Semester – IV

18MBU411 **IMMUNOLOGY – PRACTICAL** **(4H – 2C)**

Instruction Hours / week:L: 0 T: 0 P: 4 **Marks:** Internal: **40**External: **60** Total: **100**

End Semester Exam: 6 Hours

COURSE OBJECTIVES

- To strengthen the knowledge of students in immunodiagnostics.
- To learn the latest trends in immunology.
- Rapid diagnosis and Immune reaction.

COURSE OUTCOME

Introducing the science of immunology and to study various types of immune systems their classification structure and mechanism of immune activation.

EXPERIMENTS

1. Identification of human blood groups.
2. Perform Total Leukocyte Count of the given blood sample.
3. Perform Differential Leukocyte Count of the given blood sample.
4. Separate serum and plasma from the blood sample (demonstration).
5. Perform immunodiffusion by Ouchterlony method.
6. Perform DOT ELISA.
7. Perform immunoelectrophoresis.

SUGGESTED READINGS

1. Goldsby RA, Kindt TJ, Osborne BA. (2007). Kuby's Immunology. 6th edition, W.H. Freeman and Company, New York.
2. Delves P, Martin S, Burton D, Roitt IM. (2006). Roitt's Essential Immunology. 11th edition, Wiley- Blackwell Scientific Publication, Oxford.
3. Murphy K, Travers P, Walport M. (2008). Janeway's Immunobiology. 7th edition, Garland Science Publishers, New York.
4. Abbas AK, Lichtman AH, Pillai S. (2007). Cellular and Molecular Immunology. 6th edition, Saunders Publication, Philadelphia.
5. Peakman M, and Vergani D. (2009). Basic and Clinical Immunology. 2nd edition Churchill Livingstone Publishers, Edinberg.
6. Richard C and Geiffrey S. (2009). Immunology. 6th edition. Wiley Blackwell Publication.

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Experiment – 1 **Identification of human blood groups**

Aim

To determine the blood group and Rh factor of an individual.

Introduction

Blood grouping is the classification of blood based on the presence or absence of two inherited antigenic substances on the surface of red blood cells (RBCs). The ABO and Rh are the major, clinically significant and the most important of all the blood group systems. The ABO blood group system was first discovered by Karl Landsteiner in 1900. The human ABO blood group system is divided into the following four major groups depending on the antigen present on the surface of their red blood cells:

- "A" group
- "B" group
- "AB" group
- "O" group

Table 1: ABO Blood Group System

ens on the surface of Red Blood Cells	Antibodies in the Serum	ABO Blood Group	Genotype
A	Anti B	A	AA or AO
B	Anti A	B	BB or BO
A and B	Neither Anti A nor Anti	AB	AB
Neither A nor B	Anti A, Anti B, Anti AB	O	OO

The associated Anti A and Anti B antibodies usually belong to IgM class of immunoglobulins. The Rhesus system (Rh) is the second most important blood group system in humans. The most significant and immunogenic Rhesus antigen is the RhD antigen. The individuals carrying the Rh antigen are considered to have positive blood group whereas those individuals that lack this antigen are considered to have negative blood group.

Principle

The ABO and Rh blood grouping system is based on agglutination reaction. When red blood cells carrying one or both the antigens are exposed to the corresponding antibodies they interact with each other to form visible agglutination or clumping. The ABO blood group antigens are O-linked glycoproteins in which the terminal sugar residues exposed at the cell surface of the red blood cells determine whether the antigen is A or B. Blood group A individuals have A antigens on RBCs and anti-B antibodies in serum. Similarly, blood group B individuals have B antigens on RBCs and anti-A antibodies in serum. Blood group AB individuals have both A and B antigens on RBCs and neither anti-A nor anti-B antibodies in serum. Whereas, blood group O individuals have neither A antigens nor B antigens, but possess both anti-A and anti-B antibodies in serum. The Rh antigens are transmembrane

proteins in which the loops exposed on the surface of red blood cells interact with the corresponding antibodies.

Table 2: Enlists the materials required with their quantity and recommended storage.

S. No.	Materials Provided	Quantity 100 expts	Storage
1	Anti A Sera	5 ml	2-8^{o}C
2	Anti B Sera	5 ml	2-8^{o}C
3	Anti RhD Sera	5 ml	2-8^{o}C
4	Cavity slide	10 Nos.	RT
5	Mixing stick	300 Nos.	RT
6	Blood Lancet	100 Nos.	RT

Materials Required

Reagents: 70% Alcohol/ Spirit
Other requirements: Cotton

Storage

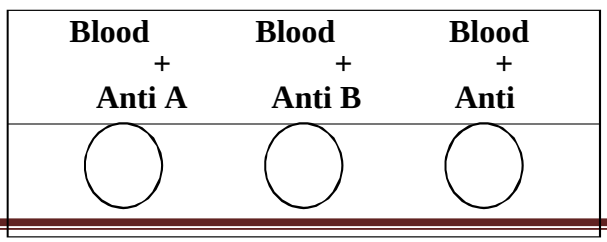
Store the Anti A Sera, Anti B Sera and Anti RhD Sera at 2-8oC. Other contents can be stored at room temperature (15-25oC).

Important Instructions

1. Before starting the experiment the entire procedure has to be read carefully.
2. Always wear gloves while performing the experiment.
3. Ensure the slide is clean and dry prior to use.
4. Do not allow the antisera reagent dropper to touch the blood sample.
5. The result of the reaction should be interpreted immediately after mixing.
6. Avoid intermixing of the antisera reagents while performing the experiment as it may give false result.

Procedure

1. Dangle the hand down to increase the flow of blood in the fingers.
2. Clean the fingertip to be pierced with spirit or 70% alcohol (usually ring or middle finger).
3. With the help of the sterile lancet, pierce the fingertip and place one drop of blood in each of the cavities.
4. Add one drop of antiserum into each cavity as shown below:



1. Mix each blood drop and the antiserum using a fresh mixing stick.
2. Observe agglutination in the form of fine red granules within 30 seconds. Anti RhD takes slightly longer time to agglutinate compared to Anti A and Anti B.

3. Note: Proper care should be taken while disposing the lancet and mixing sticks.

Interpretation

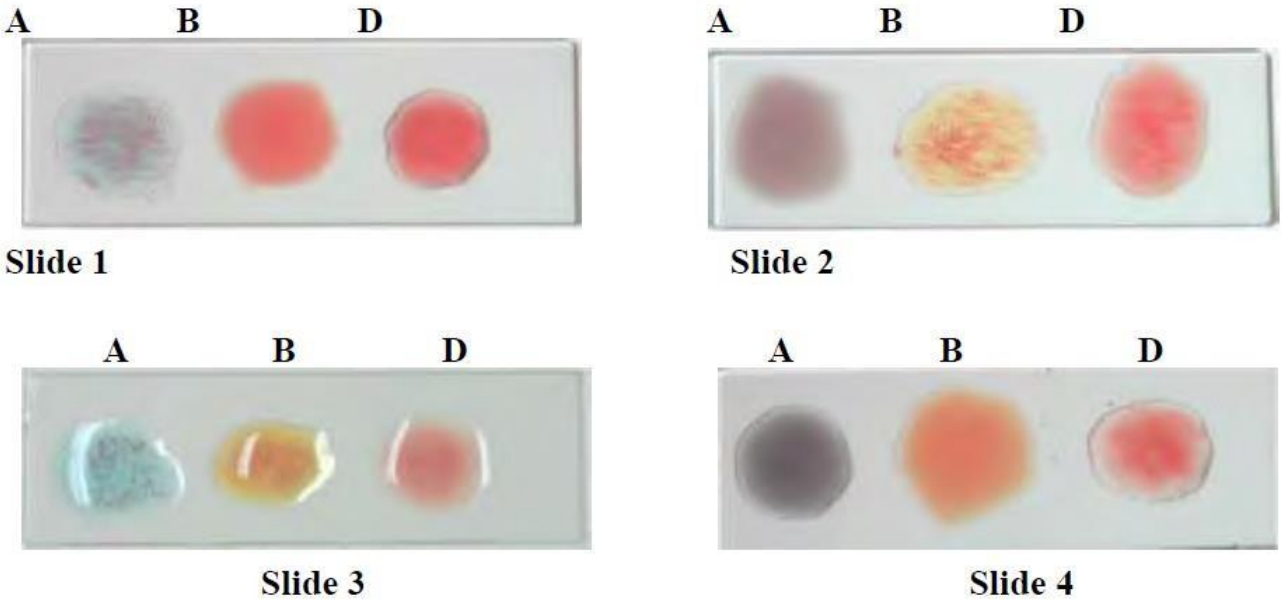


Table 3: Determination of blood group and Rh factor based on agglutination seen

Sr. No.	Anti A	Anti B	Anti RhD	Blood Group
Slide 1	□	X	□	**A**
Slide 2	X	□	□	**B**
Slide 3	□	□	□	**AB +ve**
Slide 4	X	X	□	**O**

□: Agglutination
X: No agglutination

•If agglutination is observed when blood is mixed with Anti A reagent, then the individual is said to have blood group "A".

•If agglutination is observed when blood is mixed with Anti B reagent, then the individual is said to have blood group "B".

•If agglutination is observed when blood is mixed with Anti A and Anti B reagent, then the individual is said to have blood group "AB".

•If no agglutination is observed when blood is mixed with Anti A and Anti B reagent, then the Individual is said to have blood group "O".

•If agglutination is observed when blood is mixed with Anti RhD reagent, then the individual is said to have "+ve" Rh factor.

•If no agglutination is observed when blood is mixed with Anti RhD reagent, then the individual is said to have "-ve" Rh factor.

Observations and Results

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Experiment – 2 Total Leukocyte Count of the given blood sample

Aim:

To perform Total leukocyte count of the given blood sample.

Introduction:

Total leukocyte count (TLC) refers to the number of white blood cells in 1 µl of blood (or in 1 liter of blood if the result is expressed in SI units). There are two methods for estimation of TLC:

- Manual or microscopic method
- Automated method

A differential leukocyte count should always be performed along with TLC to obtain the absolute cell counts.The purpose of carrying out TLC is to detect increase or decrease in the total number of white cells in blood, i.e. leukocytosis or leukopenia respectively. TLC is carried out in the investigation of infections, any fever, hematologic disorders, malignancy, and for follow-up of chemotherapy or radiotherapy.

Principle

A sample of whole blood is mixed with a diluent, which lyses red cells and stains nuclei of white blood cells. White blood cells are counted in a hemocytometer counting chamber under the microscope and the result is expressed as total number of leukocytes per µl of blood or per liter of blood.

Equipment

(1) Hemocytometer or counting chamber with coverglass: The recommended hemocytometer is one with improved Neubauer rulings and metallized surface. There are two ruled areas on the surface of the chamber. Each ruled area is 3 mm × 3 mm in size and consists of 9 large squares with each large square measuring 1 mm × 1 mm. When the special thick coverglass is placed over the ruled area, the volume occupied by the diluted blood in each large square is 0.1 ml. In the improved Neubauer chamber, the central large square is divided into 25 squares, each of which is further subdivided into 16 small squares. A group of 16 small squares is separated by closely ruled triple lines. Metallized surface makes background rulings and cells easily visible. The 4 large corner squares are used for counting leukocytes, while the central large square is used for counting platelets and red blood cells. Only special coverglass, which is intended for use with hemocytometer, should be used. It should be thick and optically flat. When the special coverglass is placed on the surface of the chamber, a volumetric chamber with constant depth and volume throughout its entire area is formed. Ordinary cover slips should never be employed since they do not provide constant depth to the underlying chamber due to bowing When the special cover glass is placed over the ruled area of the chamber and pressed, Newton's rings (colored refraction or rainbow colored rings) appear between the two glass surfaces; their formation indicates the correct placement of the cover glass.

(2) Pipette calibrated to deliver 20 µl (0.02 ml, 20 cmm): WBC bulb pipettes, which have a bulb for dilution and mixing (Thoma pipettes) are no longer recommended. This is because blood and diluting fluid cannot be mixed adequately inside the bulb of the pipette. Bulb pipettes are also difficult to calibrate, costly, and charging of counting chamber is difficult. Tips of pipettes often chip easily and unnecessarily small volume of blood needs to be used.

(3) Graduated pipette, 1 ml.

(4)Pasteur pipette

(5) Test tube (75 × 12 mm).

Reagent

WBC diluting fluid (Turk's fluid) consists of a weak acid solution (which hemolyzes red cells) and gentian violet (which stains leucocyte nuclei deep violet). Diluting fluid also suspends and disperses the cells and facilitates counting. Its composition is as follows:

- Acetic acid, glacial 2 ml
- Gentian violet, 1% aqueous 1 ml
- Distilled water to make 100 ml

Specimen

EDTA anticoagulated venous blood or blood obtained by skin puncture is used. (Heparin should not be used since it causes leukocyte clumping). While collecting capillary blood from the finger, excess squeezing should be avoided so as not to dilute blood with tissue fluid.

Method

(1) Dilution of blood: Take 0.38 ml of diluting fluid in a test tube. To this, add exactly 20 µl of blood and mix. This produces 1:20 dilution. Alternatively, 0.1 ml of blood can be added to 1.9 ml of diluting some of the fluid.

(2) Charging the counting chamber: Place a coverglass over the hemocytometer. Draw some of the diluted blood in a Pasteur pipette. Holding the Pasteur pipette at an angle of 45° and placing its tip between the coverglass and the chamber, fill one of the ruled areas of the hemocytometer with the sample. The sample should cover the entire ruled area, should not contain air bubbles, and should not flow into the side channels. Allow 2 minutes for settling of cells.

(3) Counting the cells: Place the charged hemocytometer on the microscope stage. With the illumination reduced to give sufficient contrast, bring the rulings and the white cells under the focus of the low power objective (× 10). White cells appear as small black dots. Count the number of white cells in four large corner squares. (To reduce the error of distribution, counting of cells in all the nine squares is preferable). To correct for the random distribution of cells lying on the margins of the square, cells which are touching the left hand lines or upper lines of the square are included in the count, while cells touching the lower and right margins are excluded.

Calculation of TLC:

$$TLC/\mu l = \frac{Nw \times Cd \times Cv}{NLS}$$

$$= \frac{Nw \times 20 \times 10}{4}$$

$$= Nw \times 50$$

Where Nw is the number of WBCs counted, Cd is the correction of dilution, Cv is the correction of volume and NLS is the number of large squares counted.

(a) TLC/L = Number of WBCs counted × 50 × 10_6 (10_6 is the correction factor to convert count in 1 µl to count in 1 liter). Example: If 200 WBCs are counted in 4 large squares, TLC/µl will be 10,000/µl and TLC/liter will be 10.0 × 10_9/liter.

If TLC is more than 50,000/ml, then dilution of blood should be increased to 1:40 to increase the accuracy of the result.

If TLC is less than 2,000/ml then lesser dilution should be used.

Expression of TLC*:* Conventionally, TLC is expressed as cells/µl or cells/cmm or cells/mm^3. In SI units, TLC is expressed as cells × 10_9/liter. Conversion factors for conventional to SI units is and SI to conventional units is 1000.

Correction of TLC for nucleated red cells: The diluting fluid does not lyse nucleated red cells or erythroblasts. Therefore, they are counted as leukocytes in hemocytometer. If erythroblasts are markedly increased in the blood sample, overestimation of TLC can occur. To avoid this if erythroblasts are greater than 10 per 100 leukocytes as seen on blood film, TLC should be corrected for nucleated red cells by the following formula:

$$C_{TLC} = \frac{TLC \times 100}{N_{RBC} + 100}$$

Where C_{TLC} is the Corrected TLC/µl, TLC is the Total Leukocyte Count and N_{RBC} is the Nucleated RBCs per 100 WBCs.

REFERENCE RANGES

Adults	4000-11,000/µl
At birth	10,000-26000/µl
1 year	6,000-16,000/µl
6-12 years	5,000-13,000/µl
Pregnancy	up to 15,000/µl

CRITICAL VALUES

TLC < 2000/µl or > 50000/µl.

Experiment – 3 Differential Leukocyte Count of the given blood sample

Aim:

To perform differential leukocyte count (DLC) of the given blood sample

Introduction:

The leukocytes are also called white blood corpuscles (WBC) and formally known as white cells of the blood but these are not white colour, these are colourless. The white blood cells are protect our body against any diseases by fighting with infections (bacterial, viral, protozoan, parasitic etc.), antigens and also against malignancy. These are two types; Granulocytes (Neutrophil, Eosinophil and Basophile) and Agranulocytes (Monocyte and Lymphocyte). After staining the blood film with Leishman stain, the blood smear examine in the microscope under oil immersion objective (100X). Under oil immersion objective the leukocytes are seen as follows:

Morphology and function of leukocytes Neutrophil

Neutrophils are round shape, 10 – 15µ in diameter. The cell contains cytoplasm and nucleus. The nucleus shows variable numbers of lobes, 2 – 7 lobes hence called polymorphonuclear leukocytes. The nucleus stain purple blue and the chromatin are coarse and ropy. The cytoplasm contains two types of granules – Primary granules and Secondary granules. When the cell stained with Leishman stain, only secondary granules are stained and these are violet colour granules, which are amphophilic. The cytoplasm takes pink colour.

Neutrophils are called first line defenses as they move first to fight the invading micro-organisms (bacteria etc.). Neutrophil with their enzymatic armory are superb killers. The activated neutrophils engulf the bacteria (phagocytosis) and released different enzymes into the phagocytic vesicles, which killed the bacteria and then digest it.

Eosinophil

Eosinophils are round shaped, 10 – 15µ in diameter. The cell contain cytoplasm packed with coarse brick red colour granules which takes acidic stain (eosin) hence called eosinophil. And the nucleus of the cell consists of 2 – 3 lobes. The nucleus stains purple blue colour and chromatin is course and ropy. Eosinophil contains a Major Basic Protein (MBP) which damages the larvae of the parasites. There is one eosinophilic cation protein which probably neutralizes heparin anticoagulant. They have a property that prevents anaphylaxis (anti allergic action). They are motile and phagocytic. Chemotoxis is also shown by the eosinophils.

Basophile

Basophils are round shape, 10 – 15µ in diameter occasionally seen in the blood smear from a healthy person. The cell contain ‘bilobed’ or ‘S’ shaped nucleus stains purple blue and chromatin is coarse and ropy. The cytoplasm of the cell packed with course blue basophilic granules. On an average all granules are of equal size, which obscured the nucleus.Basophils release the histamine resulting in

immediate hypersensitivity reaction and also have role in inflammation. They contain heparin, protease and other mediators of inflammation. They are motile and phagocytic.

Monocyte

Monocytes are large, round, 10 – 20µ in diameter The cell contains a large kidney shaped, light purple blue stained nucleus and pale blue colour cytoplasm contain no granules. Some time few fine purple granules and vacuoles are seen in the cytoplasm.

The monokines secreted by monocytes stimulated T-cells, take part in inflammation, act as pyrogen and stimulate formation of acute phase proteins. They converted into macrophases and phagocytosis the microorganism, dead tissues etc.

Lymphocyte

In the blood circulation two types of lymphocyte are found, these are small lymphocyte and large lymphocyte. The small lymphocytes are believed to be the resting phase and when active they become large.

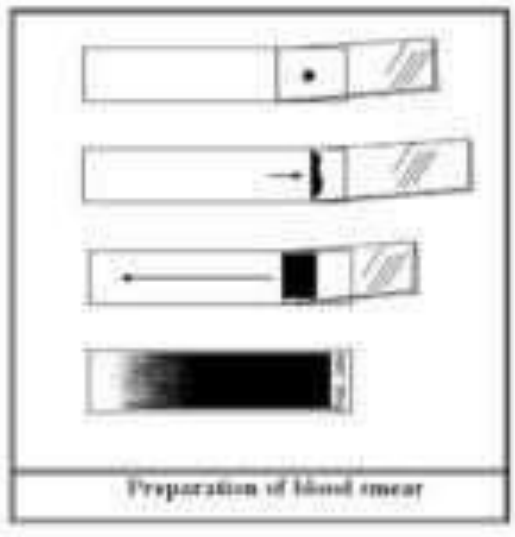
- **Small Lymphocyte :**

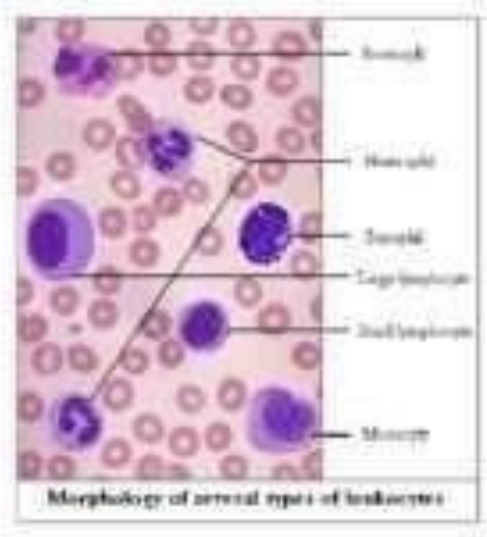
Small lymphocytes are small; round shaped, 8 – 10µ in diameter agranulocyte. The cell contains a large nucleus that occupied almost the total cell and scanty cytoplasm is seen. The cell contain cytoplasm takes blue colour and nucleus stains deep blue colour.

- **Large Lymphocyte :**

Large lymphocytes are round shaped, 10 – 18µ in diameter agranulocyte. They contain a round shaped deep blue nucleus with course and ropy chromatin and abundant clear blue cytoplasm with out any granules.

They are involver in the very important defense mechanism, called immunity. The B- lymphocytes are responsible for Humoral immunity and T-lymphocytes are responsible for Cell mediated immunity.





Clinical significance of DLC

Differential Leukocyte Count is useful to identify changes in the distribution of white blood cells

which may be related to specific type of disorders. It also gives idea regarding the severity of the disease and the degree of response of the body.

Neutrophilia

Increase in the percentages of neutrophils in the blood is called neutrophilia.

Causes :

All the physiological causes that produces leukocytosis give rise to neutrophilia.

Physiological

- Exercise.
- Pregnancy.
- Neonatal period.
- Exposure to cold etc.

Drugs

- Epinephrine.
- Steroids etc.

Pathological

- Infection with pyogenic organisms.
- Non-infective inflammations.
- Myocardial infarction, Pulmonary embolism, Haemorrhage.
- Trauma and following surgery.
- Uremia, Hepatic come, Chemicals.
- Leukaemia, Neoplasm.

Neutropenia

Decrease in neutrophils below normal in the blood is called neutropenia.

Causes :

- Starvation and debility.
- Toxaemia in old people.
- Infections like typhoid, measles, malaria, kala-azar, hepatitis, influenza etc.
- Hyperspenism.
- Bone marrow failure : Aplastic anaemia, Leukaemia, Megaloblastic anaemia etc.
- Drugs : Sulphonamides, Diuretics etc.
- Chemotherapy etc.

Lymphocytosis

Increase in the percentages of lymphocytes in the blood is called lymphocytosis.

Causes :

Absolute

- Tuberculosis, brucellosis, syphilis, pertussis, toxoplasmosis.
- Mumps, rubella, infectious mononucleosis.
- Leukaemia.
- Thyrotoxicosis.

Relative

- All causes of neutropenia.
- Infective hepatitis.
- Convalescence from acute infections.
- Infants with infections.
- Malnutrition and avitaminosis.

Lymphocytopenia

Decrease in lymphocytes below normal in the blood is called lymphocytopenia.

Causes :

- Severe bone marrow failure.
- Immunosuppressive drugs.
- Hodgkin's disease.
- Irradiation.

Viral infection like HIV.

Eosinophilia

Increase percentage of eosinophil present in the blood is called eosinophilia.

Causes :

Allergic

- High fever.
- Urticaria.
- Bronchial asthma.
- Food sensitivity.

Parasitic infections

- Hook worm.
- Hydatid cyst.
- Amebiosis.
- Filariasis etc.

Collagen disease : Periarteritis nodosa.

Recovery from acute infection.

Skin disease : Psoriasis, Pemphigus, Drug rash etc.

Monocytosis

Increase percentage of monocytes present in the blood is called monocytosis.

Causes :

- Chronic bacterial infection.
- Protozoan diseases.
- Hodgkin's disease.
- Chronic neutropenia.
- Monocytic or myelomonocytic leukemia.

Basophilia

Increase percentage of basophils present in the blood is called basophilia.

Causes :

Chronic myeloid leukemia.

Polycythaemia Vera.

NORMAL VALUES

- Neutrophils : 40 – 75 % (mean : 57 %)
- Band Formed : 2 – 6 % (mean : 3 %)
- Segmented : 50 – 70 % (mean : 54 %)
- Eosinophils : 1 – 4 % (mean : 2 %)
- Basophils : 0 – 1 %
- Lymphocytes : 20 – 45 % (mean : 37 %)
- Monocytes : 2 – 8 % (mean : 6 %)

PRINCIPLE OF DLC

The polychromic staining solution (Leishman stain) contains methylene blue and eosin. These basic and acidic dyes induce multiple colours when applied to cells. Methanol acts a fixative and also as a solvent. The fixative does not make them adhere to the glass slide. The basic components of white blood cells (cytoplasm) are stained by acidic dye and they are describing as eosinophilic or acidophilic. The acidic components (nucleus & nucleic acid) of the cells take blue to purple shades by basic dye and they are called basophilic. The neutral components of the cell are stained by the dyes.

SPECIMEN FOR DLC

EDTA anticoagulated venous blood or free flowing capillary blood.

REQUIREMENTS FOR DLC

1. Microscopic slide and a glass Spreader slide.
2. Cedar wood oil (immersion oil)
3. Leishman stain

Leishman powder : 0.15 gm
Acetone free methanol : 1.0 liter

Preparation of stain : 0.15 gm of Leishman powder is dissolved in 100 ml of Acetone free methanol and the mixture is warmed at 50°C for 15 minutes. It is then filtrated and dye is ripened by keeping the filtrate in incubator at 37°C for 7 days. The stain is routinely used for staining the blood smear.

4. Buffer solution (P_H : 7.0)
    - Sodium Dihydrogen Phosphate (NaH_2PO_4) : 3.76 gm
    - Potassium Dihydrogen Phosphate (KH_2PO_4) : 2.10 gm

Distilled water : 1.0 liter

5. Staining rack.
6. Cotton and Tissue paper.
7. Pipette
8. Timer
9. Microscope with 10X and 100X objectives.
10. Cell counter.

PROCEDURE OF DLC

1. A thin blood film is prepared by spreading a drop of well mixed blood evenly on a clean and dry glass slide and dries the smear at room temperature. Adequate drying is essential to preserve the quality of the blood film.
2. Write the Identification number on the slide by using a lead pencil or a Marker pen.
3. Dried blood film prepared slide is placed on the staining rack.
4. Covered the smear with the Leishman staining solution by adding 10 – 15 drops on the smear by using a Pasteur pipette. Wait for exactly 1½ minutes.
5. Added equal amount of buffer solution on the slide. Mixed the reaction mixture adequately by blowing the contain on it through a pipette. Wait for 10 minutes.
6. Washed the smear by using running tap water.
7. Stand the slide in the draining rack or on the laboratory counter to dry the smear.

8. Examine the stained smear under the low power objective (10X) in the microscope for screening purpose and Chooses the proper portion of the smear. Placed one drop of Cedar wood oil (immersion oil) on the smear. Switch to the oil immersion objective (100X) and increase the light by opening the iris diaphragm. Examine the field by moving from one field to next field systematically. Record the types of leukocytes seen in each field. Count at least a total of 100 leukocytes and give the result as percentage (%) of the cells.

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Experiment – 4 **Separate serum and plasma from the blood sample**

Aim

To separate serum and plasma from the collected blood sample.

Introduction

Serum is the liquid fraction of whole blood that is collected after the blood is allowed to clot. The clot is removed by centrifugation and the resulting supernatant, designated serum, is carefully removed using a Pasteur pipette. Plasma is produced when whole blood is collected in tubes that are treated with an anticoagulant. The blood does not clot in the plasma tube. The cells are removed by centrifugation. The supernatant, designated plasma is carefully removed from the cell pellet using a Pasteur pipette.

Serum Preparation

Collect whole blood in a covered test tube. If commercially available tubes are to be used, the researcher should use the red topped tubes. These are available from Becton Dickinson (BD). BD's trade name for the blood handling tubes is Vacutainer. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Remove the clot by centrifuging at 1,000–2,000 x g for 10 minutes in a refrigerated centrifuge.

The resulting supernatant is designated serum. Following centrifugation, it is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette. The samples should be maintained at 2–8°C while handling. If the serum is not analyzed immediately, the serum should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze-thaw cycles because this is detrimental to many serum components. Samples which are hemolyzed, icteric or lipemic can invalidate certain tests.

Plasma preparation

Collect whole blood into commercially available anticoagulant-treated tubes e.g., EDTA-treated (lavender tops) or citrate-treated (light blue tops). Heparinized tubes (green tops) are indicated for some applications; however, heparin can often be contaminated with endotoxin, which can stimulate white blood cells to release cytokines. Cells are removed from plasma by centrifugation for 10 minutes at 1,000–2,000 x g using a refrigerated centrifuge. Centrifugation for 15 minutes at 2,000 x g depletes platelets in the plasma sample.

The resulting supernatant is designated plasma. Following centrifugation, it is important to immediately transfer the liquid component (plasma) into a clean polypropylene tube using a Pasteur pipette. The samples should be maintained at 2–8°C while handling. If the plasma is not analyzed immediately, the plasma should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze-thaw cycles. Samples which are hemolyzed, icteric, or lipemic can invalidate certain tests. There are other commercially available tubes for blood sample collection.

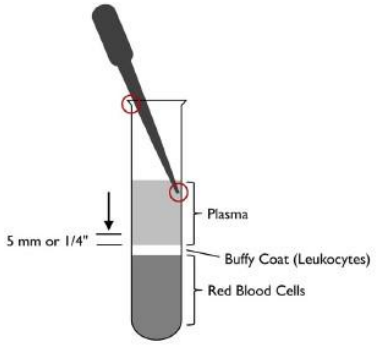


Figure 1. Separation of plasma from blood.

Table 1. Serum and plasma tubes

The commercially available serum tubes are as follows:	
Red	No anticoagulant.
Red with black	Treated with gel to help to separate the clot (not evaluated).
The commercially available plasma tubes are as follows:	
Lavender	Treated with EDTA.
Blue	Treated with citrate.
Green	Treated with heparin.
Grey	Treated with potassium oxalate/sodium fluoride (not evaluated).
Yellow	Treated with potassium oxalate/sodium fluoride (not evaluated).

Experiment-5 Immunodiffusion by Ouchterlony method

Aim

To study the reaction pattern of an antigen with a set of antibodies by Ouchterlony Double Diffusion method.

Introduction

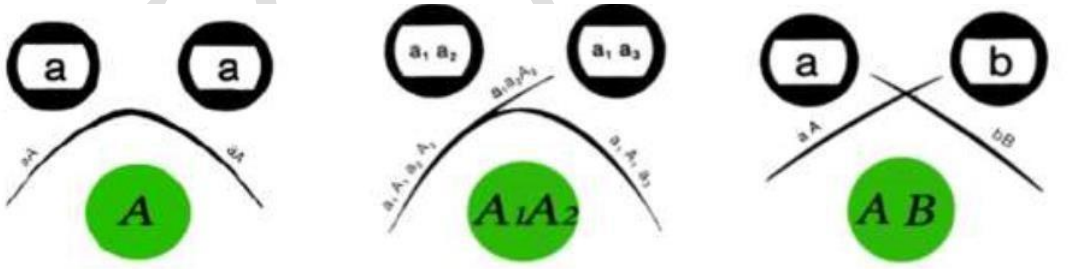
Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. Gel immunodiffusion can be classified into two groups:

- Single Immunodiffusion
- Double Immunodiffusion

In the Ouchterlony double diffusion, both the antigen and the antibody diffuse toward each other in a semisolid medium to a point till their optimum concentration is reached. Ab and of precipitation occurs at this point. The qualitative Ouchterlony Test can simultaneously monitor multiple Antibody-Antigen system and can be used to identify particular antigens in a preparation. This procedure was developed by Örjan Ouchterlony.

Principle

When soluble antigen and antibody samples are placed in adjacent wells in agarose gel, they diffuse radially into the agarose gel and set up two opposing concentration gradients between the wells. Once the gradients reach to an optimal proportion, interactions of the corresponding molecules occur and a line of precipitation will form. Using such a technique, the antigenic relationship between two antigens can be analyzed. Distinct precipitation line patterns are formed against the same anti-sera depending on whether two antigens share all antigenic epitopes or partially share their antigenic epitopes or do not share their antigenic epitopes at all. The Ouchterlony test also can be used to estimate the relative concentration of antigens. When an antigen has a relatively higher concentration, the equivalent zone will be formed a little bit away from the antigen well. When an antigen has a relatively lower concentration, the equivalent zone will be formed a little bit closer the antigen well. The pattern of lines that form can be interpreted to determine the relationship between the antigens and antibodies.



X Y Z

Pattern of Identity Pattern of Partial Identity Pattern of Non Identity

Figure 1. Antigen- Antibody Patterns formed in Ouchterlony Double Diffusion

Pattern of Identity X

Pattern of identity occurs when the antigens in the two wells are identical and specific for the antibody in the antiserum present in the third well. The concentration of the two antigens been the same, they will diffuse at the same rate resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens i.e. the two antigens are immunologically identical as shown in Figure 1.

Pattern of Partial Identity Y

Pattern of partial identity occurs when the antigens in the two wells share some epitopes which are same for both, yet each of the two antigens also have unique epitopes.

In this case antiserum contains polyclonal antibodies specific for each epitope. When one of the antigens has some of the same epitopes compared to other, the polyclonal antibody population will respond differently to the two antigens and the precipitin line formed for each antigen will be different. The 'spur' is thought to result from the determinants present in one antigen but lacking in the other antigen (refer to Figure 1). A similar pattern of partial identity is observed if the antibodies are cross reactive with an epitope on one of the antigen that is similar, but not identical to that present on the other antigen.

Pattern of Non-Identity: Z

Pattern of non-identity occurs when the antigens in the two wells are totally different. They are neither cross reactive, nor do they have any epitopes which are same. In this case the antiserum containing the antibodies is heterogeneous as some of the antibodies react with antigen in one well while some react with antigen present in the other well. So the two antigens are immunologically unrelated as far as that antiserum is concerned (refer to Figure 1).

Table 1. Enlists the materials provided with their quantity and recommended storage

S. No.	Materials Provided	Quantity 10 expts	Storage
1	Agarose	1.2 g	R T
2	10X Assay buffer	12 ml	2-8 °C
3	Antiserum X	0.1 ml	2-8°C
4	Antiserum Y	0.1 ml	2-8°C
5	Antiserum Z	0.1 ml	2-8°C
6	Antigen X1	0.1 ml	2-8°C
7	Antigen X2	0.1 ml	2-8°C
8	Antigen Y1	0.1 ml	2-8°C
9	Antigen Y2	0.1 ml	2-8°C
10	Antigen Z1	0.1 ml	2-8°C
11	Antigen Z2	0.1 ml	2-8°C
12	Glass plate	4 Nos.	R T
13	Gel puncher	1 No.	R T
14	Template	4 Nos.	R T

Materials Required

Glass wares: Measuring cylinder, Beaker

Reagents: Alcohol

Other requirements: Incubator (37 °C), Microwave or Bunsen burner, Vortex mixer, spatula, Micropipettes, Tips, Moist chamber (box with wet cotton)

Storage

Store 10X Assay buffer, Antisera and Antigens at 2-8oC. Other kit contents can be stored at room temperature (15-25°C).

Important Instructions

- Before starting the experiment the entire procedure has to be read carefully.
- Always wear gloves while performing the experiment.
- Preparation of 1X Assay Buffer: To prepare 10 ml of 1X Assay Buffer, add 1 ml
- of 10X Assay buffer to 9 ml of sterile distilled water.
- Preparation of 1% Agarose gel: To prepare 10 ml of agarose gel, add 0.1g of agarose powder to 10 ml of 1X Assay Buffer, boil to dissolve the agarose completely.
- Wipe the glass plates with cotton; make it grease free using alcohol for even spreading of agarose.
- Cut the wells neatly without rugged margins.
- Ensure that the moist chamber has enough wet cotton to keep the atmosphere humid.

Procedure

- Prepare 10 ml of 1% agarose (as given in important instructions).
- Cool the solution to 55-60°C and pour 5 ml/plate on to grease free glass plates placed on a horizontal surface. Allow the gel to set for 30 minutes.
- Place the glass plate on the template provided.
- Punch wells with the help of the gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming of rugged wells.
- Add 10 µl each of the antiserum and the corresponding antigens to the wells as shown in Fig 2.
- Keep the glass plate in a moist chamber overnight at 37°C.
- After incubation, observe for opaque precipitin lines between the antigen and
- antiserum wells.

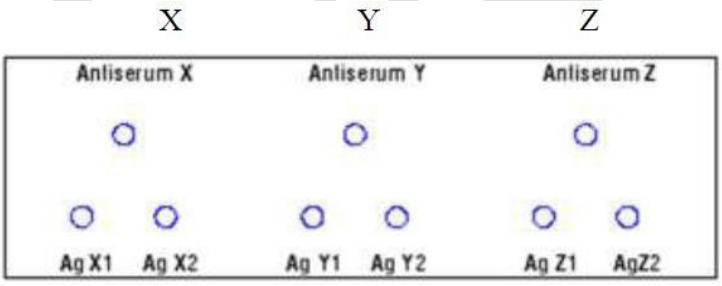


Figure 2. Template for addition of antiserum and antigen to their respective wells

Observations and Result

Observe for presence of precipitin lines between antigen and antisera wells. Note the pattern of precipitin line observed in each case.

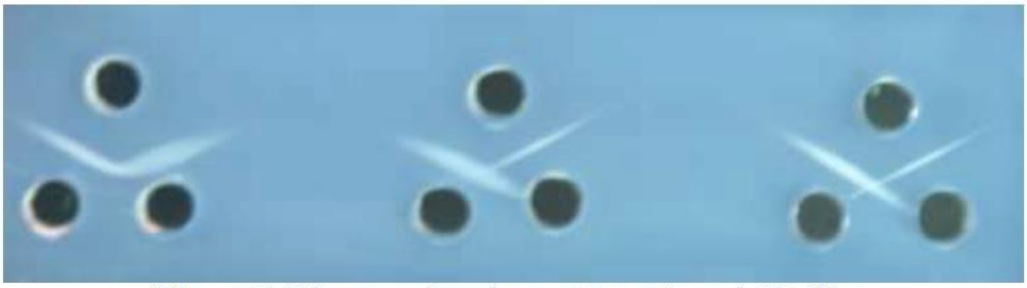


Figure 3. Diagram showing pattern of precipitin line.

Interpretation

- When antigen and antibody meet in optimal proportions a precipitation line is formed. In Ouchterlony Double Diffusion (Antigen Antibody Pattern), three patterns of precipitin lines can be observed.
- If pattern X or pattern of identity is observed between the antigens and the antiserum, it indicates that the antigens are immunologically identical.
- If pattern Y or pattern of partial identity is observed, it indicates that the antigens are partially similar or cross-reactive.
- If pattern Z or pattern of non-identity is observed, it indicates that there is no cross-reaction between the antigens. i.e. the two antigens are immunologically unrelated.

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Experiment - 6 **DOT ELISA**

Aim:

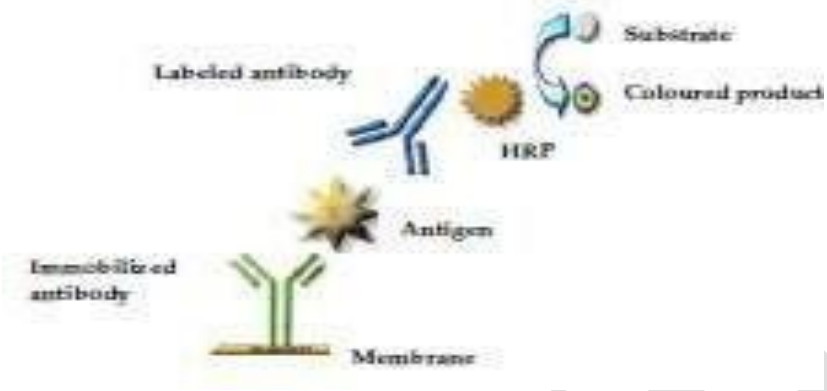
To learn the technique of Dot ELISA for the detection of an antigen.

Introduction:

Enzyme linked immunosorbent assay or ELISA is a sensitive immunological technique to detect the presence of a specific antigen (Ag) or antibody (Ab) in a biological sample. It utilizes the dual properties of antibody molecules being specific in reactivity and their ability to be conjugated to active molecules such as enzymes. An enzyme conjugated with an antibody reacts with a chormogenic colourless substrate to generate a coloured reaction product. ELISA is extensively used for diagnostic purpose which utilizes the dual. It requires an immobilized antigen/antibody bound to a solid support (e.g. microtitre plate or membrane). There are different types of ELISAs for the detection of a protein of interest in a given sample. One of the most common ELISA is dot ELISA which can visually detect the presence of an antigen very quickly. The nitrocellulose dot technique was first developed for screening large number of hybridoma antibodies in 1983.

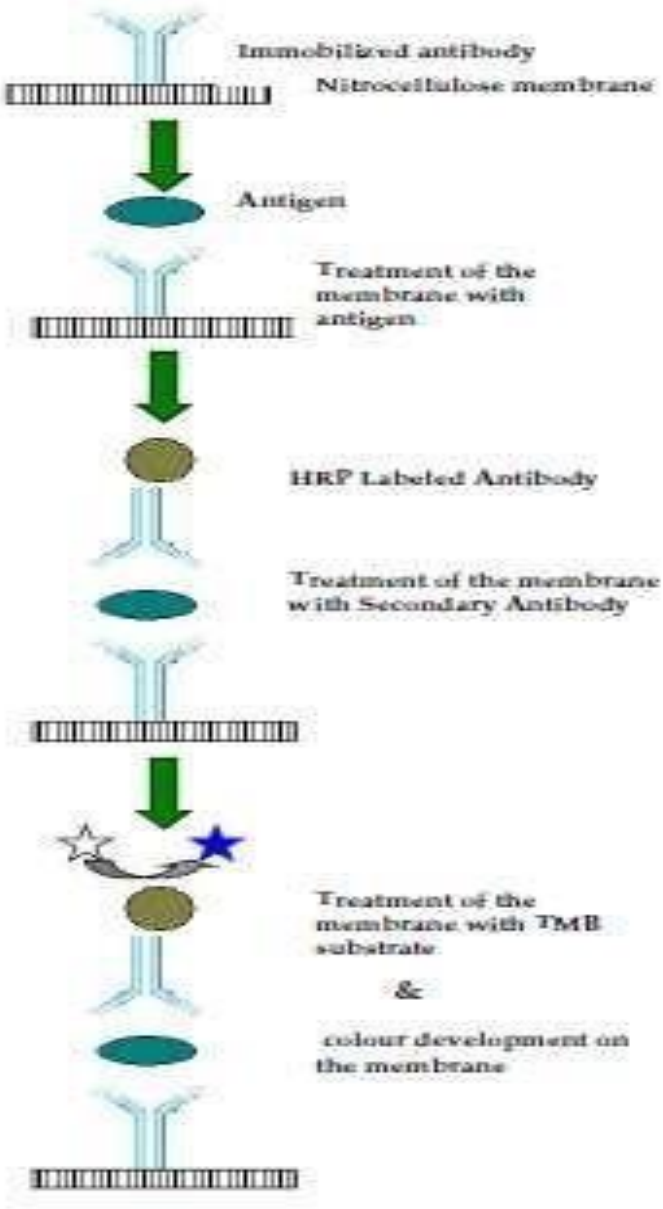
Principle:

There are various forms of ELISA for the detection of antigen or antibody based on antibody-antigen interactions. Dot ELISA, a qualitative ELISA test, can be performed very quickly with the end detection done visually. Because of its relative speed and simplicity, the dot ELISA is an attractive alternative to standard ELISA. In Dot-ELISA, small volumes of antibodies are immobilized on a protein binding membrane (Nitrocellulose) and the other antibody is linked to an enzyme Horse radish perxoidase (HRP). The test antigen at first reacts with the immobilized antibody and later with the enzyme-linked antibody. The amount of enzyme linked antibody bound is determined by incubating the strip with an appropriate substrate (Hydrogen peroxide, H_2O_2) and a chromogen [Tetramethylbenzidine (TMB)]. HRP acts on H_2O_2 to release nascent oxygen, which oxidizes TMB to TMB oxide, which gives, a blue coloured product. The latter precipitates onto the strip in the area of enzyme activity and appears as a coloured dot, hence the name Dot-ELISA. The results can be visualized in naked eye. The enzyme activity is indicated by intensity of the dot, which is directly proportional to the antigen concentration.



Procedure:

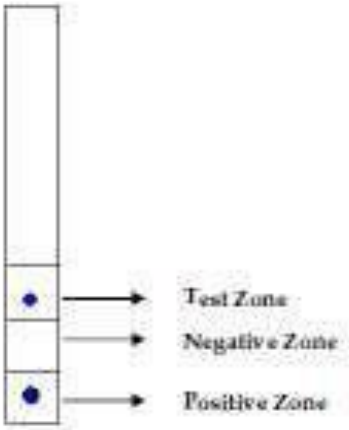
1. Take 2 ml of 1X Assay Buffer in a test tube and add 2 µl of the test serum sample. Mix thoroughly by pipetting. Insert a Dot-ELISA strip into the tube.
2. Incubate the tube at room temperature for 20 minutes. Discard the solution.
3. Wash the strip two times by dipping it in 2 ml of 1X Assay Buffer for about 5 minutes each. Replace the buffer each time.
4. Take 2 ml of 1X Assay Buffer in a fresh test tube, add 2 µl of HRP conjugated antibody to it. Mix thoroughly by pipetting. Dip the ELISA strip into it and allow the reaction to take place for 20 minutes.
5. Wash the strip as in step # 3 for two times.
6. In a collection tube (provided in the kit) take 1.3 ml of TMB/H_2O_2 and dip the ELISA strip into this substrate solution.
7. Observe the strip after 5 - 10 minutes for the appearance of a blue spot.
8. Rinse the strip with distilled water



Interpretation and Results :

Spot in the positive control zone and no spot in the negative control zone indicates proper performance of test. In the negative control zone the immobilized antibody is not present and the region is blocked with an inert protein. Therefore, there is no reaction when the reagents are added and no spot can be seen. In the test zone an antibody (specific to the test antigen, serum) is immobilized on it and then blocked with an inert protein.The test serum binds to this region and the HRP-labeled antibody binds to serum which when reacts with substrate develops blue dot. In the positive control zone, the test serum binds to the immobilized antibody and the HRP labeled antibody binds to serum which when reacts with substrate develops blue dot.

Look for the appearance of the blue dot as shown below:



Record your observations as follows:

Zone	Spot
Positive Zone	
Negative Zone	
Test Zone	

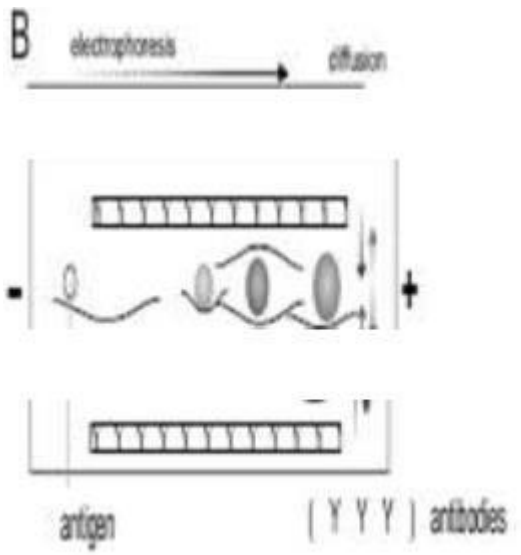
Denote +ve : on appearance of a blue spot and -ve : on absence of a blue spot.

Experiment – 7 **Immunoelectrophoresis**

Aim:
To learn the technique of Immunoelectrophoresis.

Introduction:
Immunoelectrophoresis is a powerful qualitative technique for the characterization of any antibody. In this method one antigen mixture is electrophoresed in an agarose gel that allows the separation of its different components based on their charge along the gel slide, followed by the lateral diffusion of the serum or monoclonal antibody within the gel. Antibodies specific to the antigens form white precipitation arcs which can be seen against a dark background. This technique is useful in determining the blood levels of three major immunoglobulins: IgM, IgG and IgA. The process combines antigen separation technique of electrophoresis and immunodiffusion of the separated antigen against an antibody. It is used extensively to check the presence, specificity and homogeneity of the antibodies and can detect relatively high antibody concentrations.

Principle:
In immunoelectrophoresis, the antigen mixture is first electrophoresed to separate its constituents by charge. The antiserum containing the antibodies added into the troughs diffuses with a plane front to react with the antigens. Due to diffusion, density gradient of the antigen and antibody are obtained and at a specific antigen/antibody ratio (equivalence point) huge macromolecules are formed. They form a visible white complex which precipitates as arcs in the gel. The arc is closer to the trough at the point where the antigen is in highest concentration. The method is very specific and highly sensitive because distinct zones are formed. In this method it is important that the ratio between the quantities of antigen and antibody be proper (antibody titer).



Procedure:

1. Prepare 10 ml of 1.5% agarose (as given in important instructions).

2. Mark the side of the glass plate that will be towards negative electrode during electrophoresis.

3. Cool the solution to 55-60oC and pour 6 ml/plate on to grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.

4. Place the glass plate on the template provided.

5. Punch a well with the help of the gel puncher corresponding to the markings on the template at the negative end. Use gentle suction to avoid forming rugged wells.

6. Cut two troughs with the help of the gel cutter, but do not remove the gel from the troughs.

7. Add 10 µl of the antigen to the well and place the glass plate in the electrophoresis tank such that the antigen well is at the cathode/negative electrode.

8. Pour 1X Electrophoresis buffer into the electrophoresis tank such that it just covers the gel.

9. Electrophorese at 80-120 volts and 60-70 mA, until the blue dye travels 3-4 cms from the well. Do not electrophorese beyond 3 hours, as it is likely to generate heat.

10. After electrophoresis, remove the gel from both the troughs and keep the plate at room temperature for 15min. Add 80 µl of antiserum A in one of the trough and antiserum B in the other.

11. Place the glass plate in a moist chamber and incubate overnight at 37oC. Observation for the band.

Interpretation:

The formation of precipitin line indicates the presence of antibody specific to the antigen.

1. Homogeneity of the antiserum to the antigen is denoted by presence of a single continuous precipitin line
2. Heterogeneity of the antiserum to the antigen is denoted by presence of more than one precipitin line which not only gives an indication of the number of immunodominant epitopes, but also the non identical nature of such epitopes.

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