Indira Gandhi National
Open University
School of Sciences

BZYEL-142
IMMUNOLOGY: LABORATORY

List of Exercises

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IMMUNOLOGY: LABORATORY

The laboratory course is based on theory course of Immunology which is of 4 credits. This course is worth 2 credits.

The overall objective of this course is to give the biology students experience with addressing immunological questions through experimentation.

Immunological lab course will expose students to key discipline specific techniques such as Ouchterlony's double immune diffusion method. Well designed lab Exercises that involve learners in experimental design, data analysis and science-specific communication uniquely reinforce course content, increase student appreciation of the scientific method, and refine communication skills.

Several manufacturers produce kits developed specifically for educational use. Kits are incorporated into the ELISA and Western blot units in lab course. Based on use of kits, hand-on experience, demonstration and study through slides the present lab course is divided into 8 exercises in all.

In Exercise 1, you will do the histological study of primary and secondary lymphoid organs i.e. thymus, spleen and lymph nodes. In Exercise 2, you will prepare blood smear and examine different types of WBCs i.e. eosinophils, basophils, neutrophils. The diffusion of antibody and antigen across the gel to interact is called immunodiffusion. You will prepare and observe the reaction patterns of an antigen with different types of antibodies by Outhterlony double diffusion method in Exercises 3. It is observed that when RBCs having one or both the antigens are exposed to equivalent antibodies, they form visible agglutination on interaction. You will prepare slide for ABO blood group determination in Exercise 4. The viable cells present in the suspension will be prepared by the learners from lymphoid organs. They will also able to differentiate live and dead cells in Exercise 5.

Students will be demonstrated an immunological technique for the detection and quantification of hormones, peptides, proteins and antigens i.e. ELISA in Exercise 6. In Exercise 7, you will be demonstrated immune-electrophoresis to separate and characterise a mixture of proteins and antigens.

You will be able to detect antigen in a sample using Dot ELISA kit in Exercise 8.

You are advised to thoroughly understand the principle of each exercise as it will facilitate you to conduct the Exercise and will create interest and motivation to work in laboratory. You have to be careful in the laboratory while conducting these Exercises and working with reagents hence you are advised to follow the precautions given for each Exercise.

Like all other IGNOU laboratory courses this is an intensive residential exercise requiring one week to complete it. Everyday there will be two laboratory sessions of 4 hours each. So there will be a total of 14 sessions. The first session will be introductory and the remaining 2nd to 13th sessions will be based on the exercises given in the course. A schedule for laboratory exercises will be given to you in the first session. Sessions 1 to 12 will have guided exercises under the supervision of the academic counsellor. In each session you will perform exercises for 3 hours and in the remaining 1 hour you will complete your practical note book. The last two sessions i.e., 13th and 14th will be unguided session, i.e., the term end examination.

You are aware that there is a time constraint as you will have limited access to laboratory work, therefore, you are required not to miss any of the laboratory sessions.

You will be assessed for your performance each day and on the last day you will have the term end examination. This examination will be compulsory for you to pass.

Study Guide

1. Before you enter your laboratory for performing laboratory exercises you should read the theory components of Immunology (BZYET-141).
2. You should also go through the laboratory manual and underline the important steps given in it.
3. Do not forget to carry laboratory manual and a practical record book for making and recording your observations.

Study of Slides

1. While studying permanent slides under the microscope, first focus under low power and then focus on the structure you want to study in detail and change the focus to high power. You can ask your counsellor to help you to focus the slide in the beginning or whenever you have difficulty in focusing the slide. This will prevent damage of the slides.
2. Draw diagrams in your practical book while observing the slide. You can take the help of your laboratory manual for the details of the structures and labelling.

Objectives

After completing this course, you should be able to:

- describe the organization and histology of different cells in the lymphoid organs viz: thymus, spleen and lymph nodes,
- appreciate the functional interplay of immune cells in the thymus, spleen and lymph nodes,
- identify cellular processes related to immune functions that take place in these lymphoid organs.
- prepare blood smear,
- examine different types of WBCs i.e., eosinophils, basophils, neutrophils, lymphocytes and monocytes,
- discuss structure and function of different types of WBCs,
- explain the reaction patterns of an antigen with different types of antibodies,
- determine antigen-antibody precipitation by Ouchterlony double diffusion method,
- prepare the slide for blood group determination,
- explain the principle of agglutination reaction,
- prepare cell suspension from lymphoid organs,
- differentiate live cells and dead cells,
- explain the principle and immuno-quantification technique of ELISA,

- detect and quantify peptides, proteins, antibodies and hormones,
- separate and characterize a mixture of proteins/antigens,
- describe the specificity of antigen-antibody interaction,
- detect antigen using Dot ELISA kit, and
- describe principle of Dot ELISA technique.

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EXERCISE 1

HISTOLOGICAL STUDY OF THYMUS, SPLEEN AND LYMPH NODES THROUGH SLIDES/ PHOTOGRAPHS

Structure

1.1 INTRODUCTION

Our immune system is a complex network of cells, tissues, and organs that helps the body to fight against infections and diseases. These organs can be divided into **primary** and **secondary lymphoid organs**. Primary lymphoid organs are sites for the development and maturation of lymphocytes. The two major primary lymphoid organs are **bone marrow** and **thymus**. Secondary lymphoid organs are sites where antigens are trapped for lymphocytes to interact with these trapped antigens effectively and generate immune responses. These organs include lymph nodes, spleen, and various mucosal-associated lymphoid tissues (MALT): blood vessels and lymphatic systems unite these organs to function as a complete system.

Objectives

After the end of this exercise, you should be able to:

- describe the organization and histology of different cells in the lymphoid organs viz: thymus,spleen and lymph nodes,
- appreciate the functional interplay of immune cells in the thymus, spleen and lymph nodes, and
- identify cellular processes related to immune functions that take place in these lymphoid organs.

1.2 MATERIALS REQUIRED

- Compound microscope.
- Permanent histological slides/ photographs of thymus, spleen and lymph node.

1.3 THYMUS

Thymus is a flat, bilobed organ situated just above the heart. Thymus is an encapsulated primary lymphoid organ, where the T cell development and maturation occur. It is composed of two lobes that are connected by an isthmus. The thymus is histologically split into lobules, each of which consists of a central **medulla** and a peripheral **cortex**. Each lobe is divided into lobules, which are separated by connective tissue strands called **trabeculae**.

The cortex of the thymus is densely packed with thymocytes (precursor cells of T lymphocyte), whereas the medulla is very sparsely populated by thymocytes. The network of reticular epithelial cells, macrophages, and blood vessels are also present in the cortex. The early stages of thymocyte development during which the rearrangement of receptor genes takes place on the surface of T cells occur in the cortex.

Cytokines are broad and loose category of small proteins important in cell signalling. Cytokines are peptides and cannot cross the lipid bilayer of cells to enter the cytoplasm.

A three-dimensional stromal cell network composed of epithelial cells, dendritic cells, and macrophages, which make up the organ structure and contribute to the growth and maturation of thymocytes, crisscrosses both the thymus cortex and medulla. Many of these stromal cells physically communicate with the developing thymocytes (Fig. 1.1).

In the outer cortex, some thymic epithelial cells, called **nurse cells**, having long extensions of membrane cover as many as 50 thymocytes, forming large multicellular complexes. There are long interconnecting cytoplasmic extensions of other cortical epithelial cells that form a network and have been shown to interact with various thymocytes as they crisscross the cortex.

With fewer lymphocytes and more reticular epithelial cells such as medullary epithelial cells and interdigitating dendritic cells, the medullary region is markedly different from the cortical region. In the medulla, a number of concentric bodies known as **Hassall's corpuscles** are present (Fig. 1.1).

TSLP is a protein belonging to the cytokine fanily. It plays an important role in the maturation of T Cell population through activation of antigen processing cells.

Hassall's corpuscles [or thymic corpuscles (bodies)] are named after Arthur Hill Hassall, who discovered them in 1846. They are composed of one or more granular cells at the centre, surrounded by epithelioid cells. They vary in size with diameter from 20 to more than 100μm, and tend to grow larger with age. They can be spherical or ovoid and their epithelial cells contain keratohyalin and bundles of cytoplasmic fibres. Many studies indicate that Hassall's corpuscles differentiate from medullary thymic epithelial cells.

The function of Hassall's corpuscles is currently unclear. But they are a potent source of the cytokine TSLP (Thymic stromal lymphopoietin).

The thymus grows rapidly in size until the end of the second year, after which there is slower growth, and at the age of 12th-13th years, it starts decreasing in size. Later, during old age, it is completely replaced by fat cells and connective tissue.

Functions of the thymus

The key role of the thymus is to produce and select a repertoire of T cells that will protect the body from infection. As thymocytes grow, a random mechanism that produces certain T cells with receptors capable of recognizing antigen-MHC complexes generates an immense diversity of T-cell receptors. Most T-cell receptors formed by this random method, however, are unable to recognize antigen-MHC complexes, and a small portion responds with self-antigen-MHC complex combinations. The thymus induces the death of those T cells that are unable to recognize the complexes of antigen-MHC. It also induces death to those T cells that react to self-antigen-MHC because they pose a risk of autoimmune disease. Thus, about 95 percent of all thymocytes in the thymus die by apoptosis without ever achieving maturity.The remaining lymphocytes, after development and maturation, are carried to the lymph nodes and other lymphatic tissues for their further action. **The maturing lymphocytes in the thymus are known as T- lymphocytes and are responsible for various diverse cell-mediated immune responses.** There are evidences indicating the role of the thymosin hormone released from reticular epithelial cells in the transformation of primitive lymphocytes into T-lymphocytes.

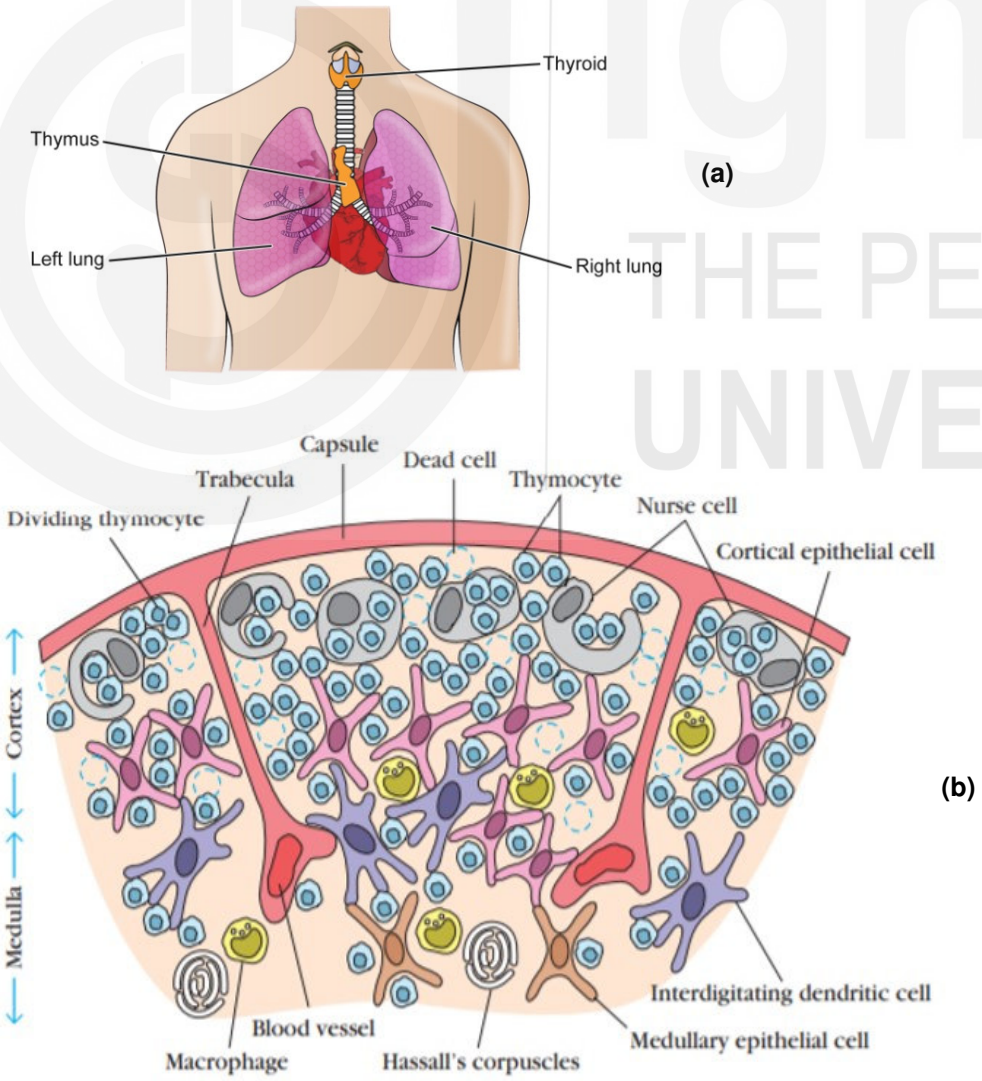


Fig. 1.1: (a) Thymus is a flat, bilobed organ situated just above the heart. (b) Diagrammatic representation of cross section of the thymus.

1.4 SPLEEN

The spleen is the largest lymphatic organ in the body. Its shape is ovoid and it is located in the upper left abdominal cavity under the diaphragm and posterior to the stomach. The spleen plays a major role in 1) mounting immune responses to blood borne antigens, 2) excluding the defective red blood cells and platelets from the circulation.

It is similar in form and function to a lymph node. However, the spleen is not supplied by lymphatic vessels, unlike the lymph nodes. Instead, blood borne antigens and lymphocytes are transported across the splenic artery into the spleen. A serous membrane known as the **peritoneum** encloses the spleen except at the region of hilum. The capsule is present under it that extends a number of projections (trabeculae) into the interior. The capsule is made up of most of the elastic fibres with some smooth muscles, and myofibroblasts.

There are two types of compartments, **red pulp** and **white pulp**, separated by a diffuse **marginal zone** (Fig. 1.2). The red pulp dominates the bulk of the stromal tissue of the spleen. It consists of rods of Billroth and splenic sinusoids which are the cellular meshwork supported by the reticular connective tissue.They appear as stripes and are made of macrophages, blood cells and plasmocytes. For different toxins, the red pulp acts as a blood filter, killing them before they enter the systemic circulation which otherwise may spread across the body and damage other organs. Many macrophages contain engulfed red blood cells or iron pigments from degraded hemoglobin within the red pulp.

The splenic white pulp carries three separate compartments: the **periarteriolar lymphoid sheath** (**PALS**), the lymphatic follicles and the marginal zone. The white splenic pulp surrounds the splenic artery branches, forming a periarteriolar lymphoid sheath (PALS) predominantly populated by T lymphocytes. Attached to the PALS are B cells which are rich primary lymphoid follicles with a germinal centre. Situated peripheral to the PALS is the marginal zone populated by lymphocytes and macrophages. Through the splenic artery, which empties into the marginal zone, the blood-borne antigens and lymphocytes enter the spleen (Fig. 1.2).

Lymphocytes in the blood after entering the sinuses of the marginal zone, move to PALS. The initial activation of the B cells happens in the T cell-rich PALS. Interdigitating dendritic cells present in the marginal zone trap the antigen and present it to T_H cells along with class II MHC molecules. These T_H cells will then activate B cells which then migrate to primary follicles in the marginal zone, along with few T_H cells. On the antigenic challenge, these primary follicles develop and enlarge into characteristic secondary follicles containing germinal centers, the site where lymphocytes mature and attain the ability to produce antibodies.

Functions of Spleen

Blood filtering is one of the most significant functions of the spleen. As it eliminates old and weakened erythrocytes from circulation, it is also known as a **"graveyard for red blood cells"**. This role is primarily due to the special composition of the red pulp present in the blood vessels and macrophages. The macrophages phagocytose and kill the haemoglobin erythrocytes, and then recycle their iron. Eventually, iron in the bone marrow ends up being processed and reused.

The spleen is a significant site of hematopoiesis during foetal development before bone marrow completely develops. The spleen also retains a certain amount of blood that can be released in a condition of acute and extreme blood loss in its blood vessels.

As the largest lymphoid organ, **the spleen is one of the key sites for activating and controlling the immune response**. In comparison to lymph nodes which are involved in protection against localized region infection, the spleen battles against systemic circulation infection and due to its complex structure, spleen is the main site for protection against encapsulated bacteria.

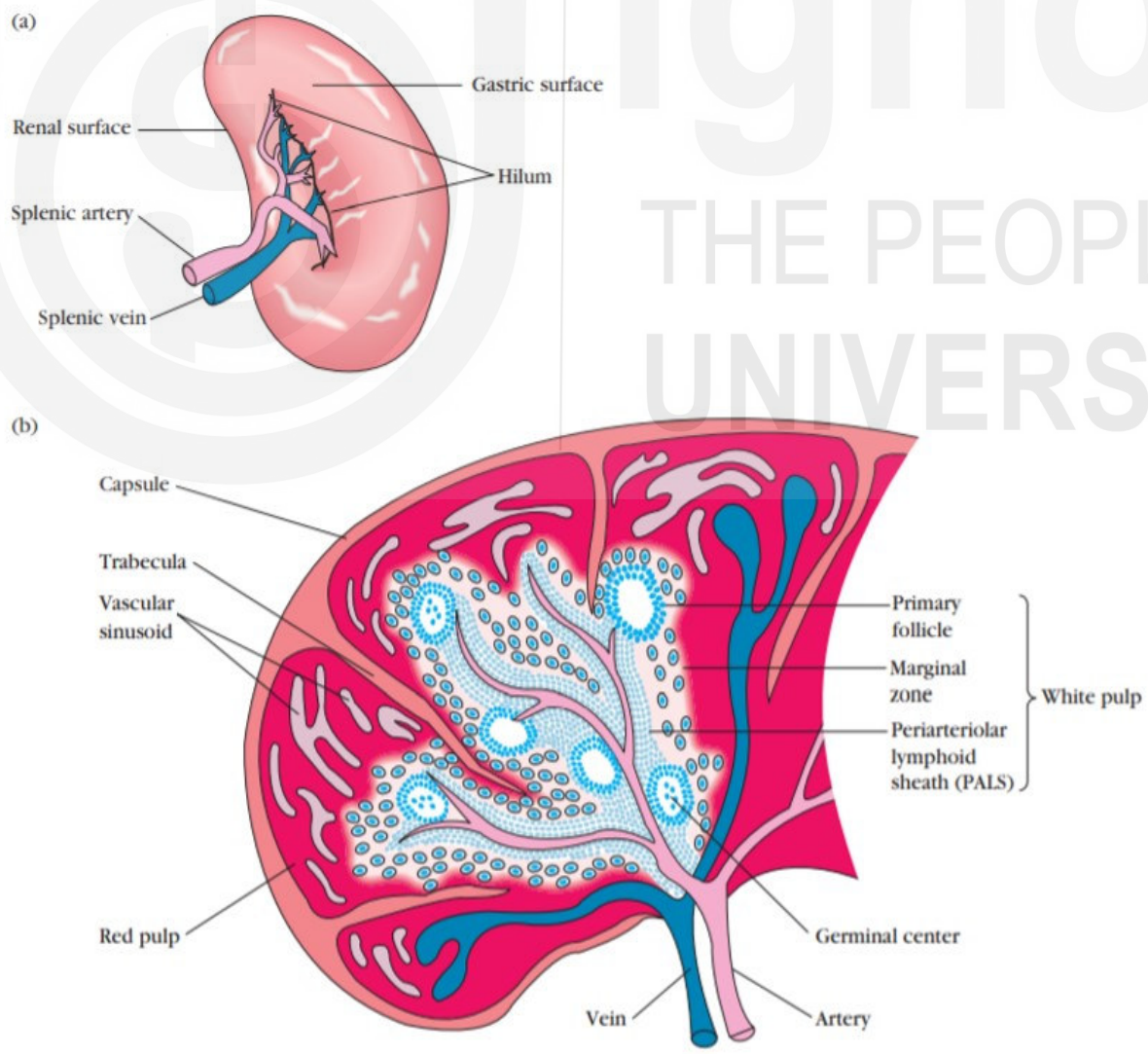


Fig. 1.2: Structure of the Spleen. (a) Spleen, is the largest secondary lymphoid organ. (b) Diagrammatic representation of cross section of the spleen.

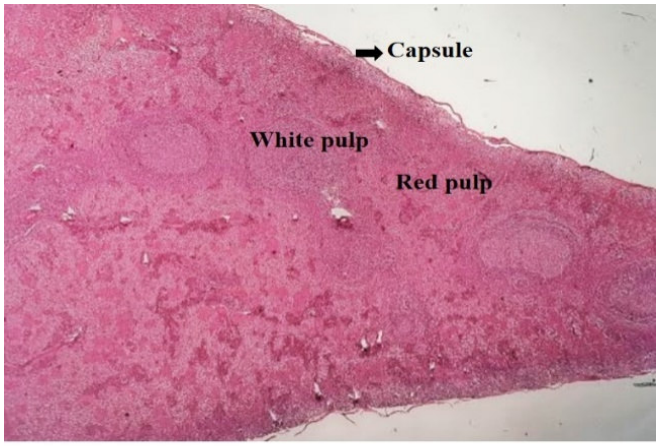


Fig. 1.2: (c) Histological section of Spleen.

1.5 LYMPH NODES

Lymph nodes are encapsulated small, oval or bean-shaped structures clustered at junctions of lymphatic vessels throughout the body. They differ in their number and position, but their histological structure and function are fundamentally similar. A lymph node is typically 1 to 2 centimetres long. An afferent lymph vessel in each lymph node guides the lymph into the node, and an efferent lymph vessel called the hilum guides the lymph on the concave side of the node out of the node. Hilum also includes the lymph node's blood supply (Figure 1.3 a and b). As the lymph percolates through a node, any particulate antigen that is taken into the lymph is trapped by the cellular network of phagocytic cells and dendritic cells (follicular and interdigitating). A lymph node's overall architecture promotes an ideal lymphocyte microenvironment to effectively encounter and react to trapped antigens.

Lymph node can be divided morphologically into three concentric regions: the cortex, the paracortex, and the medulla, each serving a different microenvironment (Fig. 1.3 c).

Lymphocytes (mostly B cells), macro-phages, and follicular dendritic cells arranged in **primary follicles** are present in the outermost layer, the cortex. Following an antigenic challenge, the primary follicles enlarge into **secondary follicles**, each containing a **germinal centre**.

The paracortex region is underneath the cortex, and is mainly populated by T lymphocytes and migrated interdigitating dendritic cells (from tissue to node). High levels of MHC class II molecules required for antigen presentation to T_H cells are expressed by these interdigitating dendritic cells. There are unusually few cells in the paracortical area in lymph nodes taken from neonatally thymectomized mice, hence the paracortex is referred to as a **thymus-dependent area** contrary to the cortex, which is a **thymus-independent area**. Lymphoid-lineage cells are more sparsely populated in the innermost layer of a lymph node, the medulla; many of those present are plasma cells that actively secrete antibody molecules.

The lymph leaving a node via its single efferent lymphatic vessel following infection or the introduction of other antigens into the body is enriched with antibodies freshly secreted by medullary plasma cells and often has a

lymphocyte concentration fifty times greater than the afferent lymph. The rise of lymphocytes in the lymph leaving a node is partially due to the proliferation of lymphocytes in response to the antigen inside the node. However, much of the increase is expressed by blood-borne lymphocytes that move into the node through the specialized endothelial cells that line the node's postcapillary venules. It is estimated that 25 percent of lymphocytes that leave a lymph node have migrated from the blood across this endothelial layer and reached the node. Whenever there is antigenic stimulation (via any foreign particle) within a node, we see a tenfold increase in the lymphocyte migration into the nodes. Factors released during antigen stimulation in the lymph nodes are assumed to promote this increased migration. This process results in the visible swelling of the lymph nodes.

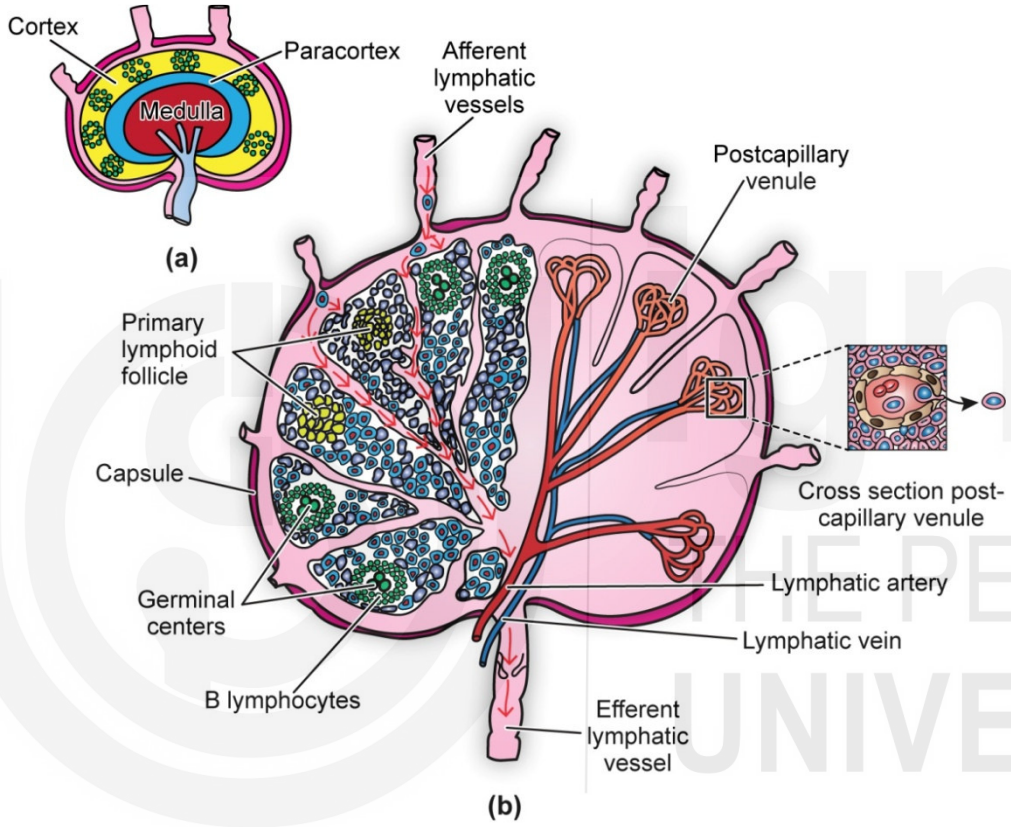


Fig. 1.3: (a) Lymph node can be divided morphologically into three concentric regions: the cortex, the paracortex, and the medulla, each serving a different microenvironment. (b) Schematic diagram of Lymph node.

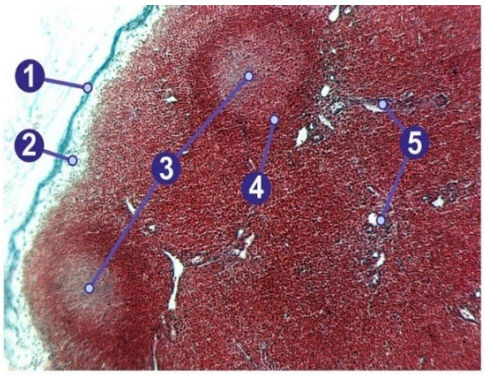


Fig. 1.3: (c) Cross-section of a lymph node with sections labeled. 1) Capsule; 2) Subcapsular sinus; 3) Germinal centre; 4) Lymphoid nodule; 5) Trabeculae.

Functions of Lymph nodes

Lymph nodes are the highly organized secondary structures clustered at the lymphatic vessel junctions. They function as filters of tissues and tissue fluids (lymph). Both endogenous and exogenous antigens are trapped, phagocytosed by macrophages, and presented to T-lymphocytes by reticular node meshwork. Furthermore, B-lymphocyte secreted antibodies also interact with antigen for an efficient immune response. The antigen appears to be trapped by a reticular meshwork of node, phagocytosed by macrophages and presented to T-lymphocytes. In addition, the antibodies secreted by B-lymphocytes also interact with appropriate antigen to mount an effective immune response.

1.6 TERMINAL QUESTIONS

1. What are the primary lymphoid organs?
2. What are Hassall's corpuscles?
3. What are the primary functions of the spleen?
4. What is lymph?
5. Why is the paracortex region of the lymph node called thymus-dependent area?

Acknowledgement of Figures

1. Fig. 1.1: Kuby, Immunology, Fourth Edition.
2. Fig. 1.2: (a, b) Kuby, Immunulogy, Fourth Edition.
3. Fig. 1.2 (c): Author.
4. Fig. 1.3: Kuby, Immunology, Fourth Edition.
5. Fig. 1.3 (c): https://en.wikipedia.org/wiki/Lymph_node#media /File: Lymphknoten_(Schwein).jpg.

EXERCISE 2

PREPARATION OF STAINED BLOOD FILM TO STUDY DIFFERENTIATED LEUCOCYTE COUNT (DLC)

Structure

2.1 INTRODUCTION

White blood cells (WBCs) are nucleated cells and heterogeneous in nature.The normal concentration of white blood cells in the blood varies from 4000 and 10,000 per micro-litre.They are the warriors against infection and help in defense.These cells are mainly involved in phagocytosis and immunity of the organism .White blood cells can be evaluated through different techniques varying in complexity and sophistication. One amongst these is the "**Differential Leucocyte Count" (DLC)**, a simple test that gives the relative percentage of each type of white blood cell. This test plays a vital role in the diagnosis and monitoring of an illness or inflammatory conditions. It also helps to determine the abnormal white blood cell populations (eg, precursor cells, immature granulocytes, and circulating lymphoma cells in the peripheral blood).

There are five types of white blood cells, and each has its own unique features:

Neutrophils (Fig. 2.1 and Table 2.1)

They are the most abundant and serve as the primary defense against infection. Their number increase in response to infection or severe injury and more number of immature forms starts appearing in the blood. These

immature cells are called **STAB** or **BAND**.The increase in the STAB number is the earliest sign of WBC response towards an infection. It is seen even before the entire count of WBC gets elevated.

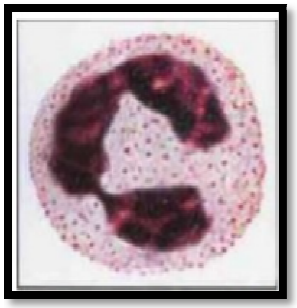


Fig. 2.1: Neutrophils.

Table 2.1: The average values of Neutrophils.

Neutrophils	Number cubic mm	%
Men	3,000-7,000	50-60
Women	1,800-7,700	50-60
Pregnancy	3,800-10,000	50-60

Eosinophils (Fig. 2.2 and Table 2.2)

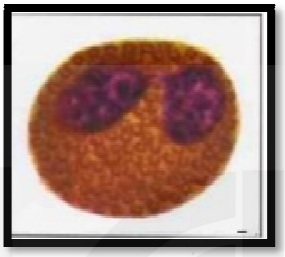


Fig. 2.2: Eosinophils.

These cells are associated with allergic disorders and in combating parasitic infections. An increase in their numbers reveals Allergic reactions, Parasite infections, Chronic skin infections and certain cancers like Lymphoma and Leukemia.However, decrease counts are related to stress, steroid exposure and anything that may suppress WBC production.

Table 2.2: The average values of Eosinophils.

Eosinophils	cubic mm	%
Men	50-250	1-4
Women	0-450	0-4
Pregnancy	0-450	0-4

Lymphoma is the cancer of the lymphatic system (the body's disease-fighting netweork).

Basophils (Fig. 2.3 and Table 2.3)

This type of WBC can destroy bacteria and other foreign bodies through phagocytosis and participate in allergic reactions. Elevation of basophil is indicative of infections,exposure to radiation, and cancers. Also, lower basophil counts reflect stress reactions,allergy and hyperthyroidism.

Table 2.3: The average values of Basophils.

Basophils	cubic mm	%
Men	25-100	0.5-1.0
Women	25-100	0.5-1.0
Pregnancy	25-100	0.5-1.0

Fig. 2.3: Basophils.

Monocytes (Fig. 2.4 and Table 2.4)

These cells are the ones which respond to allergies, infection and inflammation by phagocytosis of foreign bodies. A higher count of monocyte

reveals a viral illness, parasitic infection, collagen diseases, and some cancers. At the same time, decreased levels of monocyte reflect Rheumatoid arthritis, HIV infection, steroid exposure, and some tumour.

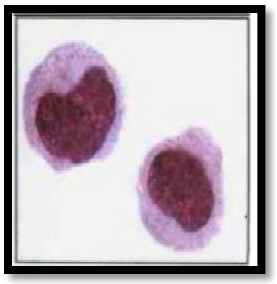


Fig. 2.4: Monocytes.

Table 2.4: The average values of Monocytes.

Monocytes	#/cubic mm	%
Men	100-600	2-6
Women	0-800	0-8
Pregnancy	0-800	0-8

Lymphocytes (Fig. 2.5 and Table 2.5)

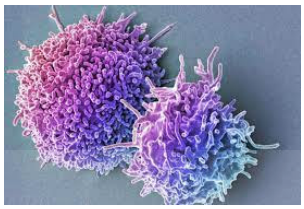


Fig. 2.5: Lymphocytes.

These cell types are vital as they play a crucial role in immediate and delayed response to infection or inflammation. An increased level is indicative of viral infection, bacterial infection, Grave's disease, and some cancers. The decreased level reflects steroid exposure, Lupus, renal failure, and immunodeficiency.

Table 2.5: The average values of Lymphocytes.

Lymphocytes	#/cubic mm	%
Men	1,000-4,000	20-40
Women	1,000-4,800	20-50
Pregnancy	1,300-5,200	20-50

Grave's disease is an autoimmure disorder that causes hyper-thyroidism. This is more common in women under the age of 40.

Objectives

At the end of this exercise, you should be able to:

- ❖ prepare blood smear,
- ❖ examine different types of WBCs i.e., eosinophils, basophils, neutrophils, lymphocytes and monocytes, and
- ❖ discuss structure and function of different types of WBCs.

Lupus is a long-term autoimmune disease in which the body's immune system become hyperactive and attacks normal healthy tissue.

2.2 MATERIAL

For a differential leucocyte count, the following material is required:

- Microscope
- Lancet (two-edged surgical knife) with sterilized needles
- Cotton
- Whole or Anti-coagulated Blood

- Distilled Water/Phosphate Buffer pH 6.5
- Romanofsky stain (any one from below)

  Leishman's stain

  Wright Stain

  Giemsa Stain
- Glass Slides
- Oil immersion
- Eye piece (100 X)

2.3 PRINCIPLE

Differential leucocyte count is performed to determine the relative number of each type of WBC present in the blood. The absolute number of each type of cell is more informative than its proportion. The absolute number can be calculated when the differential and a total number of leucocytes per unit volume are known. To identify the different WBCs, an adequately stained blood smear slide is required. To prepare a blood smear, a drop of blood is spread over a glass slide and then air-dried. It is then stained with a differential stain called **Romanofsky stain**, commonly known as Wright, Leishman or Giemsa stain. Two hundred cells are then counted and classified. Today, automated differential counts are done with the help of machines, but still manual techniques are more reliable and have better ability to discover morphological abnormalities.

2.4 PROCEDURE

This involves the following important steps:

a) Preparation of Blood Smear

This is a very crucial step as uniformly spread blood film assists in the differential count of the WBC with accuracy.The following steps need to be followed to prepare a good blood film:

1. Take three clean glass slides, free from scratch and grease.
2. Obtain capillary blood either directly from the finger using sterilized cotton and lancet or use EDTA (Ethylene diamine tetraacetic acid) anticoagulated blood.The drop of blood should be placed on the corner of the slide (Fig. 2.6).
3. Take the spreader, touch the drop of blood on the slide and push it backward so that the drop is spread evenly to the edge of the spreader.The angle between slide and spreader must be 45° (Fig. 2.7).

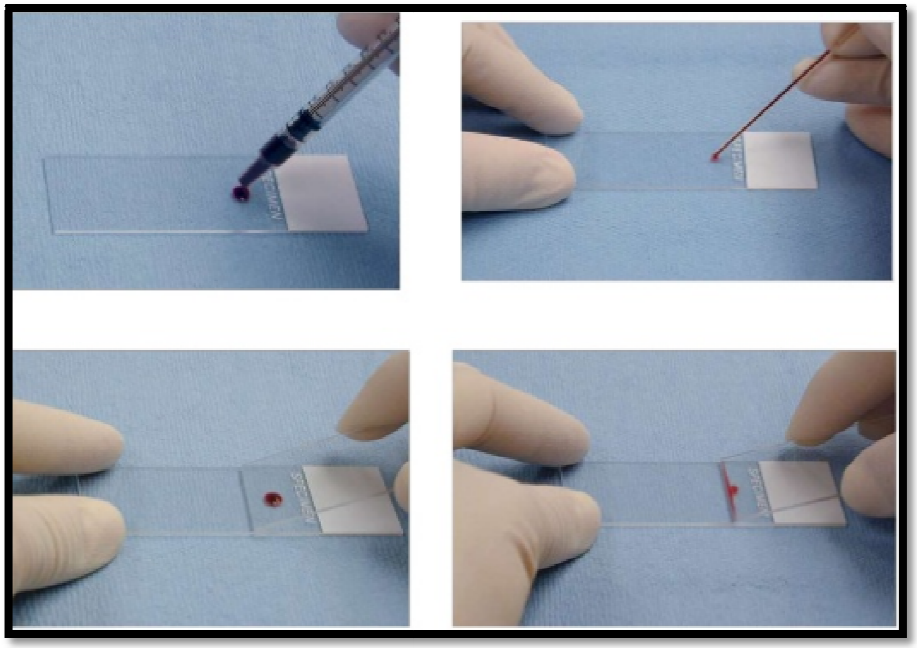


Fig. 2.6: Placing of a blood drop on slide and spreader.

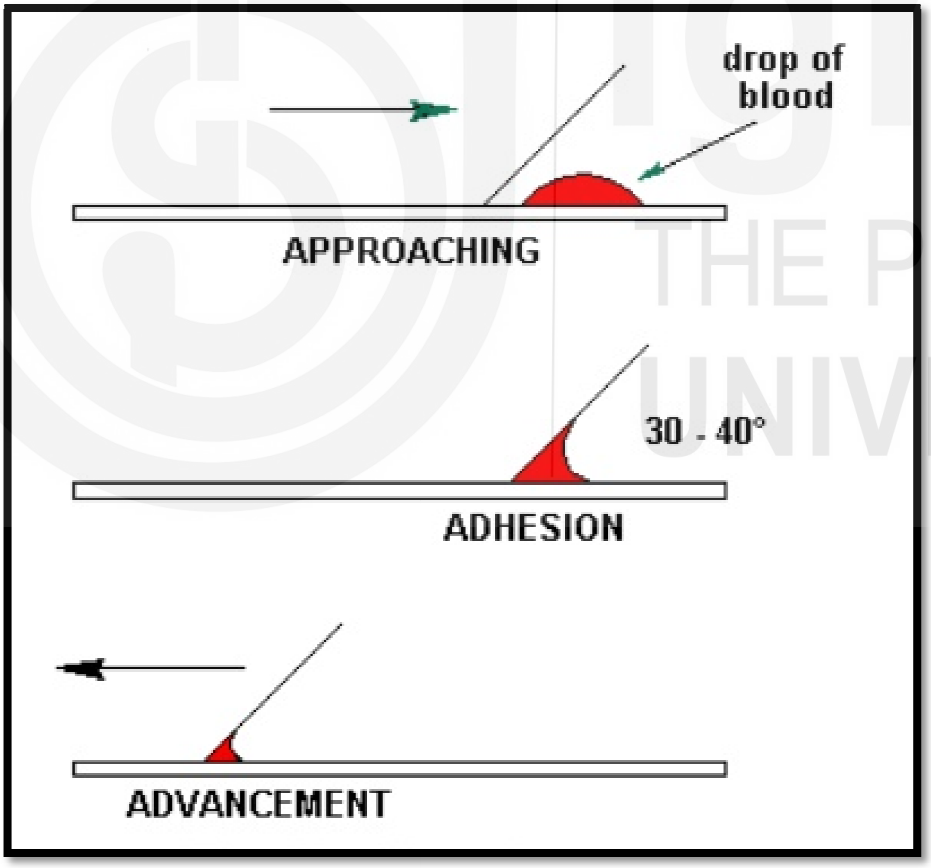


Fig. 2.7: Positioning of the spreader on the slide for smear formation.

With a careful and quick movement, push the spreader towards the other end of the slide. The blood film should be 1cm from the edge of the slide and 5 mm in width. Dry the smear at room temperature. Adequate drying is essential to preserve the quality of the blood film. Identification number on the slide by using a lead pencil or a marker pen may be marked. Place the prepared dried blood film on the staining rack (Fig. 2.8 and Fig. 2.9).

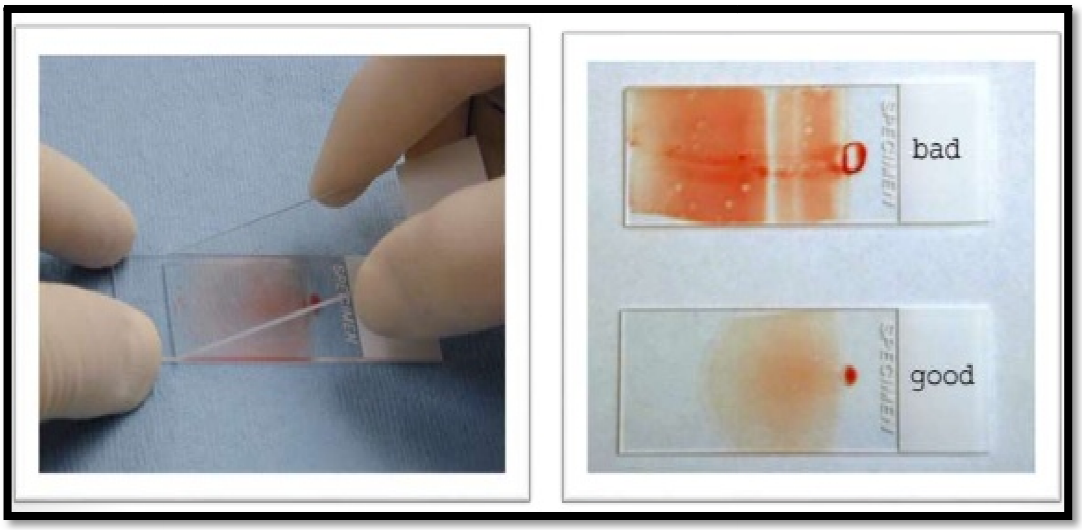


Fig. 2.8: Smear formation and comparison of good and bad film.

Fig. 2.9: Unacceptable blood films.

b) Staining of the dried blood smear slide

Now cover the smear with Leishman stain (10-15 drops without spillover) using Pasteur pipette and leave for two minutes (Fig. 2.10). Add an equal amount of double distilled water/buffer solution on the slide and blow gently to mix them. You can use a thin glass rod for this. Keep it for 10 minutes. Now wash smear by using running tap water by directing water on the hand and not directly on smear. Place the slide after washing in draining rack on the counter to dry the smear.

Romanowsky stains are universally employed and have two essential ingredients i.e.: methylene blue and eosin or azure. Methylene blue is the basic dye and has an affinity for acidic component like the nucleus, and azure/eosin is the acidic dye and has an affinity for the basic component of the cell i.e.: cytoplasm. These dyes are prepared in methyl alcohol so that they can be used in fixation and staining.

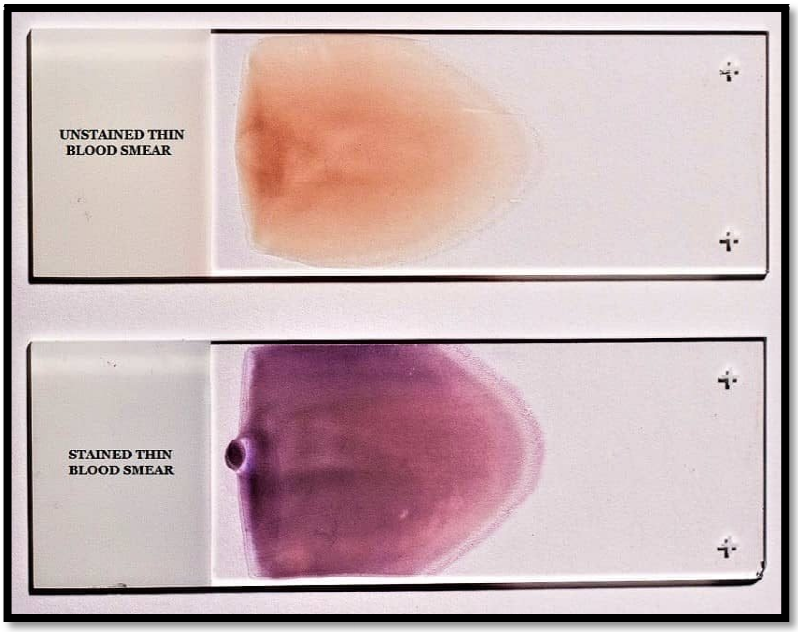


Fig. 2.10: Blood film-unstained and stained.

c) Examination of the stained slide

The prepared and dried smear is to be examined under low power objective (10X) in the microscope for screening purpose and select proper portion. Now a drop of Cedar wood oil/Immersion oil may be placed on the smear. Examine under 100X in increased light by the opening of the iris diaphragm and moving carefully from one field to another systematically. Record the types of cells observed in each field in a table drawn with ten boxes both in horizontally and vertical axis in a notebook or use hand counter. Move the objective slowly in a chain manner till at least 100 cells are counted (Fig. 2.11 and Fig. 2.12).

Fig. 2.11: Hand Counter.

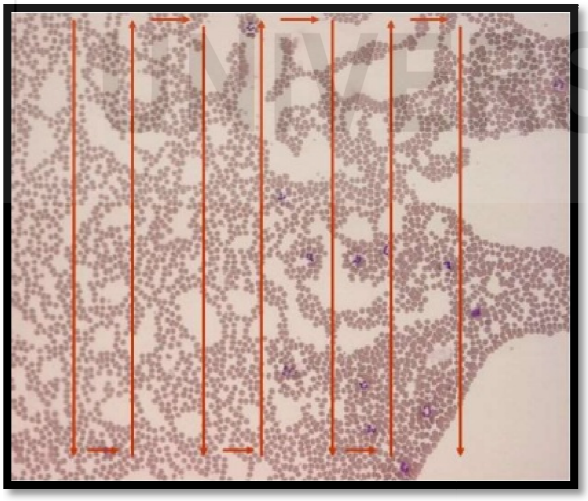


Fig. 2.12: Smear examining in chain method.

2.5 RESULT AND OBSERVATION

After staining the cells with Leishman stain, structures can be observed as depicted in Fig. 2.13 and given in Table 2.6.

The observations recorded can be compared to the normal standard range value and the result can be expressed in percentage (Table 2.7).

Table 2.6: Structure of different WBCs.

Neutrophil: Purple coloured nuclei with pink cytoplasm. Multilobed nuclei joined together by thin chromatin threads.	**Eosinophil:** Cytoplasm is faint pink, nucleus is bilobed, purple in colour and granules are orange red.
Basophil: Granules stain dark blue with purple usually bilobed nucleus.	**Monocytes:** Pink cytoplasm with kidney shaped purple colour nucleus
Lymphocyte: Large round dark blue nucleus with light blue cytoplasm.	**Platelets:** Violet coloured granules.
Red blood cells: RBCs are biconcave and thus appear white at the center and pink at the periphery.	

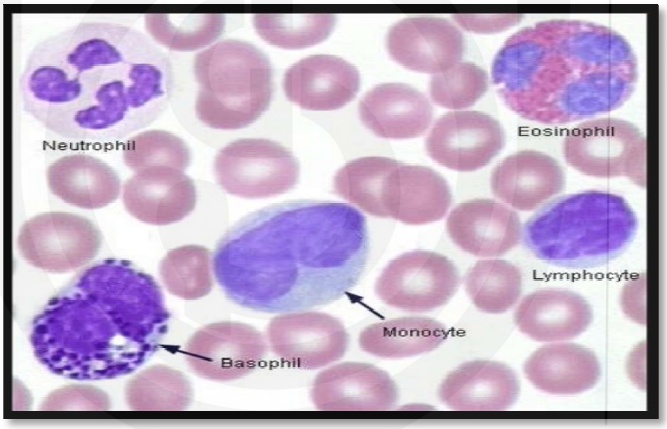


Fig. 2.13: Structure of Leucocytes.

Table 2.7: Normal Reference Range of WBCs.

Normal Reference Range

- White blood cell count 4.0–11.0 x 10^9 /l
- Differential white cell count
  - Neutrophils 2.0–7.0 x 10^9 /l (40–80%)
  - Lymphocytes 1.0–3.0 x 10^9 /l (20–40%)
  - Monocytes 0.2–1.0 x 10^9 /l (2–10%)
  - Eosinophils 0.02–0.5 x 10^9 /l (1–6%)
  - Basophils 0.02–0.1 x 10^9 /l (<1–2%)

2.6 PRECAUTIONS

1. Use of sterilized cotton and lancet to draw out blood is necessary.
2. The slides taken to prepare the smear should be clean and grease-free.
3. The angle of the spreader to slide should be followed precisely.
4. The blood film should be air- dried before staining.
5. Leishman stain, when poured on the slide for staining, should not spread off the slide.
6. While mixing of stain and water/buffers, blow very carefully and slowly.
7. The identification and recording of different cell types should be done accurately and carefully.

2.7 TERMINAL QUESTIONS

1. Differentiate among the structures of eosinophils, basophils and neutrophils.
2. Which stains are usually recommended for staining the blood smear slide?
3. What precautions should be taken for accurate identification of different types of WBCs?

EXERCISE 3

OUCHTERLONY DOUBLE IMMUNO-DIFFUSION METHOD

Structure

3.1 INTRODUCTION

Ouchterlony double diffusion is an immuno-diffusion technique developed by Orjan Ouchterlony in 1948. The antibody and antigen diffuse towards each other in a semisolid medium (gel) till the point of their optimum concentration and form a band of precipitation. This diffusion of antibody and antigen across the gel to interact is called '*Immuno-diffusion"*.Precipitation is a highly specific serological reaction involving the binding of antigen to antibody forming a complex cross-linked "*Lattice*" structure. Therefore, this technique is used for a qualitative test for the presence of a specific antigen in a sample or preparation.

Objectives

After perfoming the exercise, you will be able to:

- explain the reaction patterns of an antigen with different types of antibodies, and
- determine antigen-antibody precipitation by Ouchterlony double diffusion method.

3.2 MATERIALS REQUIRED

- **Instruments & Glass ware**

Glass slide,

Micropipette,

Gel puncher,

Measuring cylinder.

Beakers,

Incubator (37 ℃),

10ml disposable syringe,

- **Chemicals**

Agar,

Phosphate buffer (Disodium salt and monosodium salt),

NaCl,

Antiserum.

Antigen,

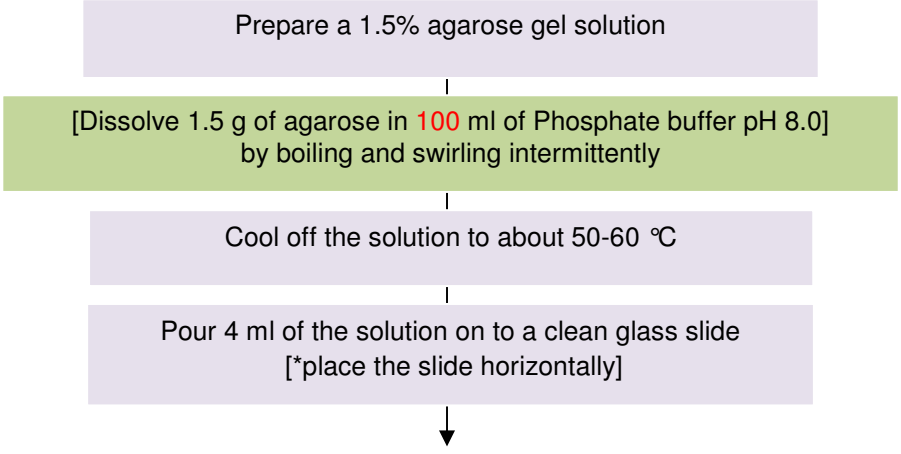
Alcohol,

Distilled water,

3.3 PRINCIPLE

When soluble antibody and antigen are placed adjacent to each other in agarose gel, they diffuse radially across the gel, and form a concentration gradient between them. The antigen-antibody reaction/ interaction occurs at the zone of optimal concentration, forming a **line of precipitation**, referred to as **Equivalence Zone**. Distinct lines termed **Precipitin lines** are formed in the gel depending on whether two antigens completely share the same antigenic epitope or partially share the same antigenic epitope or do not share the same antigenic epitope. When the concentration of either antigen or antibody is in excess, precipitation reaction is inhibited as all the binding sites of antibody would be saturated with antigen or excess antibody or binds to a single antigen, thereby preventing cross-linking. This phenomenon is called as "***Prozone Phenomenon***". Therefore, precipitin lines are formed closer to the wells in the agar gel, where the concentration of either antigen or antibody is lesser.

3.4 PROCEDURE



Allow the gel to set for 30 minutes

Fill syringe (10 ml) with Agar solution and release on to the glass slide

[Allow the gel to flow from one side to maintain a uniform agar layer] on the glass slide

Place the glass slide on to the template placed horizontally to punch wells

[Punch 3-6 wells around 1 central well]

Allow the gel to set for 30 minutes

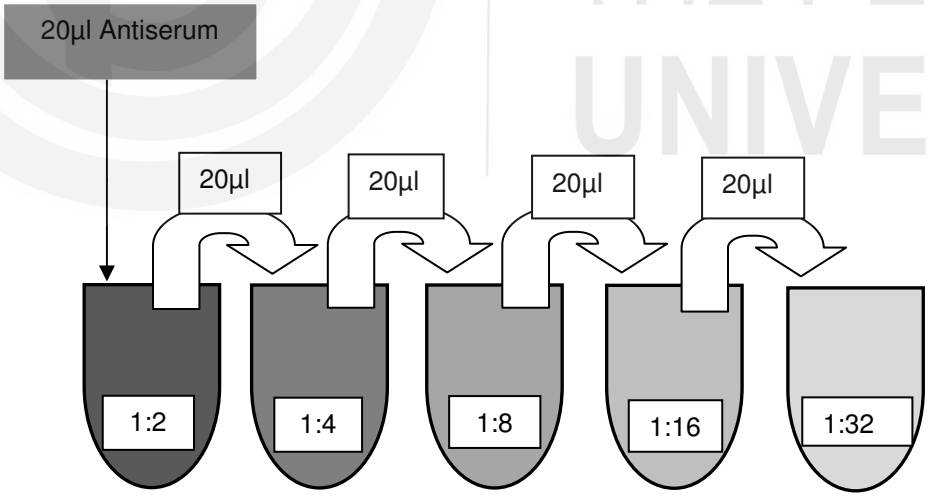
Fill the wells with 5 µl of Antigen in the central well

Fill the peripheral wells with corresponding antisera of different dilutions

[1:2, 1:4, 1:8, 1:16, 1:32 dilutions]

Incubate the glass in a moist chamber at 37 ℃

Observe for Opaque Precipitin Line between the wells



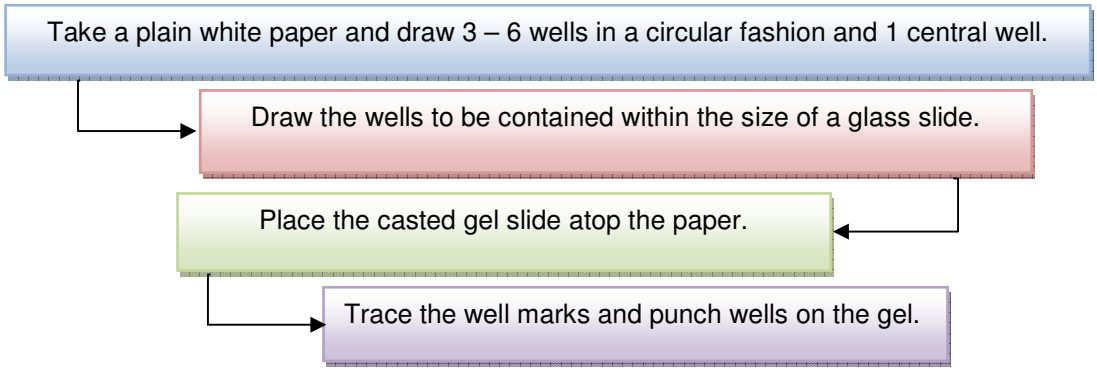
Pipette 20 µl of Antiserum to an eppendorff containing 20 µl of double distilled (DD) water/Phosphate buffer (PB buffer)

Vortex to mix properly

Draw 20 µl of the new diluted solution and dispense to an eppendorff containing 20 µl of DD water/ PB buffer

Continue the steps till you reach the desired dilution

Preparation of Template and Reaction wells (Fig. 3.1)



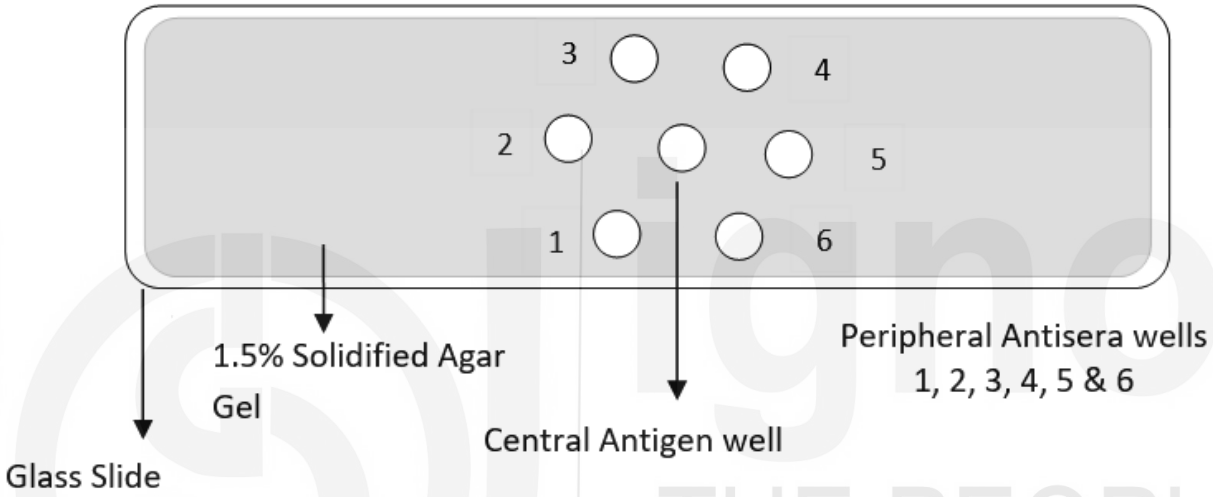


Fig. 3.1: Glass slide showing punched wells on solidified agarose gel for loading of antigen and antiserum samples into the wells. (Antibodies are present in antiserum).

3.5 OBSERVATIONS

Precipitation lines and band can be observed. To observe the precipitation band in radial immune-diffusion, different wells were punched in solidified agarose gel placed on glass slide (Fig. 3.2a). Antigen is loaded in the central well and in peripheral wells, serially diluted antisera [(e.g. neat (undiluted), 1:2, 1:4, 1:8, 1:16, 1:32)] are loaded and are allowed to diffuse. Precipitation band can be observed between central well and precipitation peripheral wells (Fig. 3.2a and Fig. 3.2b).

In Fig 3.3, different patterns of precipitation lines can be observed as follows:-

In pattern 1: Smooth line of precipitation can be seen which shows the test antigens are immunologically identical.

In pattern 2: It shows “patterns of non-identity”, which shows antigens are immunologically unrelated with each other.

In pattern 3: It shows “pattern of partial identity” which shows that the antigens share some epitopes (part of antigen which interacts with anibody) that are common to both.

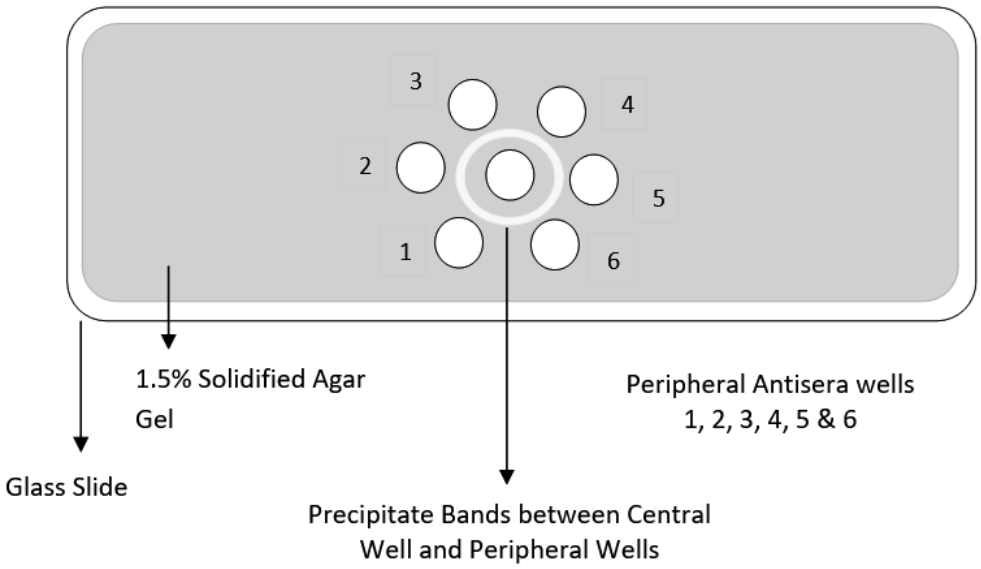


Fig. 3.2 a: Pre-coated glass slide showing precipitate bands between central well and peripheral wells. It indicates the formation of precipitation between antigen and antibodies present in antisera.

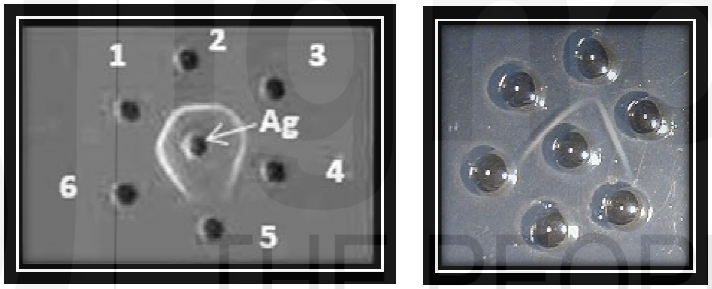


Fig. 3.2 b: Actual observation images showing radial immune diffusion. Antigens are placed in central well and serially diluted anti-sera are placed in peripheral wells.

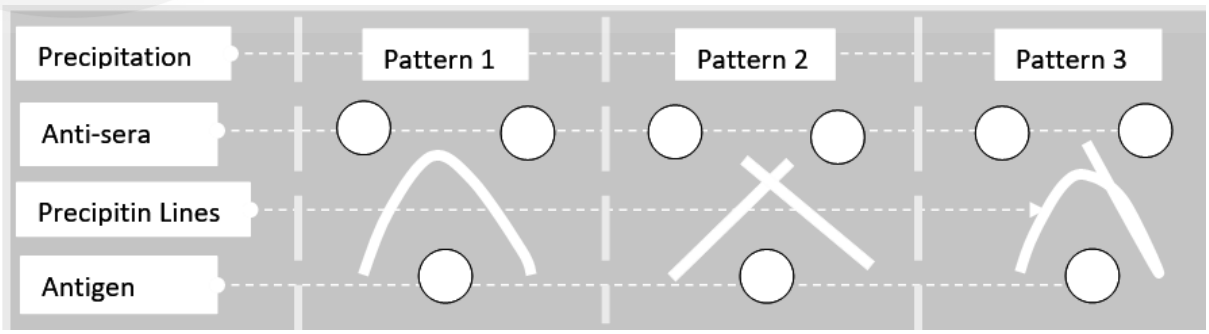


Fig. 3.3: Different precipitation patterns observed when antigen reacts with antisera.

The precipitation pattern in the slide could be of 3 types:

Precipitation pattern 1: Precipitate fuse to form an identical line.

Precipitation pattern 2: Precipitate forms independent lines.

Precipitation pattern 3: Formation of the precipitate spur.

3.5 DISCUSSION

The basis of immunology and immune defense relies solely on the interaction between antigens and antibodies. Antibodies are formed by the body's defense against bacterial and viral infections and some other toxins. . Since an antibody has affinity and specificity for a specific antigen, due to diffusion phenomenon, the movement of the antigen or antibody or both antigen and antibody molecules occur in a support medium.The maximum number of distinct antigenic substances present in the solution is indicated by the number of lines of precipitations. In conditions where the antigen and antibody in the wells are of identical shape and size, the curvature of the line of precipitin would depend on the relative molecular weight of the antigen. The line of precipitin would be straight when the antigens are of equal molecular weight. When the line of precipitin is a curve in case of unequal molecular weight, the curvature bends towards the antigen of higher molecular weight. The characteristics of the precipitin line formed depict the nature of the anti-sera and antigen of the reaction. The different reactions resulting from the nature of anti-sera and antigens are:

i) Reaction of Identity (Fig. 3.4)

The anti-sera lines grow towards each other and fuse. The concentration of the two antigens being the same, they diffuse at the same rate. Since the antibody is precipitating identical epitopes of antigen,they are said to form an identity - called precipitins.

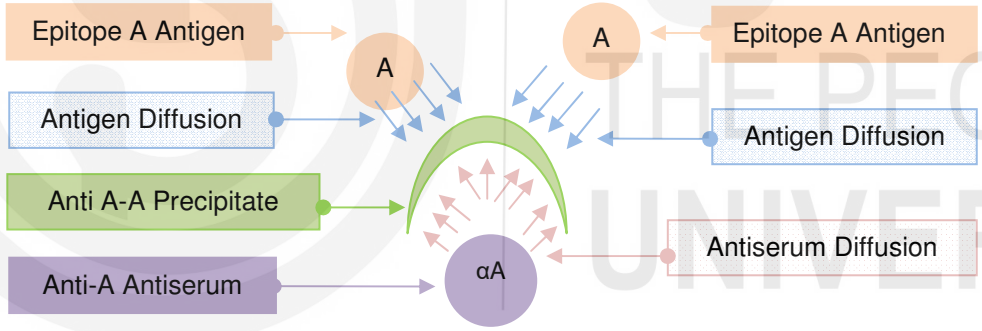


Fig. 3.4: Diagram depicting Reaction of Identity with Antigen A and Anti-A Antiserum.

ii) Reaction of Non-Identity (Fig. 3.5)

The anti-sera line forms independent lines of precipitation (often crisscross pattern) since the two antigens are unrelated or different from one another. Hence, they are said to be non-identity.

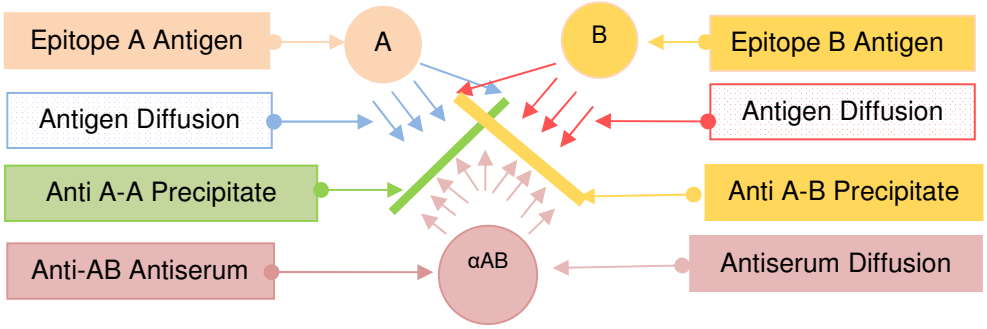


Fig. 3.5: Diagram depicting Reaction of Non-Identity with Antigen A, B and Anti-AB Antiserum.

iii) Reaction of Partial Identity (Fig. 3.6)

The antigens in the wells share some epitopes that are common to both, yet they possess individual- specific epitopes. Hence, cross-reactivity of the antigens are similar but not identical.In such a case, the anti-sera lines form a spur in the line of precipitation.

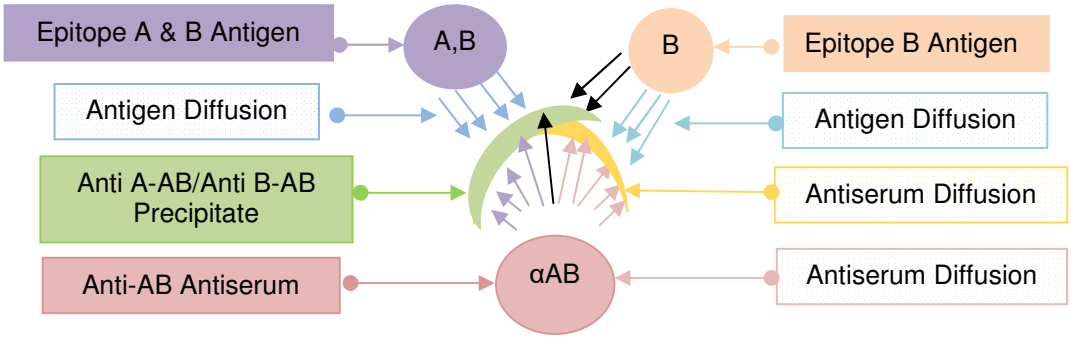


Fig. 3.6: Diagram depicting Reaction of Partial Identity with Antigen A, B and B; Anti-AB Antiserum.

3.6 PRECAUTIONS

1. Avoid air bubbles during the casting of gel on the glass slide.
2. The distribution of agar on the glass slide should be uniform.
3. Punching the wells should not touch the bottom of the glass slide.
4. Overloading of antigen and anti-sera should be avoided.

3.7 TERMINAL QUESTIONS

1. Define “Immuno-diffusion”.
2. Ouchterlony is a:
   (a) Qualitative method
   (b) Quantitative method
   (c) Both Qualitative and Qualitative
   (d) None of the above
3. How precipitation reaction is influenced by excess concentration of either antigen or antibody?
4. Discuss the Principle of the Ouchterlony double immuno-diffusion method.
5. What is zone of equivalence?
6. What do Precipitin lines indicate in Ouchterlony?
7. Describe the steps involved in anti-sera dilution using a flowchart.

Acknowledgement of Figures

Illustrations are drawn/Photographs are clicked by the authors of this Exercise.

EXERCISE 4

ABO BLOOD GROUP DETERMINATION

Sturcture

4.1 INTRODUCTION

In humans, blood contains red blood cells, white blood cells, and platelets, which play a significant role in transportation, regulation and protection. Based on the presence or absence of the antigenic substances on red blood cells or erythrocytes, it is possible to identify the blood group of an individual. This is called "**Blood Grouping**" or "**Blood Typing**". This is of prime importance when it comes to transfusion of blood in medical procedures and emergencies.

If you are rhesus positive (RhD positive), it means that a protein (D antigen) is found on the surface of the RBCs.

The blood grouping system (ABO) was discovered in the year 1900 by Karl Landsteiner. There are several blood group systems, but the ABO and RhD factor are universal with four major blood groups: A, B, AB and O. Blood group O is called the universal donor as it can donate blood to all other groups, and AB blood group is a universal recipient and can receive blood from all other groups. They are dependent upon the presence of two antigens on the surface of RBC, which are antigen A and antigen B. These antigenic components are glycoproteins or glycolipids with terminal sugar fucose, known as H antigen, under the control of A and B gene responsible for the synthesis of the specific enzyme (Fig. 4.1). O gene does not transform the H substance.

H antigen can refer to one of the various types of antigens having diverse biological funditions. It is located on the 19th chromosome in human, and has a variety of functions. It is precursor to each of the ABO blood group antigens. It is also known as substance H.

Objectives

After performing this exercise, you should be able to:

- prepare the slide for blood group determination, and
- explain the principle of agglutination reaction.

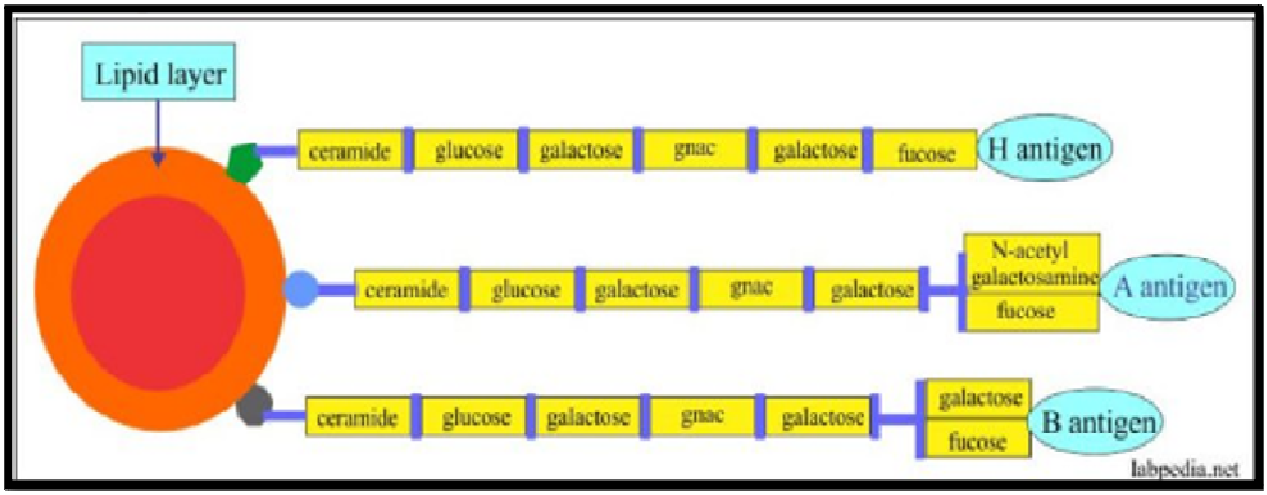


Fig. 4.1: Blood group antigen structure.

4.3 MATERIALS REQUIRED

- Alcohol Swabs
- Lancet
- Clean cavity glass slide
- Sterile cotton bolls
- Blood sample
- Toothpicks
- Monoclonal Antibodies (Anti-A, B, and D)
- Biohazard disposal container

4.3 PRINCIPLE

IgM is found mainly in blood and lymph, this is the first antibody the body makes when it finds a new infection.

The ABO and Rh blood grouping system is based on agglutination reaction. It is observed that when RBCs having one or both the antigens are exposed to equivalent antibodies, they form visible agglutination/clumping on interaction. The antigens are O-linked glycol-proteins, and the terminal sugar residues exposed at the RBCs surface determine whether the antigen is A or B. The Rh antigens are trans-membrane proteins, and the loops exposed on RBCs surface interact with the corresponding antibodies. The associated Anti A and Anti B antibodies are from IgM class of immune-globulins. Individuals with blood group A have A antigens on RBCs and anti-B antibodies in serum. Similarly, B group individuals have B antigens on RBCs and anti-A antibodies in the serum (Fig. 4.2 to Fig. 4.6). In case of AB group, both A and B antigens are on RBCs and neither of anti-A nor anti-B antibodies are in serum. Finally O group individuals have neither A antigens nor B antigens, but possess both anti-A and anti-B antibodies in serum. The corresponding antigen and antibody are never found in the same individual since, when mixed, they form antigen-antibody complexes, effectively agglutinating the blood. **The individuals carrying the Rh antigen possess positive blood group, whereas those who lack this antigen are with a negative blood group.**

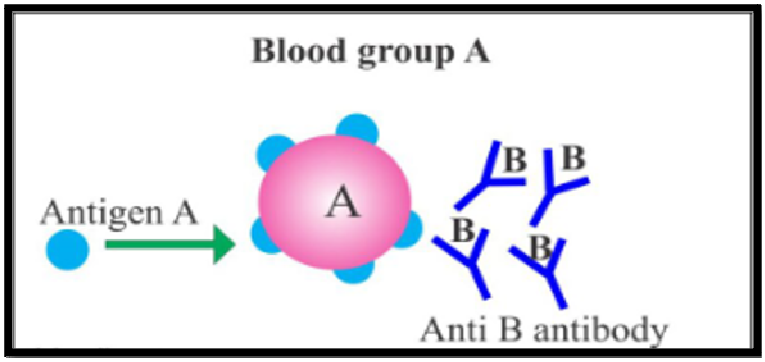


Fig. 4.2: Blood group B has B-antigen and antibody-A.

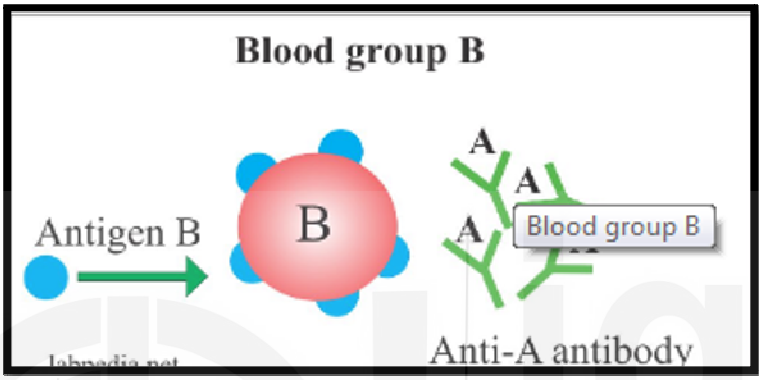


Fig. 4.3: Blood group A has antigen-A and antibody-B.

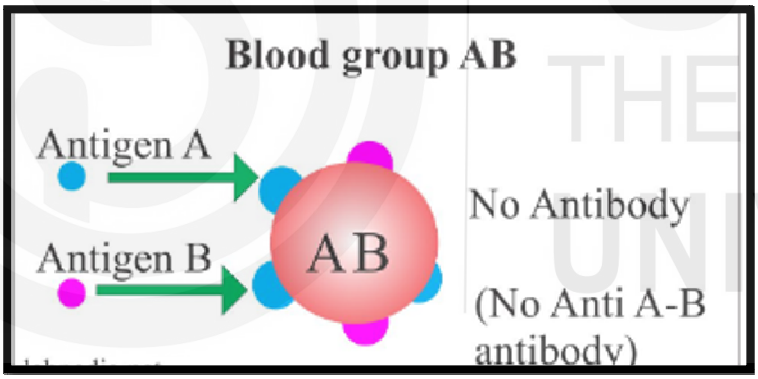


Fig. 4.4: Blood group AB has antigen-A and antigen-B and no antibodies.

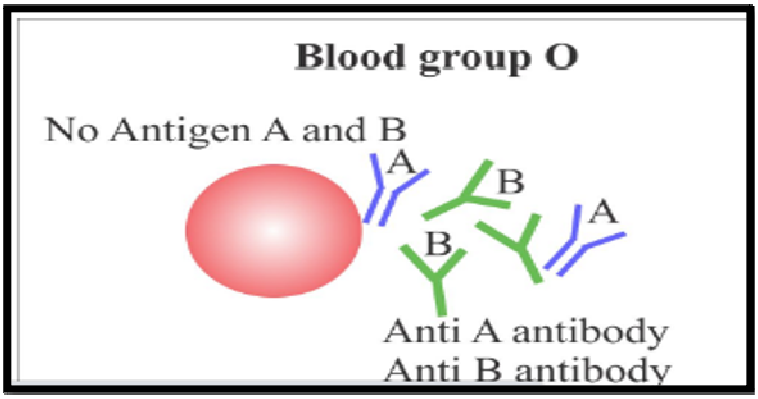


Fig. 4.5: Blood group O has no antigen and has antibody anti-A and anti-B.

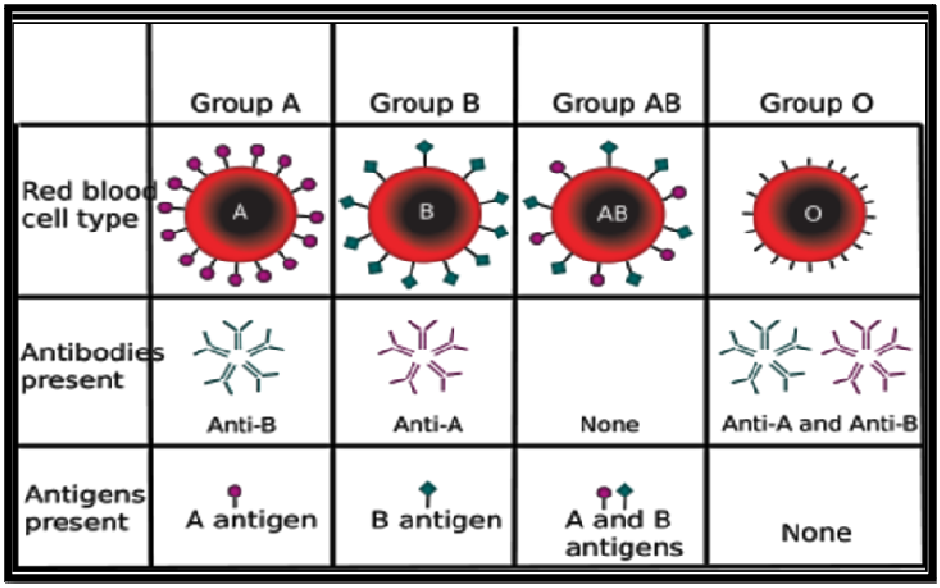


Fig. 4.6: Antigens and corresponding antibodies.

4.4 PROCEDURE

A monoclonal antibody (mAb or moAb) is an antibody made by cloning a unique white blood cell. All subsequent antibodies derived this way trace back to a unique parent cell.

1. Take clean glass slides and mark one cavity as Anti-A, the other as Anti-B and third as Anti-D.
2. Unpack the Monoclonal Antibodies (MAB) kit and with the help of a dropper add Anti-A, to first circle, Anti-B to second circle and Anti-D to third circle (Fig. 4.7).
3. Place the slide aside safely.
4. Rub the ring finger gently near the fingertip and clean with the alcohol swabs.
5. Prick the ring fingertip with the lancet at the area cleaned and wipe off the first drop of the blood.
6. As blood starts oozing out, allow it to fall on each of the three circles on a glass slide.
7. Stop the blood flow by applying the pressure and use the cotton boll if needed.
8. Mix the blood sample gently with the help of a toothpick and wait for a minute.
9. Observe the results.

Fig. 4.7: Monoclonal Antibodies (MAB) kit.

4.5 OBSERVATION AND INTERPRETATION

The results can be read by observing the slide closely. The interpretations can be made as:

- Blood group A if agglutination occurred with the Anti-A test serum,
- Blood group B if agglutination occurred with the Anti-B test serum,

- Blood group AB if agglutination occurred with both test sera, and
- Blood group O if there was no agglutination in either case.

These can be seen in slide pictures as given in Fig. 4.8.

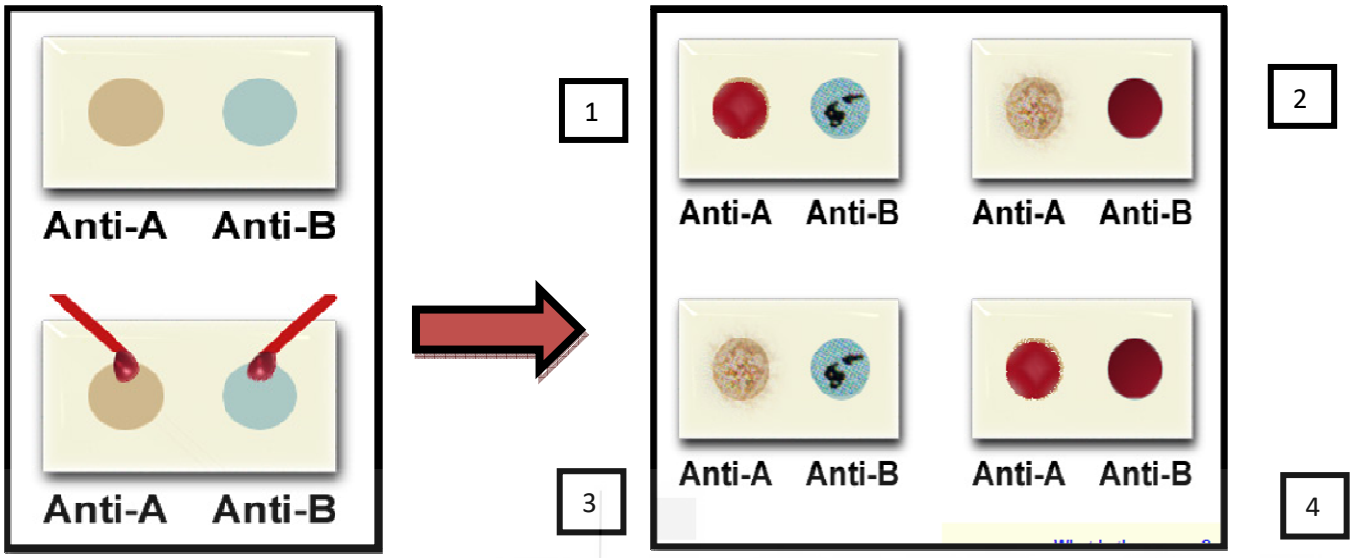


Fig. 4.8: Observation of results…..1. Type B, 2. Type A, 3. Type AB and 4. Type O.

4.6 RESULT

The blood sample collected is with blood group …… .

4.7 PRECAUTIONS

1. Clean the apparatus before use.
2. Clean the finger with an alcohol swab properly.
3. Mix the blood drop with antisera properly.
4. Read the results with great care.
5. Discard swab, lancet, cotton balls and tooth-picks after use in the biohazard container.

Acknowledgement of Figures

Fig. 4.5: https://www.labpedia.net/blood-banking-part-1.

Fig. 4.6: http://nbtc.naco.gov.in/assets/resources/training/5.pdf.

EXERCISE 5

CELL COUNTING AND VIABILITY TEST FOR SPLENOCYTES OF FARM BRED ANIMALS/CELL LINES

Structure

5.1 INTRODUCTION

A splenocyte can be any one of different WBC types as long it is situated in the spleen or purified from splenic tissue. Splenocytes consist of a variety of cell populations such as T and B lymphocytes, dendritic cells and macrophages, which have different immune functions. Trypan Blue dye exclusion test is used to determine the number of viable cells present in a cell suspension or cell line culture system. The viable cells present in the suspension are counted and expressed as a percentage of total cell present. A periodic assessment of viability test provides baseline information on the quality of cells prior to freezing or the general performance of a cell culture system.

Objectives

After completing this exercise, you will be able to:

- prepare cell suspensions from lymphoid organs, and
- differentiate live cells and dead cells.

5.2 MATERIALS REQUIRED

- **Instruments**

Haemocytometer (Fig. 5.1 and Fig. 5.2),

Tally counter,

Micropipette,

Microscope,

Scissors,

Forceps,

Mortar and pestle,

Petri dish,

Dissecting tray,

Centrifuge tube,

Centrifuge.

- **Reagents**

0.4% Trypan Blue Stain in PBS, PBS (pH = 7.5),

Farm breed Mice/ Cell Line. (*RBC Lysis Buffer: 0.1% Ammonium chloride)

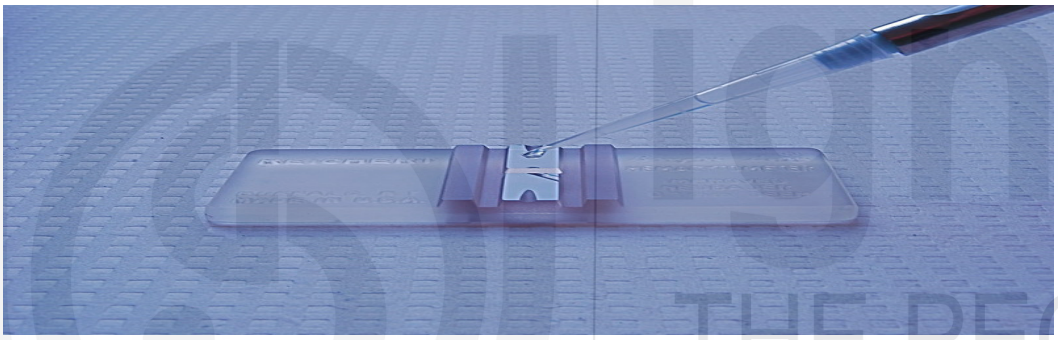


Fig. 5.1: Hemocytometer (A thick glass slide having grid in the center in which sample is loaded for counting the cells).

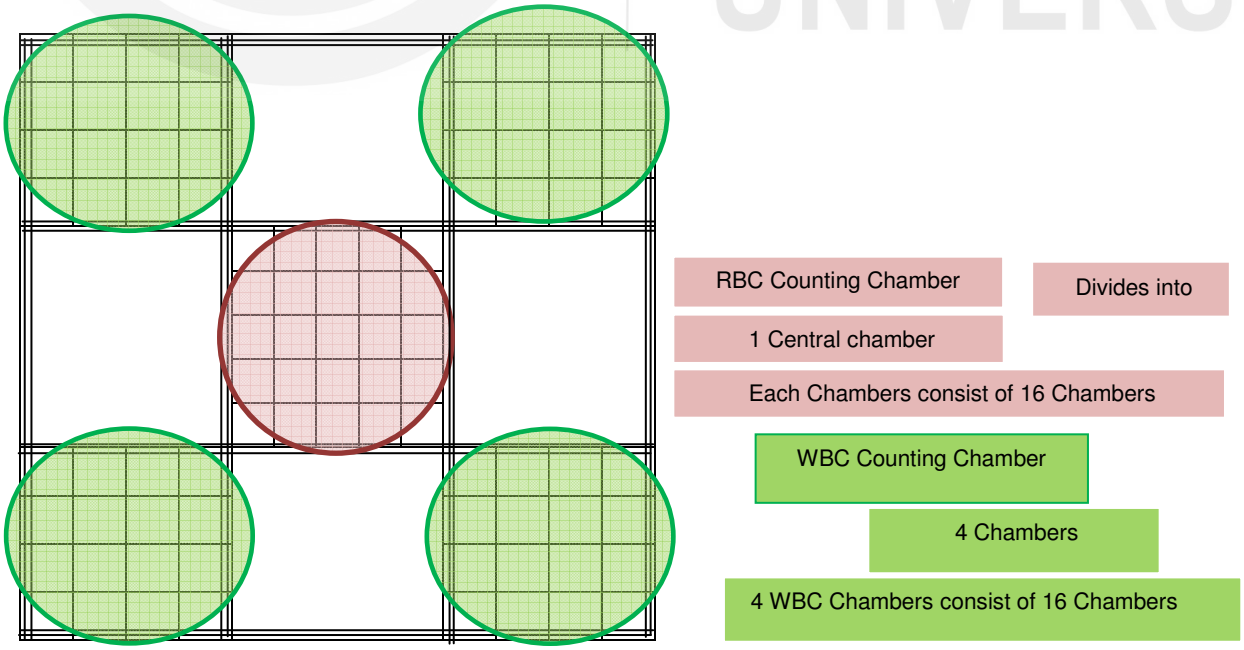


Fig. 5.2: a) Showing different WBC (Green) and RBC (Red) Counting chamber.

5.3 PRINCIPLE

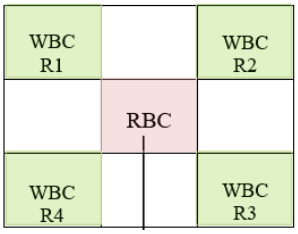


Fig. 5.2: b) Schematic representation of RBC and WBC counting chambers in a haemocytometer.

Trypan blue is a common vital stain that is used to distinguish live cells from dead cells. The splenocytes viability test is based on the ability of the live cells to metabolize the trypan blue stain and thereby appearing colourless when visualized under a microscope. However, the dead cells appear dull blue in colour from the accumulation of the stain. Trypan blue stain has a higher affinity for serum protein than cellular protein. Therefore, cells cultured in high serum conditions and/ or extraction of cells from lymphoid organs are centrifuged and suspended in an appropriate salt solution to remove serum from the sample/ suspension. This is a prerequisite for accurate reading of the cell count, as the presence of serum in the suspension would render staining of the suspension compromising the visibility of cells under the microscope. The viability of the splenocytes in trypan blue stain is time-bound. Therefore, the viability of cell count should be complete within a specific time.

5.4 PROCEDURE

i. Anesthetization of Mouse:

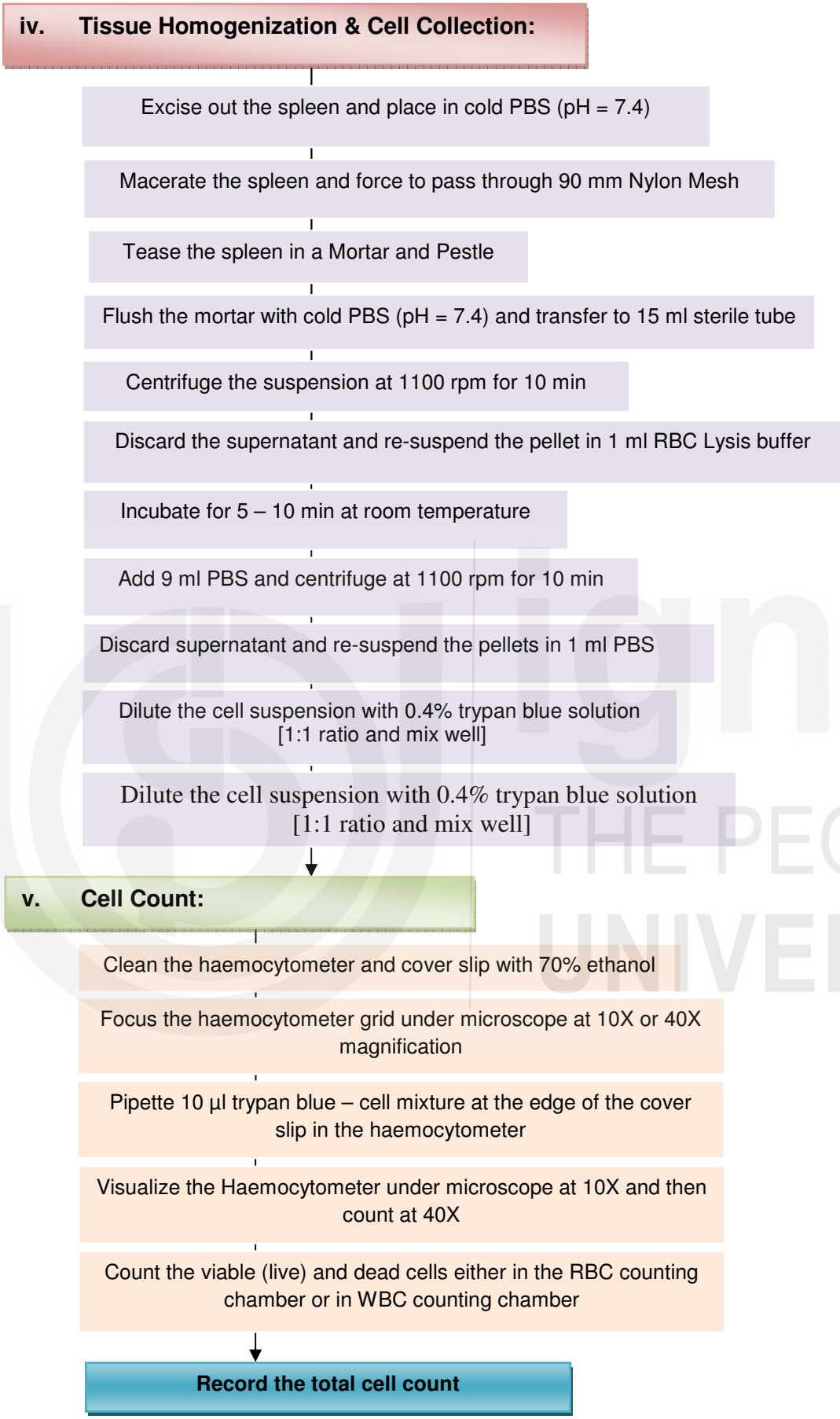
- Add 5 – 10 ml of Isofluorane on to tissue paper or cotton placed at the bottom of the anesthetizing chamber.
- Place the mouse in the chamber
- Remove the mouse when breath becomes lowered

ii. Euthanize mouse:

- Hold mouse by the nape of the neck firmly
- Now, pull tail with the other hand till you feel a pop
- Inspect the separation of cranium from the backbone.

iii. Laparotomy:

- Dissect the mice after drenching the fur with 70% alcohol
- Make a small incision in the skin (Left abdominal cavity) half way between front and hind legs
- [Use a pair of scissor and forceps]
- Remove the skin of mouse completely and open the peritoneal cavity
- Remove the spleen using forceps
- Spleen is flat and longer than kidney with the colour of kidney beans



5.5 OBSERVATIONS

Splenocytes are counted by using hemocytometer. Fig 5.3 a shows 1 WBC chamber (in the form of big large square) consists of 16 small squares where

we can observe two types of cells. One is blue stained cell (dead cell) and other one is unstained cell (live cell). Fig 5.3 b shows that which type of cells should be considered while counting the splenocytes. All the splenocytes (live/dead cells) are counted in 4 WBC squares (Fig. 5.4). For the estimation of concentration of cells per ml, the formula is used:

Average of total no. of cells counted in 4 WBC squares X Dilution factor X 10^4.

Dilution factor is taken as 2 because the cell suspension is prepared by taking equal volume of both splenocyte and trypan blue dye. In Fig. 5.5 you can observe actual images of viable and non-viable cells.

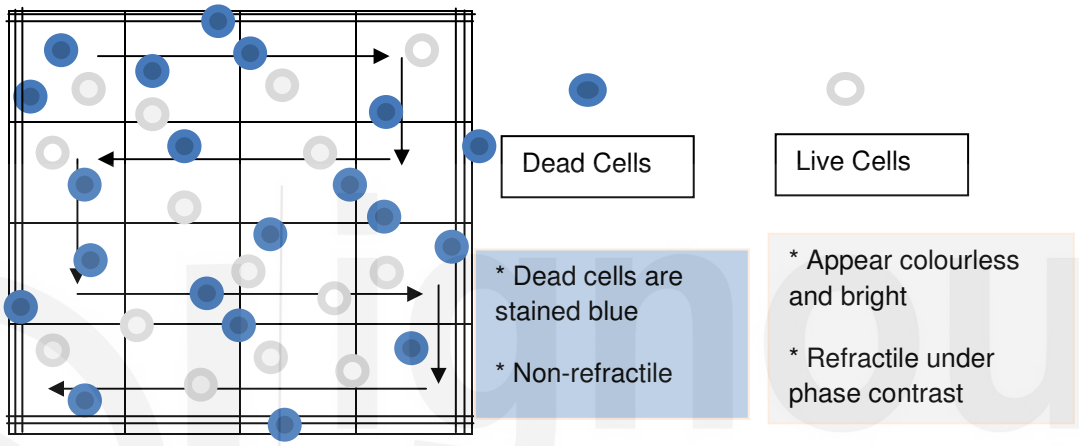


Fig. 5.3 a: Arrows show the direction of counting of cells in one of the WBC chamber (having 16 small chambers).

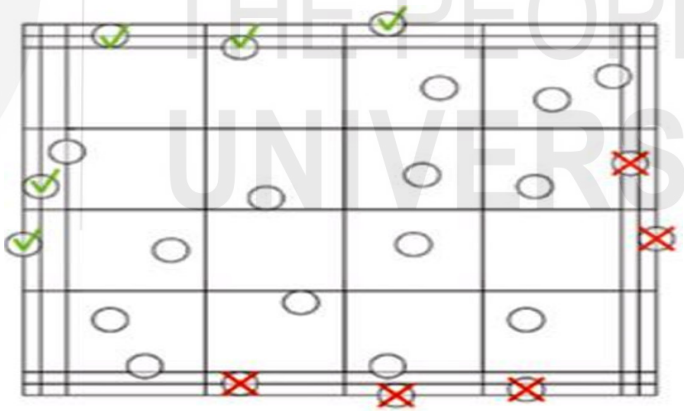


Fig. 5.3 b: WBC chamber showing which splenocyte should be considered while counting. Green tick indicates those splenocytes touching middle on top and left should be included in counting. Red cross indicates that those splenocyte touching middle line on bottom and right should be excluded.

The splenocyte viability can be calculated as follows

Concentration of cells per ml volume:

Average number of cells in one large square = x

Dilution factor = 2 (1:1)

Conversion factor of cm to ml = 10^4

Volume of 1 square = 1 mm^2 [length x breadth] x 0.1 mm [depth]

= 0.1 cm x 0.1 cm x 0.01 cm

= 0.0001 cm^3

= 10^{-4} cm^3

= 10^{-4} ml

The splenocyte viability can be calculated as.

$$\text{Cell viability} = \frac{\text{No. of viable cells counted}}{\text{Total cells counted}} \times 100$$

= % viable cells

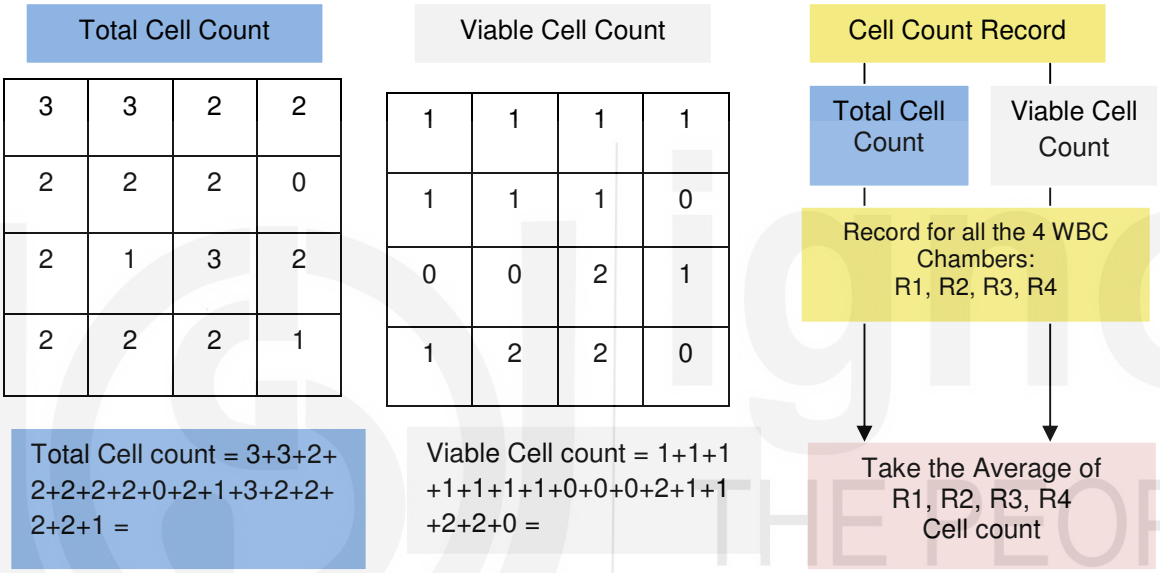


Fig. 5.4: Illustration of cell counting procedure and recording for calculation of cell viability.

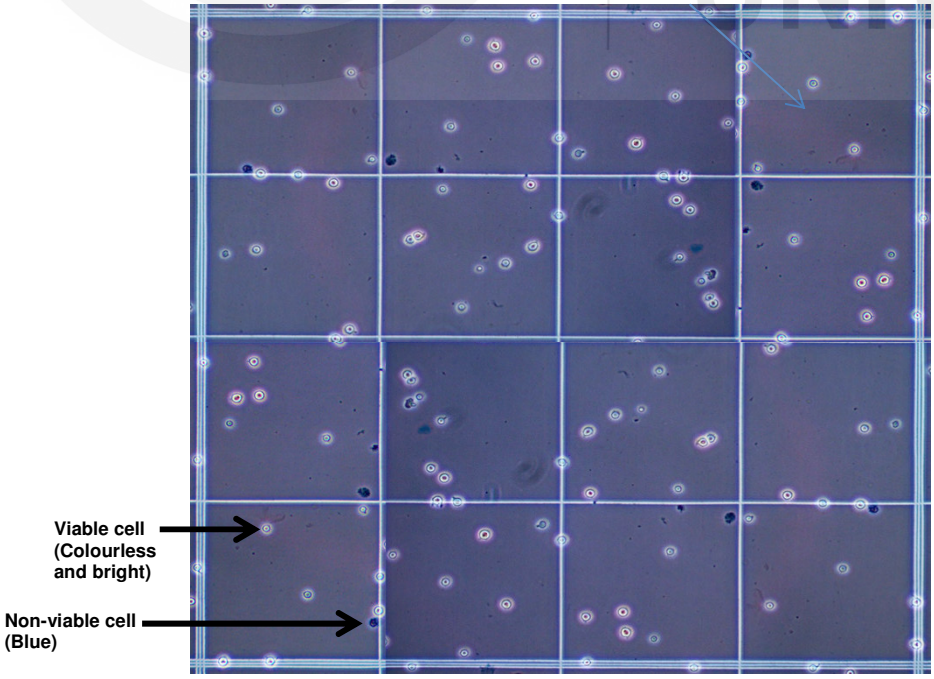


Fig. 5.5: Actual image showing one of the WBC chamber loaded with samples where different viable and non-viable cells can be observed.

5.6 DISCUSSION

Trypan blue test is a good measure to assess the optimum cell survival and growth in a cell culture set-up .In general, a viability count greater or equal to 95% is considered as excellent. Sometimes trypan blue can induce the formation of diffuse objects, which could be mistaken for cells. Such instances can lead to overestimation. Though there exist the issues of inaccurate counting and the limited window of cell counting time, the viabilities less than 80% may be considered as poor.

5.7 PRECAUTIONS

1. Use of personal protective equipment like gloves are recommended while handling the mice and trypan blue stain.
2. PBS solution should be at 4℃.
3. Place the cell suspension in ice or 4 ℃ when not in use.
4. Carefully identify spleen, do not confuse it with kidney.
5. Complete the cell count within 15 minutes.

5.8 TERMINAL QUESTIONS

1. Trypan blue is a common vital stain that is used to test cell
   (a) vitality
   (b) viability
   (c) variability
   (d) variety
2. Staining of the dead cell by trypan blue makes it appear …….... in color under microscope.
3. Why do we use trypan blue stain in the cell viability test?
4. State the purpose of assessing cell viability.
5. Differentiate between RBC and WBC/Splenocytes counting chambers.
6. How would you identify dead cells from the living ones?
7. Can we use other organs other than the spleen for performing cell viability test?
8. Why the cells extracted from the lymphoid organ are suspended in an appropriate salt solution?
9. “Trypan blue staining is a time- bound exercise” Justify.

Acknowledgement of Figures

Illustration are drawn/Photographs are clicked by the authors of this Exercise.

EXERCISE 6

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Structure

6.1 INTRODUCTION

ELISA is a sensitive immunochemical technique for the detection and quantification of hormones, peptides, proteins and antigens. It is widely utilized in medicine as diagnostics, a measure of quality control in industries and research. The assay is performed on a solid matrix using enzymes that are linked to an antibody which would serve as a marker for the detection. Therefore, ELISA is also known as solid-phase enzyme immunoassay.

Objectives

After the completion of this exercise you will be able to:

- ❖ explain the principle and immuno-quantification technique of ELISA, and
- ❖ detect and quantify peptides, proteins, antibodies and hormones.

6.2 MATERIALS REQUIRED

- ELISA plate (Fig. 6.1),
- Antigen,
- standard polyclonal anti-serum/antigen-specific monoclonal antibody,

- Low and high titer serum specific to the antigen,
- Substrate solution,
- Coating buffer (pH 9.6),
- Washing buffer (pH 7.4),
- Stop solution (1M H_2SO_4),
- 1.5 % Skimmed milk powder solution and/0.5% BSA (Bovine Serum Albumin).

Preparation of Solutions

Coating buffer (pH 9.6)

Weigh 0.29 g Sodium bicarbonate + 0.15 g Sodium chloride + 0.02 g Sodium azide and dissolve in distilled water, making up the volume to 1L.

Substrate buffer

Weigh 1.45 g disodium hydrogen phosphate + 8.0 g Sodium chloride + 0.20 g Potassium chloride + 0.20 g Potassium dihydrogen phosphate. Dissolve in 1L distilled water and add 500 µl of TWEEN – 20.

Phosphate Buffered Saline (PBS) – pH 7.2, 0.15 M

Dissolve in 500 ml Distilled Water

8 g Sodium chloride + 0.2 g Potassium chloride + 1.15 g Disodium hydrogen phosphate + 0.2 g Potassium dihydrogen phosphate

Adjust the pH to 7.2 and make up the volume to 1000 ml with distilled water.

Citrate Buffer – pH 5.0, 0.1M

33 ml of 0.1 M Citric acid + 67 ml of 0.1M Sodium citrate

Tris-HCl Buffer – pH 7.6, 0.01 M

Dissolve in distilled water1.21 g Tris in 50 ml and adjust the pH to 7.6 with HCl.

Make up the final volume to 100 ml.Dilute 10 times before Use.

ELISA Substrate

1. Diaminobenzidine (DAB):

   Dissolve 6 mg Diaminebenzidine in 10 ml 0.1M Tris-HCl buffer (pH 7.6)

   Add 10 µl of Hydrogen peroxide [just before use]

2. Ortho-Phenyl-diamine (OPD):

   Dissolve 34 mg Ortho-phenyldiamine in 100 ml of

   0.1M Sodium citrate Buffer (pH 5.0)

   Add 50 µl of Hydrogen peroxide [just before use]

Fig. 6.1: ELISA plate.

6.3 PRINCIPLE

Enzyme-Linked Immunosorbent Assay (ELISA) follows the basic principle of antibody binding to a specific epitope of antigens. The assay involves three principle reactions. First, specific immune reaction (Antigen-antibody reaction) followed by enzymatic chemical reaction of converting substrate (chromogen) to an insoluble coloured product. Third, signal detection of colour and quantification of colour intensity.

There are four major types of ELISA:

- **Direct ELISA**

Direct ELISA is the simplest form of ELISA. In direct ELISA, primary antibody labelled with a conjugated enzyme directly binds to an antigen. The addition of chromogenic substrate produces colour change on enzyme hydrolysis (Fig. 6.2).

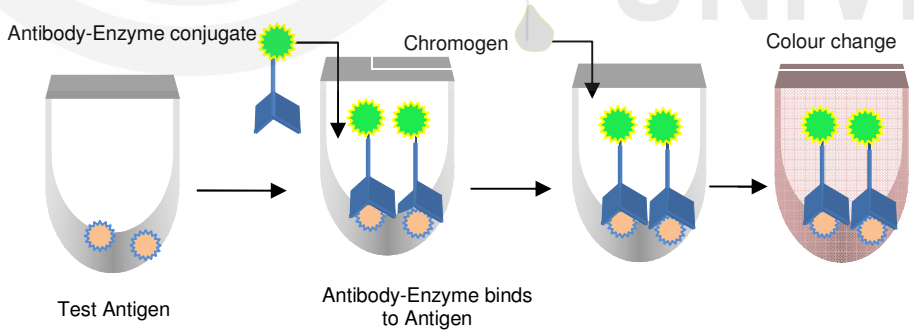


Fig. 6.2: Figure depicting the direct ELISA.For the detection of antigen in a given biological sample, enzyme (e.g. Horse reddish peroxidase) conjugated antibody is used. When antigen is detected, chromogenic susbstrate produces colour. The intensity of the colour can be measured by using a machine called ELISA plate reader at particular wavelength.

- **Indirect ELISA**

In this technique, the antigen to be detected and quantified is immobilized on to a plate using a coating antibody that specifically traps the antigen on the

solid phase. A secondary antibody, labelled with an enzyme (termed as reporter enzyme) is then directly allowed to bind with the immobilized antigen. The presence of antigen in test sample is then detected by introducing an enzyme- substrate (chromogen) to the antigen-antibody-enzyme complex, which produces the colour (Fig. 6.3)

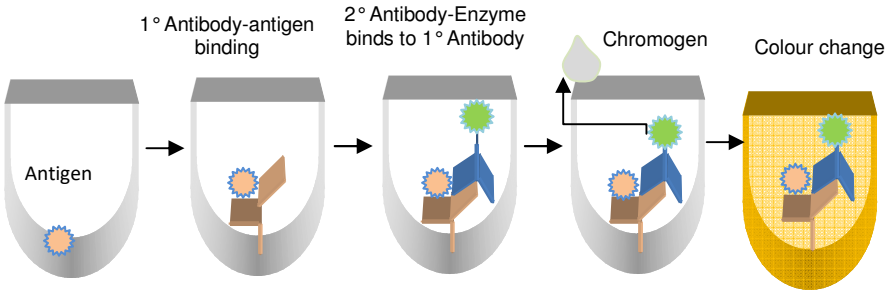


Fig. 6.3: Antigen is detected by two step process. First, primary antibody specific for antigen to be detected, are added and then secondary antibody conjugated with enzyme is allowed to bind with secondary antibody. Enzyme (e.g. Horse reddish peroxidase) works on its substrate and colour is produced for which intensity can be measured spectrophotometrically (Using ELISA plate reader at particular wavelength).

- **Sandwich ELISA**

In sandwich ELISA, the antigen is sandwiched between 1 and 2 antibody. This assay can be done in a direct way or indirect way. The primary antibody (1^{o}) is immobilized on the plate to absorb the antigen of interest. The detection antibody or secondary antibody,which is enzyme- conjugated is then introduced to bind with the antigen trapped on the 1° antibody.The addition of chromogen substrate would bring colour change on enzyme hydrolysis, linked with 2° antibody (Fig. 6.4).

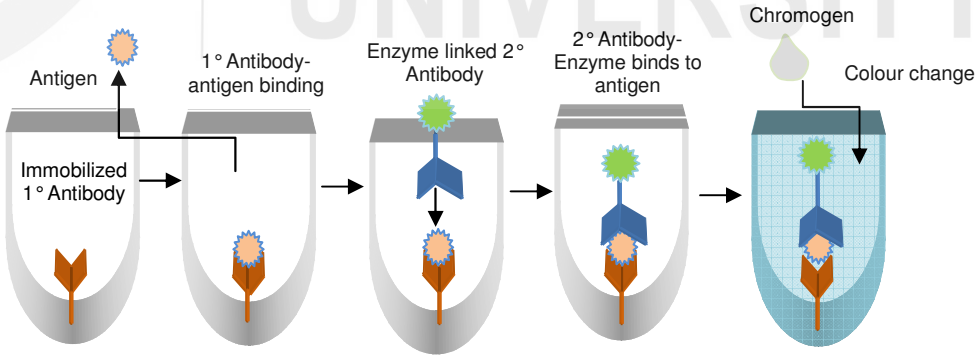


Fig. 6.4: Figure depicting sandwhich ELISA. Antigen to be measured is sandwiched between primary and secondary antibody and rest of the process remains same as previously explained.

Competitive ELISA

Competitive ELISA works on the principle that the test antigen and a conjugated version of the same antigen would compete for the limited number of specific antibody binding sites pre-coated on ELISA plate. This assay could also be done reversibly by the antibody competing for the target site of the coated antigen. The labelled antibody would compete with the native antibody

in the sample. In a competitive assay, the strength of signal emitted from the assay is inversely proportional to the concentration of antigen or antibody (Fig. 6.5).

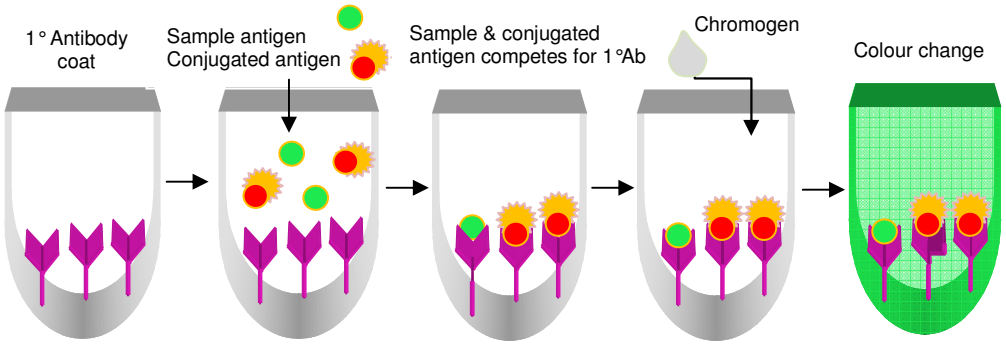


Fig. 6.5: Diagram showing competitive ELISA which provides sensitive variation for detection and measuring of antigen.

You can refer to Table 6.1 for better understanding of the differences among four types of ELISA.

Table 6.1: Comparison of the four types of ELISA methods.

	Direct ELISA	Indirect ELISA	Sandwich ELISA	Competitive ELISA
1	Fastest ELISA test –less steps, reagents and less error- prone	Economical – less requirement of the labelled antibody.	High specificity – involvement of detection and capture antibodies.	No requirement for sample processing
2	High background noise – antigen immobilization is not specific. No cross- reactivity	Likelihood of background noise from secondary antibody.	Cross-reactivity gives background noise too between captured and detection antibodies.	Cross-reactivity may occur- antibody optimization is difficult for this assay
3	Low flexibility - specific antibody for specific antigen.	High flexibility- a wide range of 1° primary antibody can be used for a single labelled 2°- antibody.	High flexibility – since both direct and indirect assay can be deployed.	Most flexible – since direct, indirect and sandwich ELISA can be deployed
4	Assay choice for analyzing the immune response to an antigen	Assay choice for determination of antibody concentration.	Assay of choice for analysing complex antigen sample	Assay of choice for detection of antigens that are not bound to 2 different antibodies.

5	Absence of signal amplification – less sensitive	High sensitivity – signal amplification	2-5 times higher sensitivity than direct and indirect ELISA	Less sensitive but more robust

6.4 PROCEDURE

1. Coating of ELISA Plate with antigen

Pipette 50 µl of dilute antigen into each of wells of the ELISA plate

Incubate it overnight @ 4 ℃ or @ 37 ℃ for 1 h on plate shaker

Wash the plate with washing buffer 3 times

[Invert the plates over plotting paper and tap the plates to remove residual solutions in the wells]

2. First Reaction

Pipette 50 µl of dilute sera to the antigen coated wells [Maintain triplicates]

Incubate @ 37 ℃ for 1 h on plate shaker

Wash the plate 3 times with washing buffer and remove residual solution

3. Second Reaction

Pipette 50 µl of diluted antimouse IgG-conjugate to the wells

Incubate @ 37 ℃ for 30 min on plate shaker

Wash the plate wells with washing buffer and remove residual solution

4. Third Reaction

Pipette 50 µl of TMB (Tetramethyl benzidine) to each wells

Incubate @ 25 ℃ for 10 min on plate shaker

5. Reaction Termination: Pipette 50 µl of 1 M H_2SO_4(Sulphuric acid)

6. Absorbance Reading: Record the ELISA absorbance reading at 450 nm.

6.5 OBSERVATION

You will observe colour on ELISA plate as shown in Fig. 6.6.

Fig. 6.6: ELISA plate showing colour on completion of reaction. The colour intensity can be measured by ELISA plate reader (See Fig. 6.7). Intensity of the colour is directly proportional to the recorded absorbance.

Fig. 6.7: ELISA Plate Reader.

In general for **calculation** of the assay value of **ELISA,** a standard curve with absorbance on Y-axis against concentration on X-axis is drawn. Then assay value (i.e. the amount of antigen or antibody in the sample) is estimated/ extrapolated from the absorbance of the sample. To achieve this, following steps are taken:

1. It is important to take ELISA samples in duplicates or in triplicates. This is done to avoid any handling error as the sample volumes added in the ELISA wells is very small (100ul) which is prone to error. Once reaction is complete absobance is taken for all the samples in ELISA plate reader at a particular optical density (OD).

2. Then calculate the average of the absorbance values for each set. of standards and samples.

3. Though the negative sample/blank/zero standard appear transparent with naked eye but it does have some absorbance value. So subtract the

average value of zero standard OD from all the other standards and the test samples (this step is unnecessary in procedure of **competitive ELISA**).

4. Then create a standard curve and extrapolate the concentration of the antigen in the test sample using its absorbance value.

5. The recorded absorbance values converted to Percentage Inhibition can be inferred as:

   - **Positive** when the percentage inhibition value is greater than 50%.
   - **Negative** when the percentage inhibiton value is lesser than 50%.

Perent inhibition can range from O to 100%. It depends upon the concentiation of the substrate and enzyme. When the inhibitor is bound, the substrate can't bind, but when the inhibitor dissociates, the substrate can bind and be converted to product.

6.6 PRECAUTIONS

1. In the entire test use proper negative control (Without the sera/or without antigen).
2. Personal protective equipment especially using gloves and eye protection gear, is recommended.
3. Avoid cross mixing of reagents.
4. Dispense the reagents in the bottom of the well, and avoid bubbles.

6.7 TERMINAL QUESTIONS

1. What is the role of antibody-linked to enzyme Horse Radish Peroxidase (HRP) in the experiment?

   (a) Antigen

   (b) Primary antibody

   (c) Secondary antibody

   (d) None of the above

2. The color intensity of spot is …….. the concentration of antigen in the sample.

   (a) Independent of

   (b) Directly proportional to

   (c) Inversely proportional to

   (d) None of the above

3. Compare direct ELISA and indirect ELISA.
4. Briefly describe the principle of ELISA.
5. In ELISA, proteins in a sample are adsorbed to an inert surface, usually a 96-well polystyrene plate. Why surface is washed with a solution of an inexpensive nonspecific protein-like, casein?

6. A patient was given an antibody, and his serum developed Ag/Ab complex. Can we measure the serum antibodies of the antibodies administered? Describe the procedure involved and the type of ELISA to be used.

Acknowledgement of Figures

Illustration are drawn/Photographs are clicked by the authors of this Exercise.

EXERCISE 7

DEMONSTRATION OF IMMUNO-ELCTROPHORESIS

Structure

7.1 INTRODUCTION

Immuno-electrophoresis is a semi-quantitative method that combines two techniques-electrophoresis and immune-diffusion. It includes the identification and characterization of proteins based on their electrophoretic and immunological properties. This method is used in clinical laboratories to examine serum abnormalities in body fluids like cerebrospinal fluid, pleural fluids and those fluids involving immunoglobulins. In research, immune-electrophoresis protocols are deployed in the detection of impurities in the purification of proteins (antigen or antibody), microbial extracts and monitoring of soluble antigens in tissues.

Objectives

After completing this exercise, you would be able to:

- separate and characterize a mixture of proteins/antigens, and
- describe the specificity of antigen-antibody interaction.

7.2 MATERIAL REQUIRED

- **Reagents**

AgaroseGel, Glassslides,

Electrophoresis buffer,

Anti-serum sample,

Anti-IgG sample,

Distilled water.

- **Instruments**

Incubation oven,

Heat Plate or Micro-wave oven,

Electrophoresis Unit with power supply,

Well Cutter,

Filter paper,

Micropipette and tips,

Glasswares (Beaker, graduate measuring cylinders)

7.3 PRINCIPLE

Individual protein molecules in a given protein mixture, when placed in a electric field, get separated on the basis of their charge to mass ratios, in horizontal gel during electrophoresis. The migrating individual proteins get accumulated at different points on the gel. The gel having separated proteins is then removed from the electric field and antibodies are introduced through the lateral trough in the gel. The antigen and antibody are then allowed to diffuse across the gel to form and precipitin lines correspond to specific antigen-antibody at the point of equivalence.

7.4 PROCEDURE

Dissolve 1.5 g off agarose in 100 ml of electrolysis buffer pH 8.5 to prepare 1.5% Agarose gel solution.

Cool of the Solution to about 50-60°C

Pour 8-10 ml of the solution on clean slide [*Avoid spillage]

Allow the Gel to solidify [Approx 30 min]

Punch a well towards the cathode for loading Antigen sample (Fig. 7.1)

Add 15 µl of Antigen Sample in the well

Place the above set up on the electrophoresis unit

Pour the electrophoresis buffer in the unit

Run the gel at 110 Volt for 30-40 min

[Till the dye crosses 3/4th of the gel length]

IMMUNE-DIFFUSION

Make 2 troughs on either side of the sample well
[*Cut the entire gel about 2/3rd of the gel thickness]

Remove the gel from the trough on either side of the slide

Allow the plate setup rest for 15 min @room temperature

Pipette 250 µl of Test Antiserum-A in one of the lateral trough

Pipette 250 µl of Test Antiserum-B on the other trough

Remove the slide from the Electrophoresis Unit
&
Incubate

[@Room Temperature for overnight in Humid Chamber]

Observe for Precipitin Arc Formation

[Antigen-Antibody Complex]

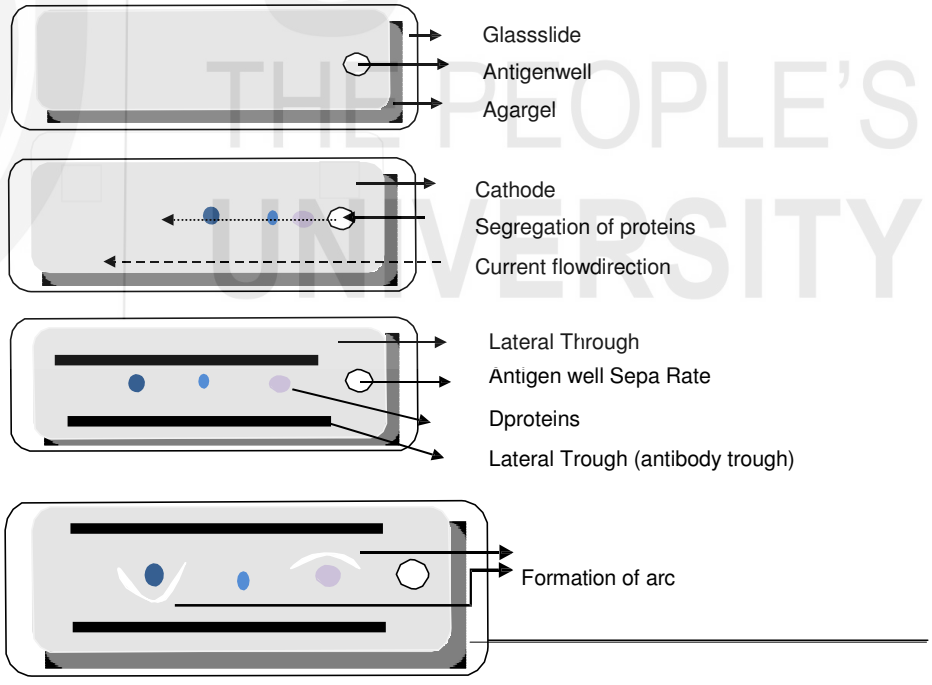


Fig. 7.1: Figure depicting the antigen sample loaded on well is separated and forms precipitin line when antigen and antibody diffuse in the agarose gel matrix.

7.5 OBSERVATIONS

1. Presence of precipitin arc/lines between antiserum trough and antigen well (Fig. 7.2).

2. The occurrence of more than one precipitin line indicates the heterogeneity of the antiserum to the antigen.
3. The presence of only one precipitin line indicates the homogeneity between antigen and antiserum.

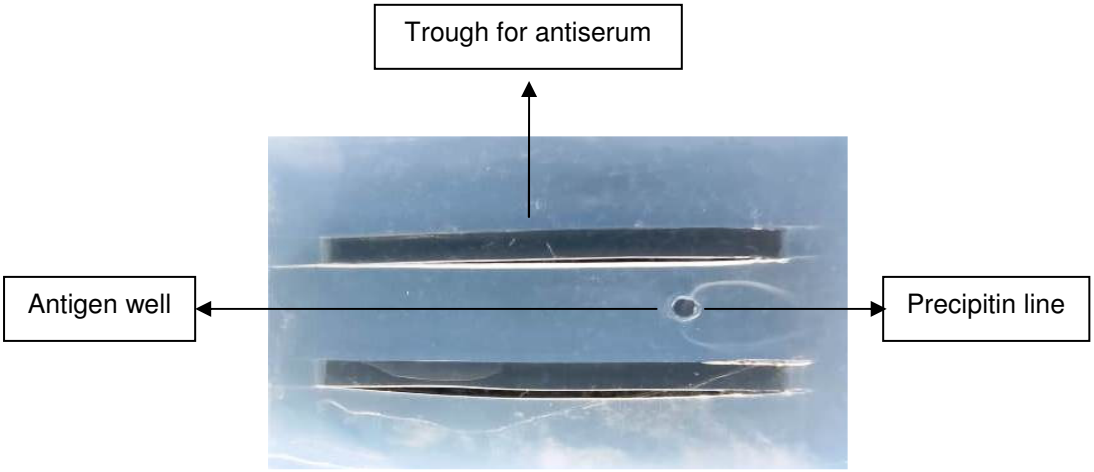


Fig. 7.2: Real observation image showing precipitin line formed between antigen well and antiserum trough.

7.6 PRECAUTIONS

1. Wear gloves (Nitrile) while conducting the experiment.
2. Maintain uniform thickness and avoid air bubbles while casting the gel.
3. Antigen wells and trough should not cut through agargel on the glassslide.
4. Do not let the sample run off from the gel during electrophoresis.

7.7 TERMINAL QUESTIONS

1. Immuno-electrophoresis is a combination of which two different techniques?
2. Immuno-electrophoresis can identify and characterize:
   (a) RNA
   (b) Proteins
   (c) DNA
   (d) Both DNA and protein
3. Mention the significance of immuno-electrophoresis in clinical laboratories.
4. What is the role of an electric field in Immuno-electrophoresis?
5. Differentiate Immuno-electrophoresis from electrophoresis.

Acknowledgement of Figures

Illustration are drawn/Photographs are clicked by the authors of this Exercise.

EXERCISE 8

Dot ELISA

Structure

8.1 INTRODUCTION

Dot Enzyme-linked Immunosorbent Assay (ELISA) is a very sensitive immunochemical technique used to detect the presence of a specific protein in given sample. It is regarded as an important diagnostic tool for medical purposes due to its sensitivity and robustness.In Dot ELISA, the antigen in the test sample is sandwiched directly between two antibodies. Therefore, the assay is a type of Sandwich ELISA (Refer to Exercise no. 6 for Sandwich ELISA). Dot-ELISA is an alternative to standard ELISA because of its relative speed and simplicity. In Dot-ELISA the result can be visualized with the naked eye as we get a big dot (that is why it is called Dot ELISA) on the test strip whereas in standard ELISA we need ELISA plate reader. Dot-ELISA is a qualitative test (detection of presence or absence but by standard ELISA test proteins can be quantified). In the current exercise on Dot-ELISA, we will test the sample qualitatively and not quantitatively.

Dot ELISA can be performed through Dot ELISA Kit which is performed on a nitrocellulose and other paper membranes which avidly bind proteins. The test membrane kit is divided into three zones:

Test zone: With pre-set immobilized antibody for specific antigen binding.

Positive control zone: With pre-set immobilized antibody bound to antigen.

Negative control zone: Immobilized antibody is not present, hence,no reaction.

Objectives

After the exercise you will be able to:

- ❖ detect antigen using Dot ELISA kit., and
- ❖ describe principle of Dot ELISA technique.

8.2 MATERIALS REQUIRED

- Dot- ELISA Kit
- 10XAssay buffer,
- Ab-HRP conjugate (Antibody-Horse Radish Peroxidase conjugate),
- TMB/ H_2O_2(Tetramethyl benzidine/Hydrogen peroxide),
- Test serum samples,
- Collection Tubes,
- DotELISA Strip

8.3 PRINCIPLE

Dot- ELISA strip is, a nitrocellulose membrane,coated with antibodies that react with two different epitopes of the same antigen. First, the immobilized antibody reacts with the antigen present in the test sample. Next the secondary antibody is linked to an enzyme that converts the specific chromogenic substrate to an insoluble product as a colour precipitate.

Here, the secondary antibody is linked to the enzyme;, Horse Radish Peroxidase (HRP). The binding of antigen (present in the test serum samples)with the immobilized antibody is detected using a secondary antibody conjugated to HRP using hydrogen peroxide as a substrate and Tetra-methylbenzidine (TMB) as a chromogen. The enzyme HRP acts on the substrate H_2O_2 to release oxygen (O_2). The product O_2 then oxidizes the chromogen TMB (Tetra methyl benzidine) to an insoluble product TMB oxide (TMBO). The product TMBO is deposited where enzymes are located,thus giving the blue coloration (Fig. 8.1).

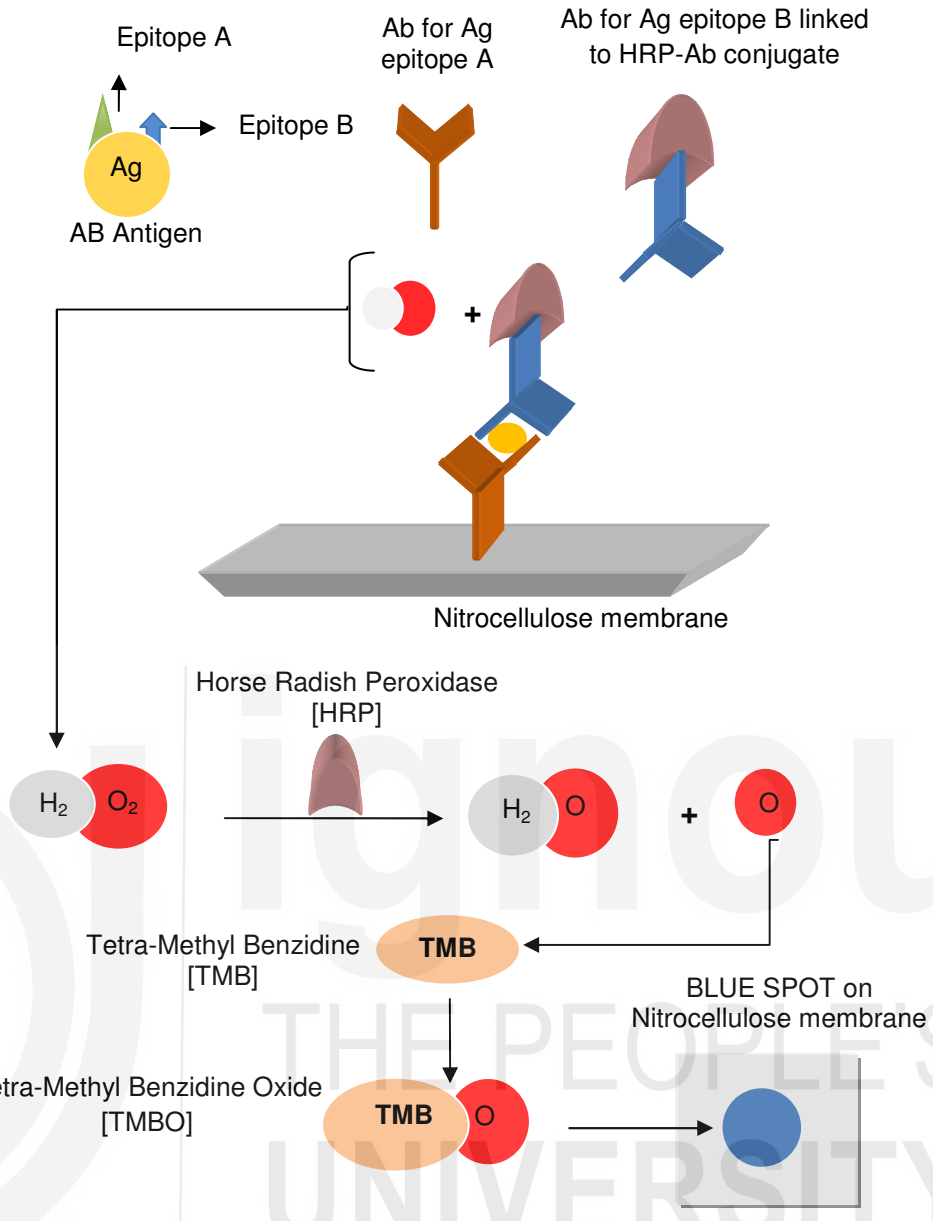
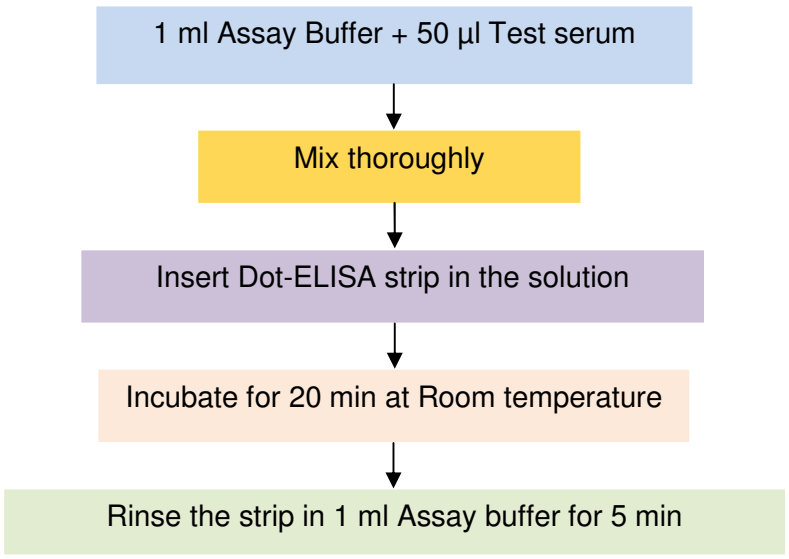


Fig. 8.1: Schematic representation of the working principle of Dot Enzyme Linked Immunosorbent Assay (ELISA)

8.4 PROCEDURE



[Repeat 3 times with new assay buffer at every wash]

↓

Dip the strip in
1 ml assay buffer + 10 µl Ab-HRP solution

[Roomtemperaturefor 20 min]

↓

Rinse the strip in assay buffer for 5 min by dipping

[Repeat 3 times]

↓

Dip the strip in
100 µl of 10x TMB/ H_2O_2+ 900 µl DDW solution

↓

Observe after 20 min for development of

BLUE SPOT

↓

Rinse the strip with DDW (Double Distilled Water) to stop the reaction

8.5 OBSERVATIONS

There are three zones in the Dot-ELISA strip viz. negative control, test, and positive control.Spot in the positive control zone and no spot in the negative control zone indicate the proper performance of the test.

Since there is no immobilized antibody in the negative control zone, therefore, no spot can be seen.Where as immobilized antibodies are there in the positive control zone so the blue dot develops. The immobilized antibody (specific to the test antigen) is present in the test zone so test serum binds to the region and the HRP-conjugated antibody binds to the serum and develops a blue dot when reacts with the substrate (Fig. 8.2).

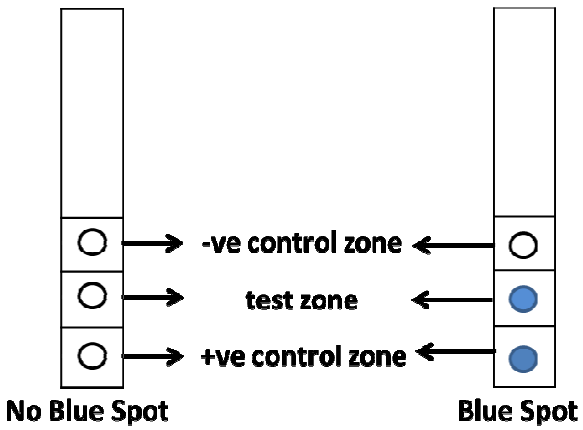


Fig. 8.2: Dot-ELISA strip showing blue dot in positive control and test zone and no dot in a negative control zone.

8.6 DISCUSSION

The appearance of the blue spot in the positive control zone and the absence of a spot in the negative zone is an indication of the correct performance of the test. The presence of a specific antigen is detected in the test zone through binding of the test or sample serum to the specific antibody immobilized that facilitates the binding of HRP conjugated antibody to the captured test serum, which then reacts to give blue coloration. The level of antigen concentration present in the test sample is specified by the intensity of the spot that can be directly correlated with the enzyme activity.

8.7 PRECAUTIONS

1. Always wear gloves while performing the exercise.
2. Dilute the 10x Assay buffer to 1x with distilled H_2O before use.
3. To avoid cross- contamination of reagents, use and throw the micro tips after use.
4. Do not leave the reagents at room temperature.

8.8 TERMINAL QUESTIONS

1. Expand: Dot-ELISA.
2. Dot-ELISA is a type of …….. ELISA.
3. ELISA is the rapid detection technique for …….. in the given sample.
   a) DNA
   b) RNA
   c) Lipid
   d) Protein (Antigen)
4. Discuss the principle of Dot-ELISA using schematic representation.
5. What are the applications of Dot-ELISA?

Acknowledgement of Figures

Illustration are drawn/Photographs are clicked by the authors of this Exercise.