.

The white blood cells (WBCS) or leucocytes are nucleated actively amoeboid and do not contain haemoglobin and ae originated purely from extravascular tissue. They are composed of nucleoproteins and varieties of enzymes. Their number is less and life span is short as compared to red blood cells. The WBCs exist in two forms viz. granulocytes and agranulocytes. Granulocytes are further classified as eosinophil, basophil, neutrophil, while agranulocytes shows lymphocytes and monocytes. These varieties possess independent morphological, functional and staining properties. The main function of white blood corpuscles is phagocytosis that is body defence mechanism against foreign particles and invading bacteria. They are also involved in antibody formation in immunological body defence mechanism. It also take part in process of repair in an area of inflammation.

The basic principle is that the blood is diluted with acid solution which removes the red cells by haemolysis and also accentuates the nuclei of the white cells; thus the counting of the white cells becomes easy. Blood cell counts can be performed using the hemacytometer.

Significance:

The white cell count is the number of white cell present in one cubic millimeter of blood. The normal values of white blood cell count vary between 5000 to 10,000 per cubic millimeter or 7-11 thousand cells/µl of blood volume in healthy individual. Variation in normal values is observed in diseased states. WBC count increases (leucocytosis) in conditions like pneumonia, leukemia, meningitis, small pox etc. while the count decreases (leucopenia) in conditions such as influenza, typhoid, infectious hepatitis etc. Moreover the count rises in pregnancy and during menstruation. Thus, white blood cell count is useful in diagnosis.

The aim of the experiment is to estimate white blood cell count of a blood specimen.

Requirements:

Neubaur chamber, WBC pipette, Cover slip, WBC diluting fluid, Needle, spirit, cotton

Procedure:

1. Sterilize the finger tip with cotton plug soaked in 70% alcohol and let it dry.

2. Take a bold prick to have free flow of blood and draw the blood in a WBC pipette up to 0.5 marks.

3. Dip the WBC pipette in WBC diluting fluid up to 11 mark and rotate the pipette equally in your hands to mix the solution well by swirling.

4. Take the haemocytometer and place it on the flat surface of the work bench. Place the cover slip on the counting chamber.

5. Allow a small drop of diluted blood, hanging from the pipette, to sweep into the counting chamber by capillary action. Make sure that there is no air bubble and there is no overfilling beyond the ruled area.

6. Leave the counting chamber on the bench for 3 minutes to allow the cells to settle. Observe the cells by placing the counting chamber on the mechanical stage of the microscope.

Focus on one of the corner squares of the counting chamber and count the white cells schematically, starting from the upper left small square of each Square. Repeat the count in all the four corners of the chamber. Apply the margin rules i.e. count the cells lying on two adjacent margins, and discard those on the other two margins.

Data analysis:

$$\frac{\text{No. of cells X Dilution factor X Depth factor}}{\text{Area count}}$$

Where: Dilution factor = 20, Depth factor = 10, Area count = 4

Differential Leukocyte Count:

The Types of Leukocytes:

1-Granulocyte cell:

a-Neutrophile: is a cell with acidophilic cytoplasm and fine pinkish red granules ,the nucleus is usually lobulated (3-5) lobes ,connected by thin chromatin filament .

- Percentage 65-70% from blood.
- Number 3000-6000 \ mm3.
- Disease case : Neutrophilia :increase Neu. In blood ex : inflammation ,intoxication.

b-Eosinophile: acidophilic cell ,usually larger than the Neu. ,it is cytoplasm contain bright red granules or orange the nucleus consist usually 2 lobes or (bilobes).

- Per. 2-4 % from blood.
- Num. 150-300 \ mm3.
- Eosinophilia :ex: Allergy, Asthma.

c-Basophile :it is a small cell granules are black or blue in color and over cover most of the cell even the nucleus or (S-shaped).

- Per. 0-1 % from blood
- Num. 0-100 \mm3
- Bosophilia : ex: Wounds

2- A granulocyte cell:

a-Lymphocyte :it is also small cell, the nucleus cover most of the cells &it is round dark violet in color ,the cytoplasm is usually blue in colour with no granules .

- Per. 32-40 % from blood.*
- Num. 1500-4000 \ mm3.
- Lum.is divided into 2 types :T-cell(cytotoxic),B- cell(production of antibodies)

Disease Case :

- Lymphocytosis :increase lymphocyte in blood ex :Brucellosis and Tuberculosis

b-Monocyte : is the largest mature leukocyte the nucleus is usually kidney shape or horse shape.

- Per. 5-8 % from blood.
- Num. 300-600 \ mm3.
- Monocytosis :ex: Malaria, Typhus.
- Function of WBC :it is the first defense line of the body against bacterial infection.
- The shape of Typical Slide: finger shape, thin smooth, not thick rapid spreader.
- The evidence of staining: To show the nucleus of the cell.
- The evidence of diluting: To fixed the stain.

Preparation and Examination of the Peripheral Blood Smear:

The preparation and examination of the peripheral blood smear is one of the most frequently requested tests in the hematology laboratory .blood Smear must be prepared correctly and examined in such a way as to provide the physician with an accurate interpretation. There are 4 methods used to prepare blood smear :

1-Slide to Slide.

2-Coverslipe to Slide.

3-Coverslipe to Coverslipe.

4-Automated Spinner.

Blood smears are prepared with EDTA anticoagulant blood to minimize degenerative changes in the blood cells. To ensure good preservation of cellular morphology, differential smears should be made as soon as possible and no later than 3 hours after collection.

The procedure of slide to slide:

1-Place a drop of blood from the finger about 2mm in diameter in the central line of a slide about 1-2 cm from one end.

2-The spreader is placed at an angle of 40 degrees to the slide and then moved back to make contact with the drop.

3-The drop should spread out quickly along the line of contact of the spreader with the slide.

4-The moment this occurred the film should be spread by a rapid, smooth, forward, movement of spreader.

5-The drop should be of such size that the film is 3-4 cm in length.

6-The film should be dried rapidly .A good blood film preparation will be thick at the drop end and thin film at the opposite end. The thickness of the spread when pulling the smear is determined by the :A-angle of spreader slide (The greater the angle ,The thicker and Shorter the smear).B-size of the blood drop .C-Speed of spreading (Slowly the thicker).while the thin smears are used for describing blood cell, the thick smear are used for detecting malarial parasites.

There are 3 stains that used in differential leukocyte count:*

1-Wright stain.
2-Leishman stain.
3-Giemsas stain.
The composition of Giemsas stain:

Giemsa powder 0.3 gm

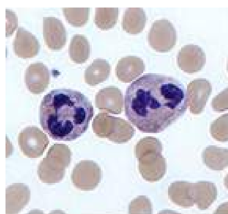
Glycerin 25 ml

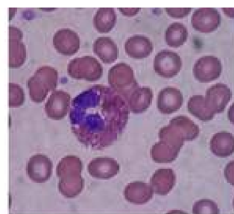
Methyl alcohol 25 ml this makes stock solution and before use it has to be diluted by adding 1 ml (stain) to 9 ml of buffered water

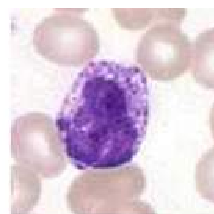
Method of staining:
1-The blood film is fixed with methyl alcohol for 2 minutes.
2-Pour Giemsa stain diluted 1:9 with buffer over the smear for 8-10 minutes.
3-Wash off with buffer and dry.

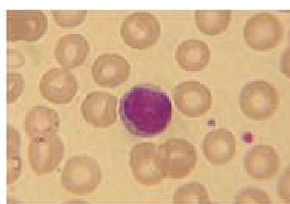
The Count :

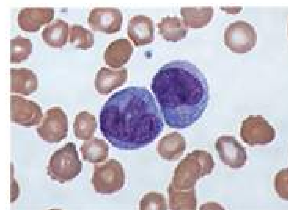
The dry and stained film examined without a coverslipe under oil immersion objective .for differential leukocyte counts choose an area where the morphology of the cells is clearly visible .do differential count by moving the slide in area including the central and peripheral and the smear .a total of 200 cells should be counted in which every white cell seen must be recorded in a table under the following heading: Neutrophile, Eosinophile, Basophile, Lymphocyte, and Monocyte then find the percentage of each type.


(1) Neutrophils


(2) Eosinophils


(3) Basophils


(4) Lymphocytes


(5) Monocytes

Erythrocyte sedimentation process:

Erythrocyte sedimentation is governed by factors that stimulate or inhibit erythrocyte aggregation and sedimentation. Normal erythrocytes are negatively charged and repel each other, which limits the sedimentation rate. Large clumps fall faster than small ones, so factors that increase aggregation will increase sedimentation. Erythrocytes usually aggregate into clumps that resemble a stack of coins, which are called rouleaux.

Westergren method:

The Westergren method requires collecting 2 ml of venous blood into a tube containing 0 .5 ml of sodium citrate. It should be stored no longer than 2 hours at room temperature or 6 hours at 4 °C. The blood is drawn into a Westergren-Katz tube to the 200 mm mark. The tube is placed in a rack in a strictly vertical position for 1 hour at room temperature, at which time the distance from the lowest point of the surface meniscus to the upper limit of the red cell sediment is measured. The distance of fall of erythrocytes, expressed as millimeters in 1 hour, is the ESR.

Wintrobe method:

The Wintrobe method is performed similarly except that the Wintrobe tube is smaller in diameter than the Westergren tube and only 100 mm long. EDTA anticoagulated blood without extra diluent is drawn into the tube, and the rate of fall of red blood cells is measured in millimeters after 1 hour. The shorter column makes this method less sensitive than the

Westergren method because the maximal possible abnormal value is lower. However, this method is more practical for demonstration purposes.

Wintrobes method .....

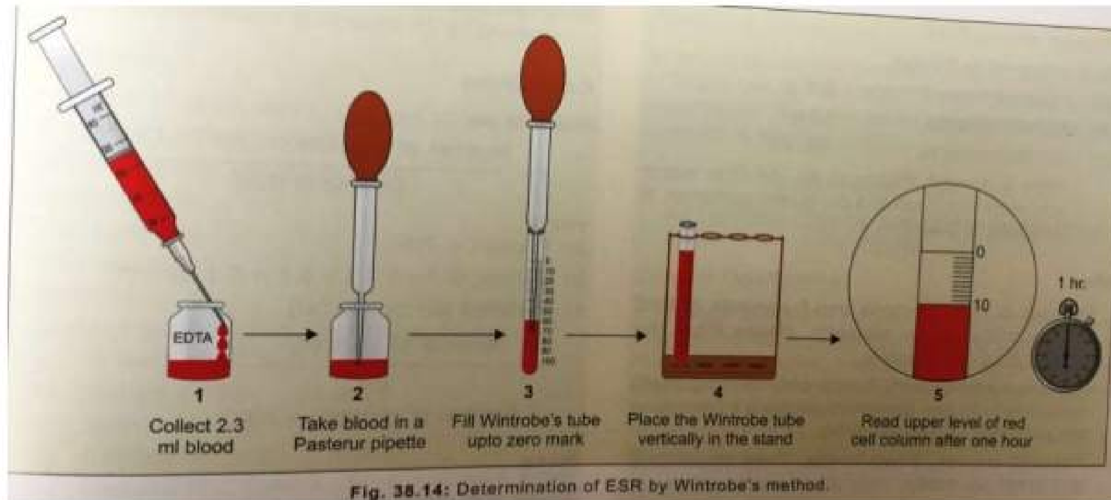


Fig. 38.14: Determination of ESR by Wintrobe's method.

The commonly used methods are Sahli's/ acid hematin method and Cyanmethemoglobin method. The details of these methods are described below.

Sahli's/acid hematin Method

Principle:

Blood is mixed with N/10 HCl resulting in the conversion of Hb to acid hematin which is brown in color. The solution is diluted till it's color matches with the brown colored glass of the comparator box. The concentration of Hb is read directly.

Equipment required:

Hemocytometer which consists of

- comparator box which has brown colored glass on either side
- Hb pipette which is marked upto 20mm3(0.02ml blood)
- Tube with markings of Hb on one side z glass rod
- dropper

Reagents required N/10 HCl Distilled water Sample: Venous blood collected in EDTA as described earlier Procedure

1. Add N/10 HCl into the tube upto mark 2 g%

2. Mix the EDTA sample by gentle inversion and fill the pipette with 0.02ml blood. Wipe the external surface of the pipette to remove any excess blood.

3. Add the blood into the tube containing HCl. Wash out the contents of the pipette by drawing in and blowing out the acid two to three times. Mix the blood with the acid thoroughly.

4. Allow to stand undisturbed for 10min.

5. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop stirring with the glass rod till it's color matches with that of the comparator glass. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.

6. Remove the stirrer and take the reading directly by noting the height of the diluted acid hematin and express in g

Determination of clotting time

Background

Whenever a great blood vessel ruptures bleeding continues. In a few minutes blood looses its fluidity and sets into a semisolid mass. The mass is referred as clot and the phenomenon as coagulation. Clotting time is defined as the time interval in between onset of bleeding and appearance of semisolid mass i.e. clot. Normal value of clotting time is 3-4 minutes. Clotting time is determined using two methods viz. Capillary glass method and Wrights Coagulometer.

The aim of the experiment is to determine the clotting time of the subject. REQUIREMENTS: Spirit, cotton, needle, capillary tube, stop watch.

Procedure:

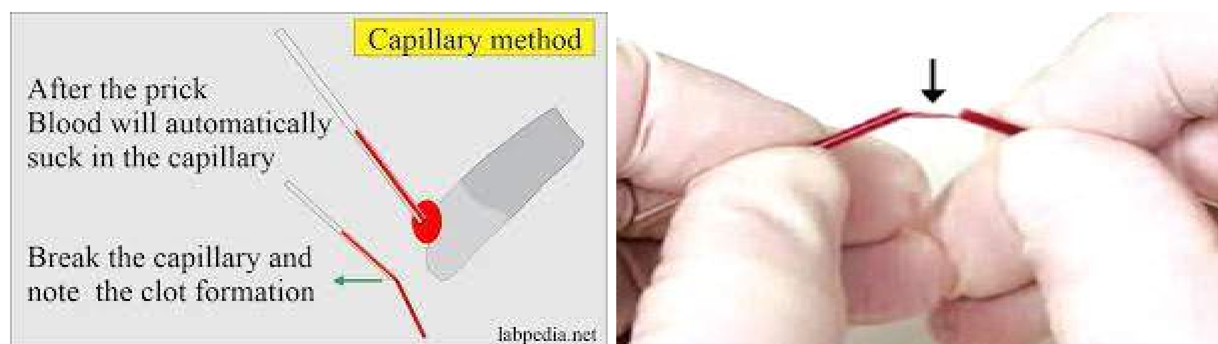
Capillary glass method:

1. The finger tip of the subject Is sterilized and a bold prick Is made in the finger tip with a sterilized needle to have free flow of blood.

2. The blood coming out of the puncture Is sucked into a capillary glass tube of 15 cm long. 3. Then the tube is kept undisturbed horizontally for about 1-2 minutes.

4. A small bit of the glass tube is broken off every 30 seconds until a fine thread of clotted blood appears while the capillary tube is broken.

5. When the thread appears the stop watch is stoped. This gave us the clotting time. The period in between appearance of blood in finger and formation of clot was taken as clotting time.



Determination of bleeding time

Background

Definition:

The bleeding time is the time required for a small cut to stop bleeding. When a blood vessel is injured, blood comes out for some time and then it stops because of the formation of platelet plug. The duration of bleeding is the bleeding time. Normal value for bleeding time is 1-3 minutes.

Significance:

The bleeding time is mainly used in the diagnosis and treatment of the haemorrhagic diseases. The bleeding time is also useful just before operations such as tonsillectomy. In such cases it may point out an abnormal bleeding process. This will aware the physician to take proper precaution. The bleeding time may be performed by following methods: Duke Method, Ivy Method, Macfarlane Method.

The aim of the experiment is to determine the bleeding time of the subject.

Requirements:

Spirit, cotton, needle, piece of filter or bloating paper, stop watch.

Procedure:

Duke method for bleeding time:

1) The finger tip of the subject is sterilized with spirit and a bold prick is made with a sterile needle to have free flow of blood.

2) The stop watch is started and time is recorded.

3) A piece of bloating paper is folded into half and exactly at every 15 seconds interval the blood coming out from the puncture is wiped.

4) The above step is repeated until blood ceases to flow.

5) The time at which blood ceased to flow is recorded.

6) The bleeding time is determined from the recorded time data.

Hemoglobin Colorimetric

Quantitative determination of Hemoglobin

IVD *For in vitro diagnostic use only*

Store at 2-8°C

PRINCIPLE OF THE METHOD

Hemoglobin is oxidized by potassium ferricyanide into methaemoglobin, which is converted into cyanomethaemoglobin, by potassium cyanide.

The intensity of the color formed is proportional to the Hemoglobin concentration in the sample.

CLINICAL SIGNIFICANCE

The Hemoglobin is a protein that contains iron and that the red color to the blood. The Hemoglobin is in red globules and it is the one in charge of oxygen transport by the blood from the lungs to weaves.

When the level of Hemoglobin appears underneath the normal levels is describing an anemia that can be of different origins: primary anemia, cancer, pregnancy, renal diseases, and hemorrhages.

If the Hemoglobin levels appear high it can be due to. cardiopathies. dehydratation and stays in places of much altitude Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

PRECAUTIONS

HAEMOGLOBIN

Harmful: Harmful by inhalation, in contact with skin and if swallowed.. Keep container tingly close. After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell, seek medical advice immediately.

Cyanide (poison): The amount of cyanide in the Reagent Concentrate (40x) is appreciably less than the minimum lethal dose for an adult. Gaseous hydrogen cyanide will be released on contact with acids.

REAGENT:

- 15g/dl Hemoglobin Standard.

PREPARATION

Ready to use.

STORAGE AND STABILITY

All the 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 540 nm > 0.01.

ADDITIONAL EQUIPMENT

- Spectrophotometer or colorimeter measuring at 540 nm
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.

SAMPLES

Venous or capillary blood

Use anticoagulants like EDTA, heparin or oxalate.

Stability of the sample' 1 week at 2-8°C.

PROCEDURE

1. Assay conditions.
   Wavelength .... 540 nm
   Cuvette .... 1 cm. light path
   Temperature .... 15-25°C
2. Adjust the instrument to zero with distilled water
3. Pipette

	Blank	Standard	Sample
R(ml)	5.0	5.0	5.0
Hemoglobin Cal (µl)	-------	20	-------
Sample (µl)	-------	-------	20

4. Mix and incubate for 3 min. at room temperature (15-25°C).
5. Read the absorbance (A) of the samples and Standard, against the Blank.

CALCULATIONS

(A)Sample x 15 (Standard conc.) = g/dl Hemoglobin (A) Standard in the sample

QUALITY CONTROL

Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

REFERENCE VALUES

Men 14 - 18 g/dl= 8.7 — 11.2 mmol/L

Women 12-16g/dl= 7.5— 9.9mmol/L

These values are for orientation purpose, each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS

Measuring range:

From *detection limit* of 0.1 g/dl to *linearity limit* of 20 g/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.

Precision:

	Intra-assay (n=20)		Intra-assay (n=20)	
Mean (g/dl)	8.00	15.2	7.81	15.1
SD	0.29	0.33	0.19	0.26
CV (%)	3.59	2.19	2.51	1.74

Sensitivity:

1 g/dl = 0,027 A

Prothrombin Time (PT)
(LIQUID REAGENT)
IVD For In-Vitro diagnostic use only

Store at: 2-8°C

INTENDED USE

Prothrombin Time (PT) is commonly used for screening for extrinsic factor deficiency, monitoring oral anticoagulant therapy and quantitative determination of the extrinsic coagulation factors.

PRINCIPLE

Tissue thromboplastin, in the presence of calcium ions and Factor VII, activates the extrinsic pathway of coagulation. When a mixture of tissue thromboplastin and calcium ions is added to normal anticoagulant plasma, the clotting mechanism is initiated and a clot will form within a specified time period. If a deficiency exists within the extrinsic pathway, the time required for clot formation will be prolonged. The degree of prolongation is proportional to the severity of single factor deficiency, or in a cumulative deficiency of all the factors involved.

MATERIALS

MATERIALS PROVIDED

- PT Reagent: ISI 1.0

MATERIALS NEEDED BUT NOT PROVIDED:

- Plasma Normal and Abnormal Control.

STORAGE AND STABILITY

- Do not mix or use the components of this kit with the components of any other kit with different lot numbers.
- Throughout testing all test tubes, syringes and pipettes should be plastic.
- Throughout testing all test tubes, incubation time should be kept in constant and incubation temperature at 36.5-37.5°C.
- If testing is delayed for more than 4 hours, plasma may be stored at 2-8°C.
- Each laboratory should establish a Quality Control program that includes both normal and abnormal control plasmas to evaluate instrument, reagent tested daily prior to performing tests on patient plasmas. Monthly quality control charts are recommended to determine the mean and standard deviation of each of the daily control plasma. All assays should include controls, and if any of the controls are outside the established reference ranges, then the assay should be considered invalid and no patient results should be reported.

SPECIMEN COLLECTION AND PREPARATION

1. Plasma obtained from whole blood samples that had been collected in a tube with 0.109M sodium citrate as an anticoagulant, nine parts of freshly collected whole blood should be immediately added to one part of anticoagulant. Centrifuge the whole blood specimen at 2500xg for 15 minutes. Separate the plasma using a plastic pipette and place it in a plastic test tube. Perform the Prothrombin Time assay within 4 hours.
2. Reconstitute the control plasmas (normal control plasma, abnormal control plasma) according to the package insert included with the control.

PROCEDURE

A. Manual Method

1. Bring all reagents, controls and sample to room temperature 15 minutes prior to testing.
2. Pre-warm PT reagent at 37°C for 5 minutes.
3. Pipette 100µl of PT reagent to each tube.
4. Add 50 µl of sample, controls to the tubes prepared in step 3, start stop watch , mix in a water bath (37°C) for 8 seconds , then record the time required for clot formation .

B.Automated Method

To perform this test, refer to the appropriate Instrument Operator's Manual for detailed instructions.

RESULTS

$$\text{Prothrombin Time Ratio (PTR)} = \frac{\text{Clot time of the test plasma}}{\text{Clot time of the control plasma}}$$

INR = PTR^{ISI}

Example: for a PTR of 2.0 and an ISI of 1.0

INR = $2.0^{1.0}$ = 2.0

REFERENCE VALUES

Normal control sample: (11-16 seconds)

PTR: 1.0±0.15; INR: 0.8-1.24

- These values should only serve as guidelines.
- Because differences may exist between instruments, laboratories, and local populations, it is recommended that each laboratory establish its own reference range of expected Prothrombin Time LIQUID results.
- The results of the Prothrombin Time LIQUID tests should be reported to the nearest tenth of a second. Results greater than the upper limits of the range should be considered abnormal and follow-up testing should be performed. PT values below the lower limits of the range may indicate a compromised sample, and a new sample should be collected.

Clinical Immunology: Paper-VIII, Unit-16

Experiment 1: Determination of 'ABO' blood grouping and 'Rh' typing.

AIM: Qualitative analysis of ABO blood grouping and Rh typing.

PRINCIPLE: The human blood could be broadly divided into 4 major groups, which are usually distinguished as A, B, AB and O. Group A will have only anti –B antibody in the plasma. Group B will have only anti –A antibody in the plasma. Group AB will have neither anti – A nor anti B antibodies in the plasma. Group O will have both anti –A and anti B antibodies in the plasma.

Table 1: A profile of natural IgM antibodies (= coagglutinins) in the plasma/serum of human.

Blood group	Natural antibody profile	
	Anti - A	Anti- B
A	-	+
B	+	-
AB	-	-
O	+	+

- = absent; + = present

The erythrocytes from group A will agglutinate if mixed with anti –A serum, but not with anti – B serum. Group B cells will agglutinate if mixed with anti - B serum, but not with anti – A serum. Group AB cells will agglutinate if mixed with either anti –A or anti –B serum. Group O cells will not agglutinate if mixed with either anti –A or anti –B serum (table 2). These features not only reveal the blood group of an individual but also demonstrate the specificity and the reactivity of antibodies.

MATERIALS: Lancet, alcohol / ether, cotton, glass slides, anti-A and anti- B sera (commercially obtained), wooden toothpick or pin heads.

Table 2: Reactivity of human RBC types to antisera A and B.

	Agglutination of human RBC			
Antiserum	A	B	AB	O
Antiserum-A	+	-	+	-
Antiserum-B	-	+	+	-

- = no agglutination; + = agglutination

PROCEDURE:

1. Keep a few clean glass slides ready.
2. Sterilize the fingertip with alcohol/ ether.
3. Allow the fingertip to dry.
4. Sterilize a lancet.
5. Pierce the fingertip with lancet by maintaining aseptic conditions.
6. Allow the blood to flow normally.
7. Wipe off the first drop of blood with cotton.
8. Allow the next two drops to fall on the left and right of the clean glass slide respectively.
9. Add one drop of anti-A serum to the right side drop.
10. Add one drop of anti-A serum to the left side drop.
11. Precaution: make sure that the tip of the pipette used for transferring antiserum does not touch the blood.
12. Mix the drop of blood + antiserum with a clean wooden toothpick or pinhead.
13. Examine the slides for evidence of agglutination of red cells.
14. Observe the result visual and microscopically within 2 minutes for occurrence of agglutination of RBC.

RESULTS:

Record your blood group as given in the Table 2.

REMARKS /INFERENCE:

Comment on specificity in the reactivity of the two antisera tested with the blood samples.

Experiment 2: Antibody measurement by Radial immuno-diffusion (RID) technique.

AIM: To quantify the concentration of unknown antigen in a sample using radial immunodiffusion.

PRINCIPLE: The formation of antigen-antibody complexes is the first step in removing infectious agents from the body. Because each antibody can bind more than one antigen and each antigen can be bound by more than one antibody molecule, very large macromolecular complexes can form. These complexes form precipitates which can be cleared from the body through various means. These precipitates are also useful for laboratory and diagnostic tests. When antibodies and antigens are inserted into different areas of an agarose gel, they diffuse toward each other and form opaque bands of precipitate, at the interface of their diffusion fronts. Precipitation reactions of antibodies and antigens in agarose gels provide a method of analyzing the various antibody antigen reactions in a system. Radial immunodiffusion represents a hallmark in the evolution of immunology because it represents the first successful attempt to develop a precise quantitative assay suitable for routine use in the diagnostic laboratories. Radial immunodiffusion received its designation from the fact that a given antigen is forced to diffuse concentrically on a support medium to which antiserum has been incorporated. A polyclonal antiserum, known to precipitate the antigen, is added to molten agarose and an agarose plate containing the antibody is then prepared. Identical wells are cut in the antiserum containing agar and those wells are filled with identical volumes of samples containing known amounts of the antigen (calibrators) and of unknown samples to which the antigen needs to be assayed. After 24 to 48 hours it is possible to measure the diameters of circular precipitates formed around the wells, where antigens were placed. Those diameters are directly proportional to antigen concentration.

MATERIALS: Small size petridish, antigen, agarose, antiserum, well cutter, filter paper

Sample

1. Collect whole blood without anticoagulant and allow to clot at room temperature.
2. Separate serum by centrifugation within 2-3 hours after collection.
3. Remove plates from refrigerator to room temperature approximately 30 minutes before filling wells. Do not open bag until ready for use.
4. If excess moisture is present, remove plate from its bag and remove cover until evaporation has dried the surface and wells. Replace cover until used.
5. For best results, three wells should be filled with reference sera for each plate.

Preparing the standards (serial dilution)

1. Label four micro test tubes: 1:2, 1:4, 1:8, and 1:16.
2. Using a micropipette, add 50 microliters of Buffer to each tube.
3. With a fresh pipet tip, add 50 microliters of “Standard” to the tube labeled 1:2, mix. Transfer 50 microliters of the 1:2 dilution to the tube labeled 1:4, Mix, and so on.

PROCEDURE:

1. Deliver specimen or standard (5 µl) to well by placing the pipette tip at the bottom of the well. Allow the well to fill to the top of the agar surface. Avoid bubbles to ensure proper volume and diffusion of sample. Visualization may be aided by placing the plate on dark background.
2. Mark time of completion on plate cover and replace cover.
3. Replace plate in bag and reseal carefully.
4. Incubate plates upright on a flat surface at room temperature (20 to 24 ºC) over 48 hours for End Point readings.

Calibration:

1. Using the reference sera determine their ring diameters to the nearest 0.1mm.
2. Using regular graph paper plot the concentration on the X axis and the zone diameters squared on the Y axis for each protein for End point readings.
3. Draw a straight line of "best fit" between the four points.

RESULTS:

1. Measure diameters of precipitin zones to within 0.1mm. Variations in incubation times of more than 30 minutes will produce changes in diameters, especially those at higher levels of antigen except when plates have reached endpoint.
2. Determine the concentration of each unknown or specimen protein from the reference curve find the corresponding concentration.

INTERPRETATION:

When an unknown diameter exceeds that of the top standard, the specimen should be diluted with saline and rerun.

When an unknown diameter is smaller than that of the lowest standard, concentration should be reported as "less than" the concentration of the reference serum. If available, "low level" radial immunodiffusion plates may be utilized.

Experiment 3: A. Antigen-Antibody reaction testing by precipiting ring. B. Antigen-Antibody reaction testing by Ouchterlony test.

3A. Antigen-Antibody reaction testing by precipiting ring.

AIM: To analyze qualitative occurrence of precipitation reaction between antigen and antibody in solution by ring/interfacial/precipitation test.

PRINCIPLE: When a solution of antigen is carefully overlaid or mixed on the solution of antiserum containing antibody (IgG), diffusion of molecules occurs between the two components until equilibrium is reached. The reaction between antigen and antibody commences simultaneously with their diffusion, resulting in formation of antigen-antibody complexes. At the point of equilibrium, a maximum number of such complexes are formed which rapidly aggregate to form visible precipitate of antigen-antibody complexes. Such precipitates appear as a white opaque ring of precipitation near the interface between the antigen and antiserum or in the bottom of the tubes. The precipitation reaction occurs predominantly in the antibody (= antiserum) layer, since antigen – antibody complexes remain soluble in the presence of excess antigen.

MATERIAL: Commercially available kit or narrow glass tubes (inner diameter: 2 mm), syringe with needle (23 G), antigen (human serum) and anti–human serum (commercially available).

PROCEDURE:
As per instruction given in the commercially supplied kit or manually we may follow:

1. Introduce 5- 10 µl of antiserum at the level of about 1 cm from the top of a narrow glass tube using the syringe.
2. Overlay 5-10 µl of antigen solution carefully on antiserum by vertically holding the tube.
3. Keep the tube upright without disturbance.
4. Observe the tube for the appearance of a ring of precipitate near the interface.

Note: The visible precipitation reaction appears within 45 min.

RESULT: Record your observations and draw a neat diagram indicating the exact position of precipitation ring formed within the reaction mixture.

REMARKS: Discuss the merits and limitations of this test.

3B. Antigen-Antibody reaction testing by Ouchterlony double immune-diffusion technique

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 4: Quantitative assay of Immunoglobins in plasma (IgG, IgM).

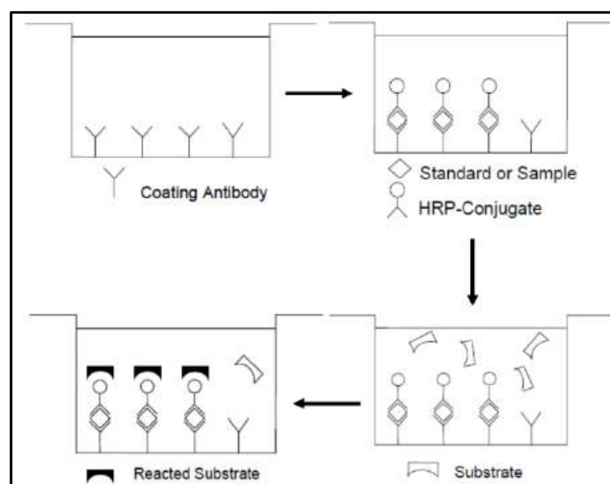
4A. Quantitative assay of IgG Immunoglobins in plasma/Serum

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:

- 1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human total IgG.
- 1 vial (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:

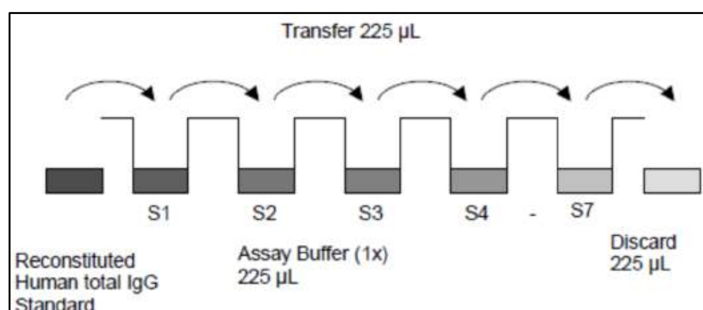
Number of Strips	HRP-Conjugate (mL)	Assay Buffer (1x) (mL)
1-6	0.03	2.97
1-12	0.06	5.94

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:

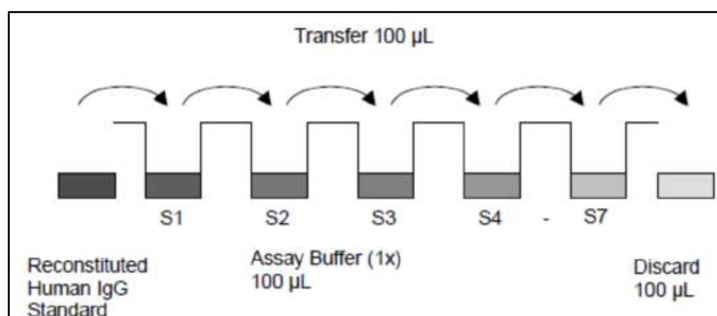
1. Pre-dilute samples before starting with the test procedure. Dilute serum and plasma samples 1:500,000 with Assay Buffer (1x) according to the following scheme:
    10 μL sample + 990 μL Assay Buffer (1x) = Pre-dilution A
    10 μL Predilution A + 990 μL Assay Buffer (1x) = Pre-dilution B
10 μL Predilution 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	Standard 4	Sample 4	Sample 4

	0.013 µg/mL	0.013 µg/mL		
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 establi shes 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 0.224	0.219
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

4B: 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.

Serology: Paper-IX, Unit-18

Experiment 1: Study of precipitation, agglutination and coagulation test.

1A. 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:

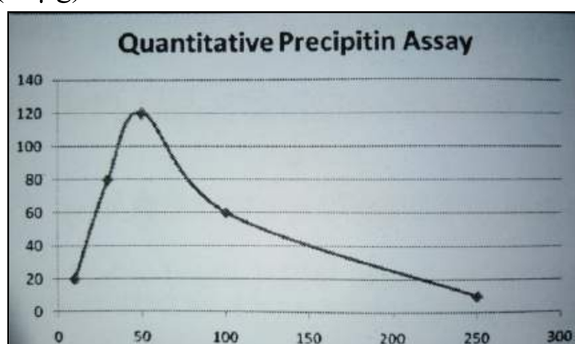
Calculate the protein content of al tubes from the following formula:

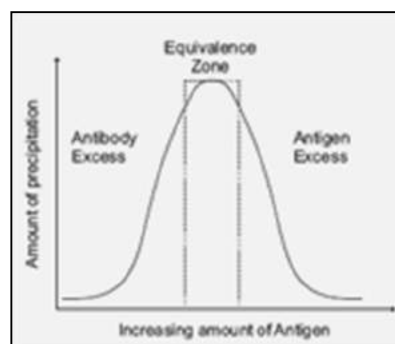
Protein content in the precipitate = (A_{280} X M)/1.4 mg
Where M is the amount of NaOH added in "ml" and 1.4 is the Extinction Coefficient.

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 A_{280} 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 = (P_m – Ag)/V
Where; P_m = 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.

1B. 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.

Experiment 2: VDRL test, WIDAL test, RPR test, ASO test.

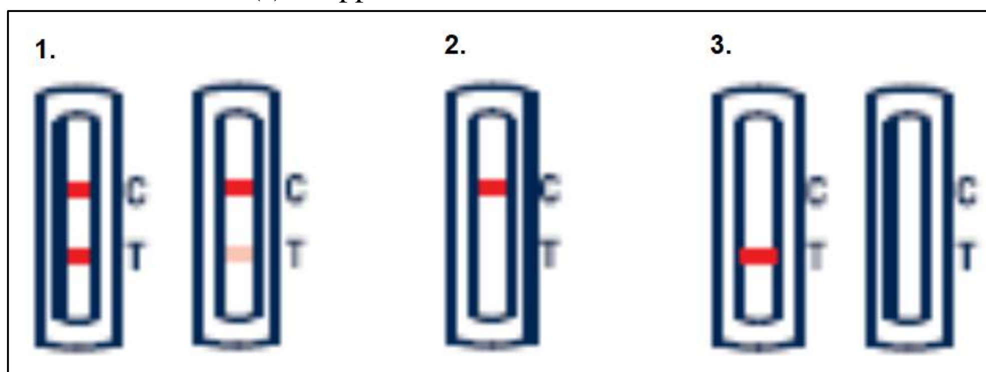
2A. VDRL test:

AIM:
To rapid detect the syphilis in patient sample.

INTRODUCTION:
The venereal disease research laboratory (VDRL) test is used to detect if a person has been infected with the bacteria causing syphilis, which is a sexually transmitted disease. The test detects the presence of antibodies against the bacteria *Treponema pallidum*. This test is only indicative, and if positive, it must be followed up with another blood test to make a definitive diagnosis of syphilis.

PRINCIPLE:
The Syphilis Ultra Rapid Test Device is a rapid chromatographic immunoassay for the qualitative detection of antibodies (IgG and IgM) to *Treponema pallidum* (TP) in whole blood, serum or plasma to aid in the diagnosis of Syphilis. Recombinant Syphilis antigen is immobilized in the test line region of the device. After a specimen is added to the specimen well of the device, it reacts with Syphilis antigen coated particles in the test. This mixture migrates chromatographically along the length of the test strip and interacts with the immobilized Syphilis antigen. The double antigen test format can detect both IgG and IgM in specimens. If the specimen contains TP antibodies, a colored line will appear in the test line region, indicating a positive result.

PROCEDURE:
1. Add two drops of serum or plasma into the sample window using provided disposable dropper.
2. For Venipuncture Whole Blood specimens: Add 1 drop (20µl) of whole blood to the specimen well (S), then add 2 drops of buffer.
3. For Finger stick Whole Blood: Allow 1 hanging drop of finger stick whole blood specimen to fall into the center of the specimen well (S), then add 2 drops of buffer.
4. Wait for the colored band(s) to appear. The result should be read at 20 minutes.



RESULTS & INTERPRETATION:

1. Positive: Two lines appear. One coloured line should be in the control line region (C) and another apparent coloured line should be in the test line region (T).
2. Negative: One coloured line appears in the control line region(C). No line appears in the test line region (T).
3. Invalid: No visible band at all or test band without control band.

2B. 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.

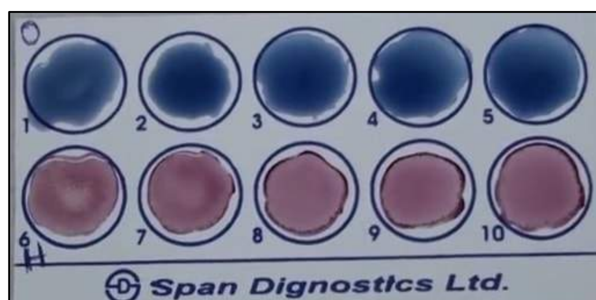
Preparation of Widal Antigens

- 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.

PROCEDURE:

SLIDE TEST

1. Place one drop of positive control on one reaction circles of the slide.
2. Pipette one drop of Isotonic saline on the next reaction cirlcle. (-ve Control).
3. Pipette one drop of the patient serum tobe tested onto the remaining four reaction circles.
4. Add one drop of Widal TEST antigen suspension ‘H’ to the first two reaction circles. (PC & NC).
5. Add one drop each of ‘O’, ‘H’, ‘AH’ and ‘BH’ antigens to the remaining four reaction circles.
6. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
7. Rock the slide, gently back and forth and observe for agglutination macroscopically within one minute.

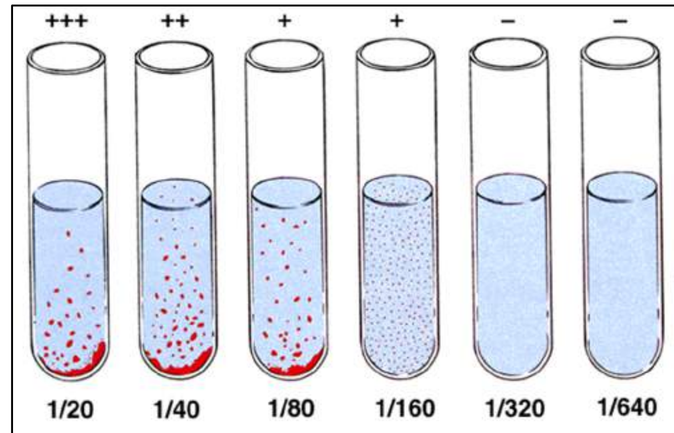


SEMI-QUANTITATIVE METHOD

1. Pipette one drop of isotonic saline into the first reaction circle and then place 5, 10, 20, 40, 80 ul of the test sample on the remaining circles.
2. Add to each reaction circle, a drop of the antigen which showed agglutination with the test sample in the screening method.
3. Using separate mixing sticks, mix the contents of each circle uniformly over the reaction circles.
4. Rock the slide gently back and forth, observe for agglutination macroscopically within one minute.

STANDARD TUBE TEST METHOD

In Widal Test, two types of tubes were originally used:
(1) Dreyer's tube (narrow tube with conical bottom) for H agglutination and
(2) Felix tube (short round-bottomed tube) for O agglutination.
Now a days 3 x 0.5 ml Kahn tubes are used for both types of agglutination.



1. Take 4 sets of 8 Kahn tubes/test tubes and label them 1 to 8 for O, H, AH and BH antibody detection.
2. Pipette into the tube No.1 of all sets 1.9 ml of isotonic saline.
3. To each of the remaining tubes (2 to 8) add 1.0 ml of isotonic saline.
4. To the tube No.1 tube in each row add 0.1 ml of the serum sample to be tested and mix well.
5. Transfer 1.0 ml of the diluted serum from tube no.1 to tube no.2 and mix well.
6. Transfer 1.0 ml of the diluted sample from tube no.2 to tube no.3 and mix well. Continue this serial dilution till tube no.7 in each set.
7. Discard 1.0 ml of the diluted serum from tube No.7 of each set.
8. Tube No.8 in all the sets, serves as a saline control. Now the dilution of the serum sample achieved in each set is as follows: Tube No. : 1 2 3 4 5 6 7 8 (control) Dilutions 1:20 1:40 1:80 1:160 1:320 1:640 1:1280.
9. To all the tubes (1 to 8) of each set add one drop of the respective WIDALTEST antigen suspension (O, H, AH and BH) from the reagent vials and mix well.
10. Cover the tubes and incubate at 37° C overnight (approximately 18 hours).
11. Dislodge the sedimented button gently and observe for agglutination.

INTERPRETATION:

SLIDE TEST

- Agglutination is a positive test result and if the positive reaction is observed with 20 ul of test sample, it indicates presence of clinically significant levels of the corresponding antibody in the patient serum.
- No agglutination is a negative test result and indicates absence of clinically significant levels of the corresponding antibody in the patient serum.

TEST TUBE

- The titre of the patient serum using Widal test antigen suspensions is the highest dilution of the serum sample that gives a visible agglutination.
- The sample which shows the titre of 1:100 or more for O agglutinations and 1:200 or more for H agglutination should be considered as clinically significant (active infection). **Example: In the above figure, titre is 160.**
- Demonstration of 4-fold rise between the two is diagnostic.
- H agglutination is more reliable than O agglutinin.
- Agglutinin starts appearing in serum by the end of 1st week with sharp rise in 2nd and 3rd week and the titre remains steady till 4th week after which it declines.

2C. 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:

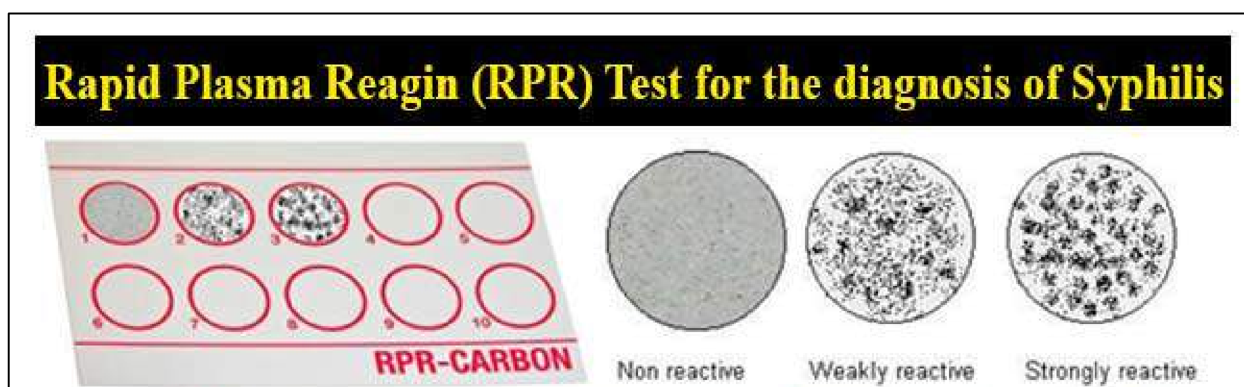
<u>Qualitative Test</u>

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.

<u>Quantitative Test</u>

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.

2D. 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:

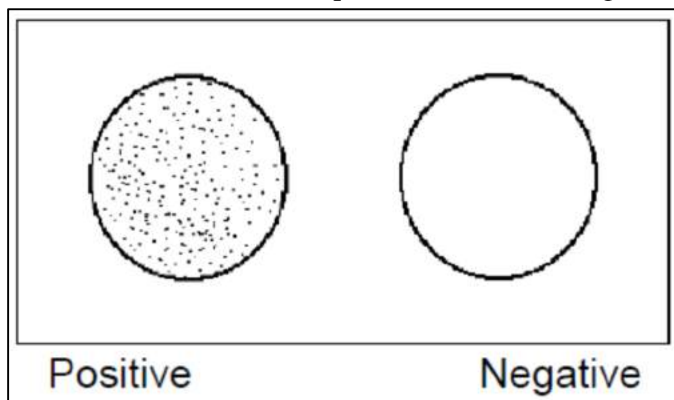
1. Positive and negative controls should be included in each test batch.
2. Acceptable performance is indicated when a uniform milky suspension with no agglutination is observed with the ASO Negative Control and agglutination with large aggregates is observed with the ASO Positive Control.
3. Results 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.

EXPERIMENT 3. CRP test, RA test, AIDS & STS test.

3A. 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.

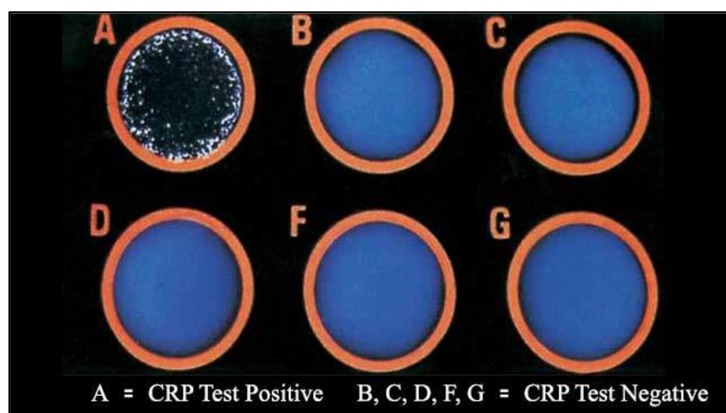
PROPCEDURE:

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



A = CRP Test Positive B, C, D, F, G = CRP Test Negative

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.

3B. RA (Rheumatoid Arthritis) test:

AIM:

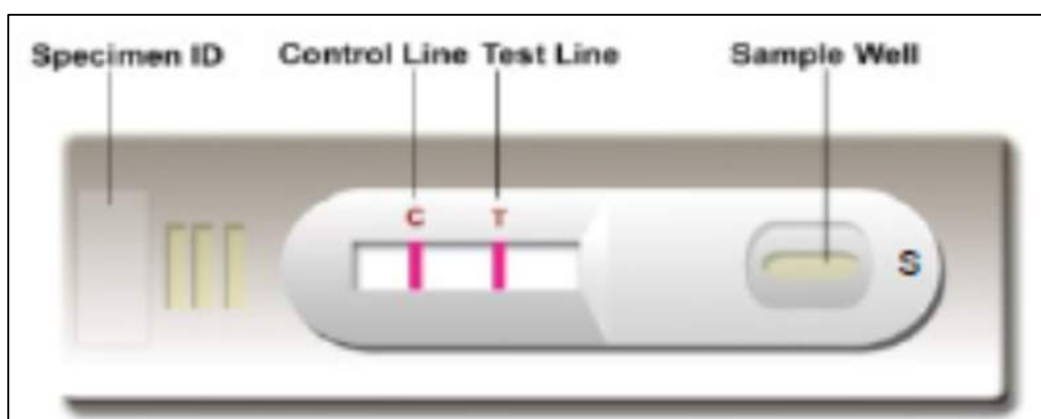
To rapid test the qualitative detection of all subtypes of rheumatoid factor (RF) in human serum, plasma or whole blood at a sensitivity of 8 IU/mL. by a lateral flow immunoassay.

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.

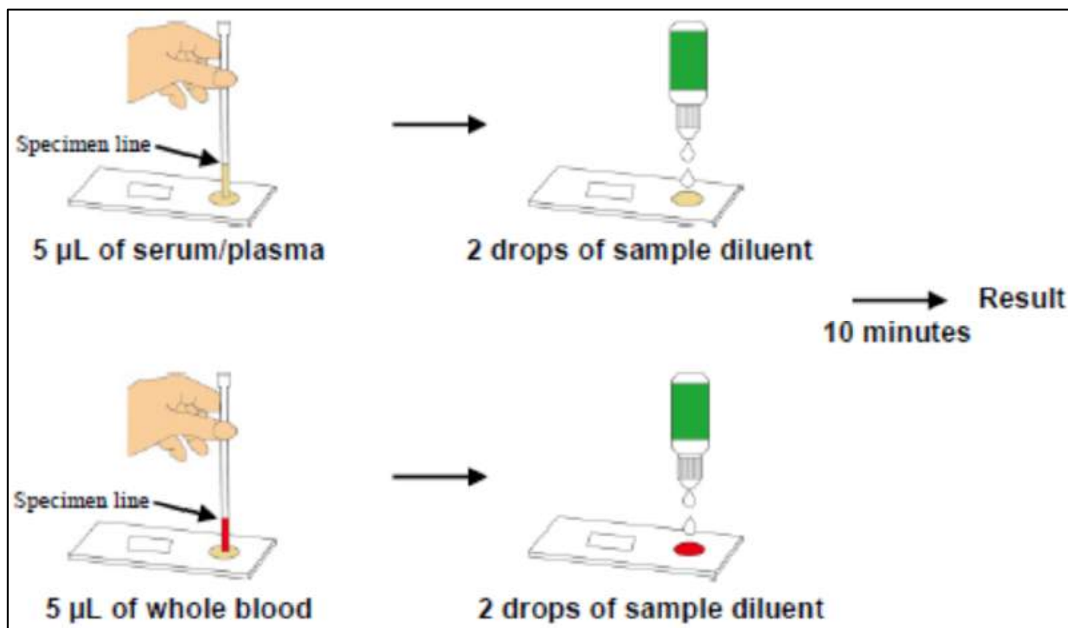
2: When ready to test, open the pouch at the notch and remove the device. Place the test device on a clean, flat surface.

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.

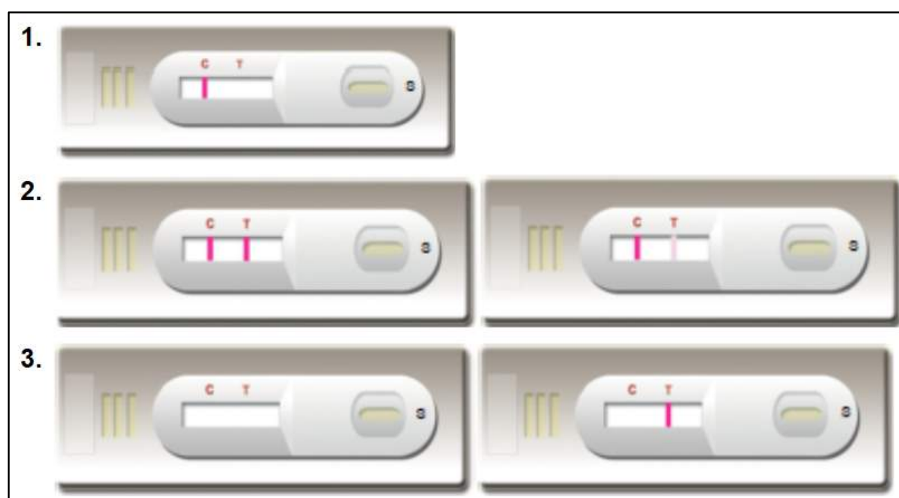


RESULT & INTERPRETATION:

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.

3C. AIDS test & STS 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*:**

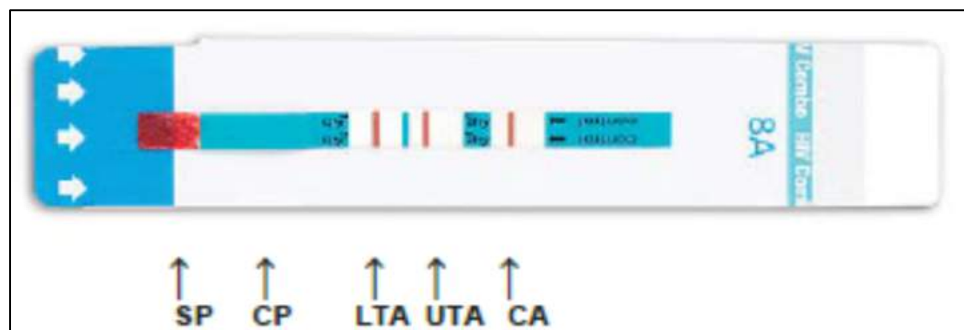
HIV–1/2 Ag/Ab Combo is an immunochromatographic test for the simultaneous and separate qualitative detection of free HIV-1 p24 antigen and antibodies to HIV-1 and HIV-2. The test device is a laminated strip that consists of a Sample Pad containing monoclonal biotinylated anti-HIV-1 p24 antibody, a Conjugate 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

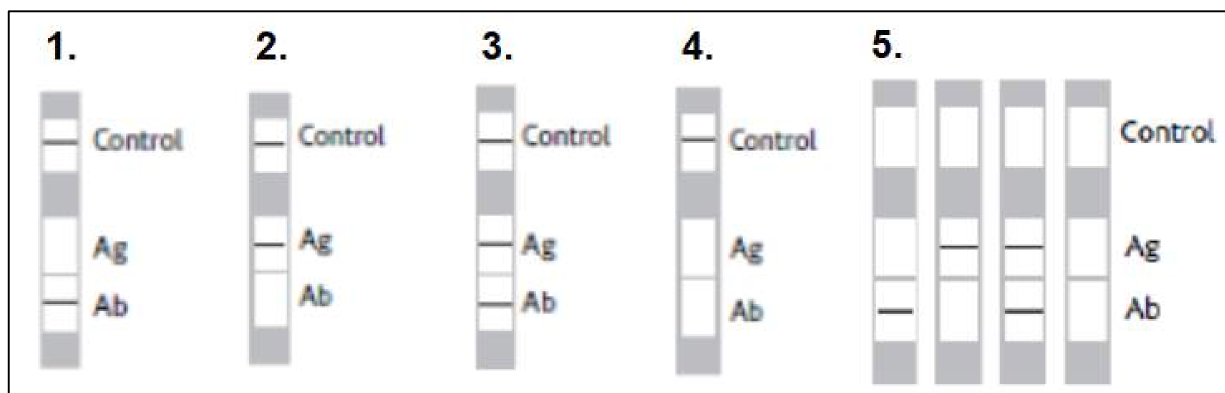
detected in the specimen. The test result is interpreted as PRELIMINARY POSITIVE for HIV-1 and/or HIV-2 antibodies.

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.



EXPERIMENT 4. Immunological test for pregnancy

AIM:
The hCG Card Pregnancy Test is a rapid chromatographic immunoassay for the qualitative detection of human chorionic gonadotropin in urine to aid in the early detection of pregnancy.

INTRODUCTION:
Human chorionic gonadotropin is a glycoprotein hormone produced by the developing placenta shortly after fertilization. In normal pregnancy, hCG can be detected in both urine and serum as early as 7 to 10 days after conception. hCG levels continue to rise very rapidly, frequently exceeding 100 mIU/mL by the first missed menstrual period, and peaking in the 100,000-200,000 mIU/mL range about 10-12 weeks into pregnancy. The appearance of hCG in both the urine and serum soon after conception, and its subsequent rapid rise in concentration during early gestational growth, make it an excellent marker for the early detection of pregnancy.
The hCG Card Pregnancy Test is a rapid test that qualitatively detects the presences of hCG in urine specimen at the sensitivity of 25 mIU/mL. The test utilizes a combination of monoclonal and polyclonal antibodies to selectively detect elevated levels of hCG in urine. At the level of claimed sensitivity, the hCG Card Pregnancy Test shows no cross-reactivity interference from the structurally related glycoprotein hormones hFSH, hLH and hTSH at high physiological levels. The hCG Card Pregnancy Test has a sensitivity of 25 mIU/mL, and is capable of detecting pregnancy as early as 10 days after a possible conception.

PRINCIPLE:
The hCG Card Pregnancy Test is a rapid chromatographic immunoassay for the qualitative detection of human chorionic gonadotropin in urine to aid in the early detection of pregnancy. The
test utilizes a combination of antibodies including a monoclonal hCG antibody to selectively detect elevated levels of hCG. The assay is conducted by adding a urine specimen to the specimen well of the test device and observing the formation of colored lines. The specimen migrates via capillary action along the membrane to react with the colored conjugate. Positive specimens react with the specific antibody-hCG-colored conjugate to form a colored line at the test line region of the membrane. Absence of this colored line suggests a negative result. To serve as a procedural control, a colored line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

MATERIALS & REAGENTS:
1. The test card contains anti-hCG particles and anti-hCG coated on the membrane. The kit can be stored at room temperature or refrigerated (2-30°C). The test card is stable through the expiration date printed on the sealed pouch. The test card must remain in the sealed pouch until use.
2. Urine Specimen collection container.
3. Dropper, Timer.

<u>SPECIMEN: URINE ASSAY:</u>

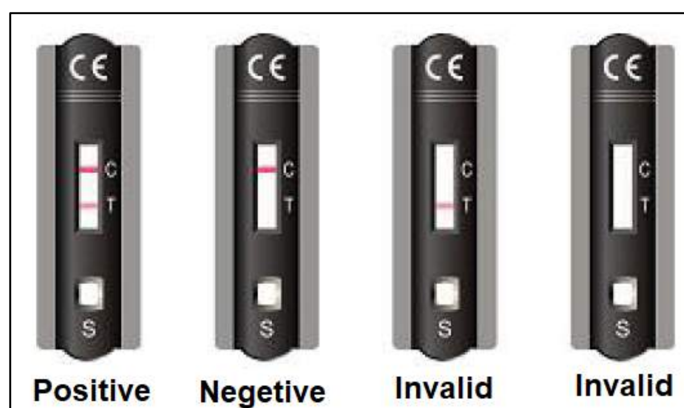
A urine specimen must be collected in a clean and dry container. A first morning urine specimen is preferred since it generally contains the highest concentration of hCG; however, urine specimens
collected at any time of the day may be used. Urine specimens exhibiting visible precipitates should be centrifuged, filtered, or allowed to settle to obtain a clear specimen for testing.

PROCEDURE:

1. Allow the test device, urine specimen and/or controls to equilibrate to room temperature (15-30°C) prior to testing.
2. Bring the pouch to room temperature before opening it. Remove the test device from the sealed pouch and use it as soon as possible.
3. Place the test device on a clean and level surface. Hold the dropper vertically and transfer 3 full drops of urine (approx. 180 µl) to the specimen well (S) of the test device, and then start the timer. Avoid trapping air bubbles in the specimen well (S).
4. The result should be interpreted between 3-5 minutes.

RESULTS & INTERPRETATION:

1. **Positive: Two distinct red lines appear**. One line should be in the control region (C) and another line should be in the test region (T). The intensity of the red color in the test line region (T) will vary depending on the concentration of hCG present in the specimen. Therefore, any shade of red in the test region (T) should be considered positive.
2. **Negative: One red line appears in the control region (C)**. No apparent red or pink line appears in the test region (T).
3. **Invalid: Control line fails to appear.** Insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure. Review the procedure and repeat the test with a new test device.
4. Please confirm negative results at 10 minutes. Do not interpret result exceeding 10 minutes.



EXPEIMENT 5: Montouxe test.

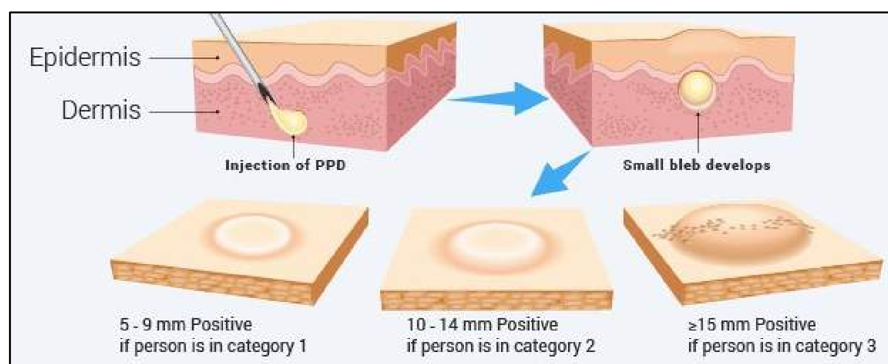
AIMS:
To rapid detect of tuberculosis.

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:
  - ≥5 mm is positive in patients with baseline of 0 mm
  - ≥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.