SEMESTER III

17BTU313 IMMUNOLOGY PRACTICAL 4H - 2C

Total hours/week: L:0 T:0 P:4 Marks: Internal: 40 External: 60 Total: 100

Practical

1. Differential leucocytes count
2. Total leucocytes count
3. Total RBC count
4. Haemagglutination assay
5. Haemagglutination inhibition assay
6. Separation of serum from blood
7. Double immunodiffusion test using specific antibody and antigen.
8. ELISA.

References

1. Abbas, A.K., Lichtman, A.H., & Pillai, S. (2007). *Cellular and Molecular Immunology* (6th ed.). Philadelphia: Saunders Publication.
2. Delves, P., Martin, S., Burton, D., & Roitt, I.M. (2006). *Roitt's Essential Immunology*. (11th ed.). Wiley-Blackwell Scientific Publication, Oxford.
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KARPAGAM ACADEMY OF HIGHER EDUCATION, COIMBATORE
DEPARTMENT OF BIOTECHNOLOGY

LIST OF PRACTICAL

Name of the Faculty: Dr. S. Selvakumar	**Department: Biotechnology**
Course name: Immunology Practical	**Course code: 17BTU313**
Academic Year: 2019-2020	**Semester: Third**
Class: II year B.Sc.,	**Section: B1**

1. Differential leucocytes count
2. Total leucocytes count
3. Total RBC count
4. Haemagglutination assay
5. Haemagglutination inhibition assay
6. Separation of serum from blood
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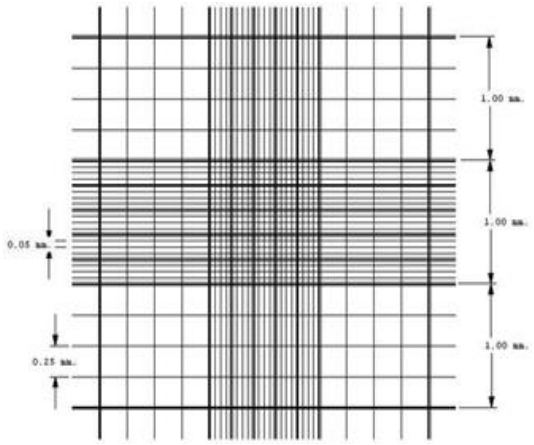
Exp No: 1 Total Leucocyte Count

AIM: To count the total number of leucocytes from the blood sample.

Introduction: Although a variety of automated cell counting instruments have been developed, Hemocytometer remains the most common method used for cell counting around the world. The most frequently used haemocytometer is the Neubauer (or 'Improved Neubauer') chamber. Other haemocytometers include the Burker, Thoma and Fuchs-Rosenthal. Using these, the particles (e.g., leucocytes, erythrocytes, thrombocytes, bacteria, fungus spores, pollen) are visually counted under a microscope.

Neubauer's chamber is a thick glass plate with the size of a glass slide (30x70x4mm). The counting region consists of two square shaped ruled areas. There are depressions or the moats on either side or in between the areas on which the squares are marked thus giving an "H" shape.



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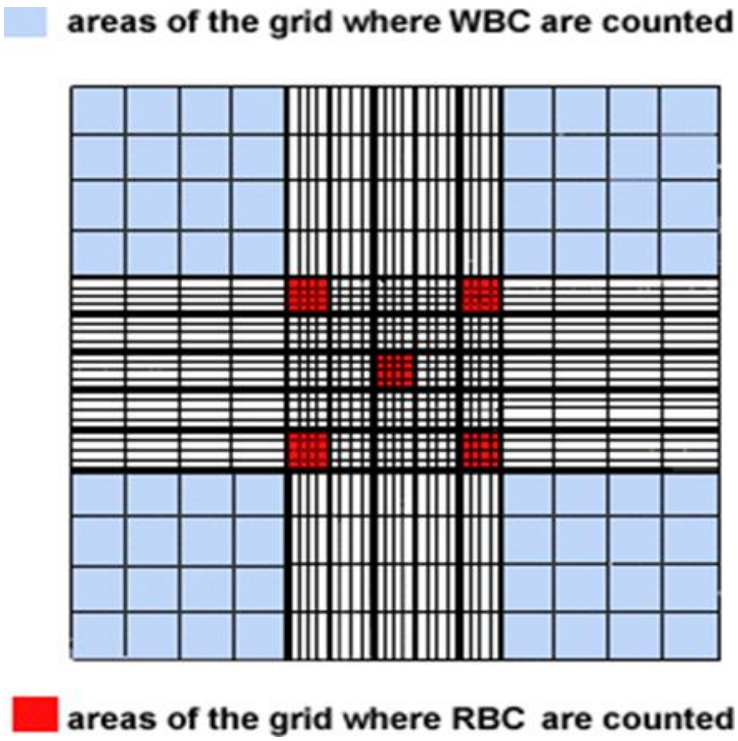
The ruled area is 3mm2 divided into 9 large squares each with a 1 mm^2 area. The large central square (which can be seen in its entirely with the 10X objective), is divided into 25 medium squares with double or triple lines. Each of these 25 squares are again divided into 16 small squares with single lines, so that each of the smallest squares has an area of 1/400 mm^2.

1. Sample Preparation:

The glass cover is a squared glass of width 22 mm. The glass cover is placed on the top of the Neubauer chamber, covering the central area. The ruled area is 0.1 mm lower than the rest of the chamber. So that when a cover slip is kept on the counting region, there is a gap of 0.1mm (1/10mm) between the cover slip and the ruled area.

CELL COUNTING AREAS IN NEUBAUER CHAMBER

The counting can be done either in the central large square or in the corner squares, depending on the size of the cells under study.



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WBC Counting Area

The four large sqaures placed at the corners are used for white blood cell count. Since their concentration is lower than red blood cells a larger area is required to perform the cell count.

RBC Counting Area

The large center square is used for RBC counts. As already stated, this area is subdivided into 25 medium sqaures, which in turn are each divided into 16 squares. Of the 25 medium sqaures, only the four corner squares and the center square within the large center square are used to perform RBC counts.

Platelet Counting Area

The large center square is used to count platelets. Platelets in all 25 squares within the large center square are counted.

Microscope focusing and Cell Counting

- ✓ Place the Neubauer chamber on the microscope stage. Using the 10X objective, focus both onto the grid pattern and the cell particles.
- ✓ As 10X is appropriate for WBC counting, count the total number of cells found in 4 large corner squares.
- ✓ To count the RBCs and Platelets, the microscope must be switched to 40X objective. Count the cells in the respective areas as stated early.
- ✓ Write down the amount of cells counted.

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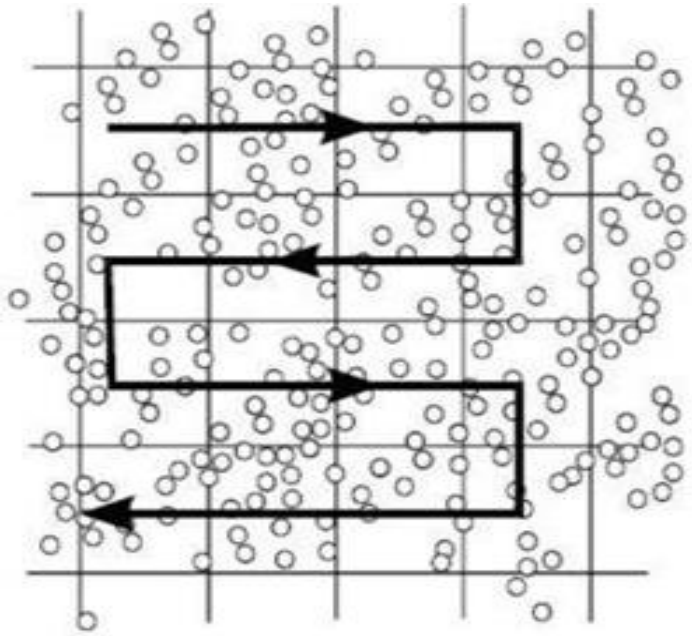

CALCULATING THE CELL COUNTS

The total number of cells per microliter of sample can be calculated from the number of cell counted and area counted. This is because the ruled areas of the chamber contain an exact volume of diluted sample. Since only a small volume of diluted sample is counted, a general formula must be used to convert the count into the number of cells/microliter.

$$\text{Particles per µl volume} = \frac{\text{Counted particles}}{\text{Counted surface (mm}^2\text{)} \cdot \text{Chamber depth (mm)} \cdot \text{Dilution}}$$

The dilution factor used in the formula is determined by the blood dilution used in the cell count. The depth used in the formula is always 0.1. The area counted will vary for each type of cell count and is calculated using the dimensions of the ruled area.

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Exp No: 2 Total RBC Count

Aim: Learn to count RBCs per µl (mm^3) using a haemocytometer.

Introduction: The Hemocytometer is a classic device used to measure cell numbers, particularly in blood samples. Counting is performed by introducing citrated (4% w/v sodium citrate (dihydrate), pH adjusted with citric acid, USP) blood into the counting region of the chamber. The height of the chamber is 0.1 mm. Using this we can estimate the volume occupied in the boxes marked R (for RBC). The total length of one side of 5 R-boxes is 1 mm. Using this measure and a mean count of cells in each R-box, we can estimate the number of RBC's in a unit of blood as follows: $C_{RBC} = \frac{\langle N_{RBC}^{R-box} \rangle \cdot 25}{V_{R25}} \cdot d_f$

CRBC= RBC count (cells/µl)

$\langle N_{RBC}^{R-box} \rangle$= Mean RBC-count from five R-boxes (usually the 4-corners and central)

VR25 = Vol. of the 5x5 RBC region in µl

df =Dilution factor

This count has been shown to vary between men and women. We will take one sample each to test this. Counting is done by eye.

When counting certain conventions need to be used. Cells at edges of a line are counted only in the L-shape, i.e. lower line and left-lines. This reduces over counting artefacts. Averaging over 4-5 R-boxes ensures in homogeneities in spreading or clumping of cells don't affect the final result.

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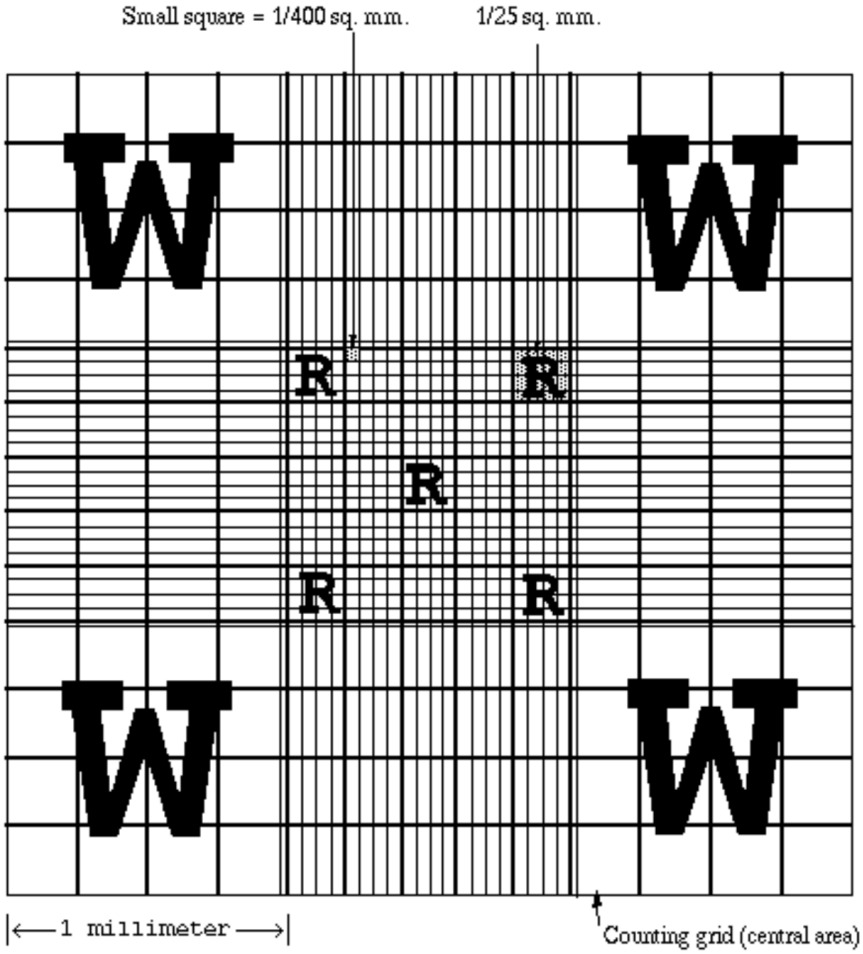

Top view of the chambers of a Neubauer's Hemocytometer for RBC (R) and WBC (W) counting with scales indicating sizes of each region. The height in the z-direction of the entire chamber is uniformly 0.1 mm.

Materials:

Biologicals:

1. Droplet of blood from capillary bleed

Glass/plastic ware:

1. RBC diluting pipette with hose and bulb

2. 50 ml beaker for waste material

3. Trash bin for lancets

4. Plastic droppers

Chemicals

1. RBC diluent 3.2 or 4% w/v Sodium Citrate ($Na_3C_6H_5O_7$)

2. A 2.5% bleach mixture for cleaning

3. 95% Alcohol for rinsing

Instruments

1. Sterile lancet

2. Hemocytometer for RBC and WBC counting

3. Microscope

Others

1. Tissue paper

2. Gloves for use while staining

METHOD

Counting RBCs (using blood donated to you)

1. As before make a pin-prick using a fresh unused lancet on the index- or ringfinger. If you have been pricked before on that finger, use another finger.

2. Bring a cleaned RBC dilution pipette tip close to the droplet of oozing blood. Using the bulb

allow (by capillarity and pressure) blood to enter up to the 0.5 mark.

3. Using a small tube of Sodium-Citrate solution, additionally aspirate this solution to reach the mark 101 (1:200 dilution).

4. Gently turn the dilution tube in your hand.

5. Introduce the diluted blood in the Hemocytometer.

6. Count as per the instructions in the finer grid.

Observations

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Exp No: 3 Hemagglutination (HA) Assay

AIM: To perform hemagglutination assay

Introduction: The hemagglutination assay is a method for titering influenza viruses based on their ability to attach to molecules present on the surface of red blood cells. A viral suspension may agglutinate the red blood cells, thus preventing them from settling out of suspension. By serially diluting a virus in a 96-well plate and adding a consistent amount of red blood cells, an estimation of the amount of virus present can be made.

Equipment and Materials Required

- Certified Biological Safety Cabinet
- Tabletop centrifuge with appropriate fittings
- Inverted microscope (optional)
- l conical tubes
- Disposable pipettes – 1 ml, 5 ml, 10 ml
- Micropipette and sterile disposable aerosol resistant tips – 160 µl
- PBS
- Turkey red blood cells in Alsevers solution purchased from a supplier such as Lampire Biological Products
- Round-bottomed 96-well dish

Turkey RBC preparation:

- 4 ml of turkey blood is pipetted into a 15 ml conical and topped off with PBS.

- Spin in tabletop centrifuge at 800 rpm for 10 minutes.
- Aspirate the supernatant without disturbing the blood cells.
- Add 12 ml PBS and mix by inverting – do not vortex.
- Spin at 800 rpm for 5 minutes and repeat wash two more times.
- Aspirate supernatant after final wash and add enough PBS to make a 10% solution of red blood cells. This solution is useable for one week.
- Make a final working solution of 0.5% RBCs in PBS.

Viral Dilution and Assay:

- A round-bottomed 96-well dish is preferred for this assay. Flat-bottomed plates will also work, but need to be placed at an incline to develop.
- To each well, add 50 µl PBS.
- In the first column, add 50 µl of virus sample.
- Mix each well and transfer 50 µl to the next well on its right. Repeat mixing and transferring 50 µl down the length of the plate. Discard 50 µl from the last well into a bleach solution.
- Add 50 µl of 0.5% red blood cell working solution to each well. Mix gently.
- Leave at room temperature for 30-60 minutes to develop. Negative results will appear as dots in the center of round-bottomed plates. Positive results will form a uniform reddish color across the well.
- The virus's HA titer is a simple number of the highest dilution factor that produced a positive reading.

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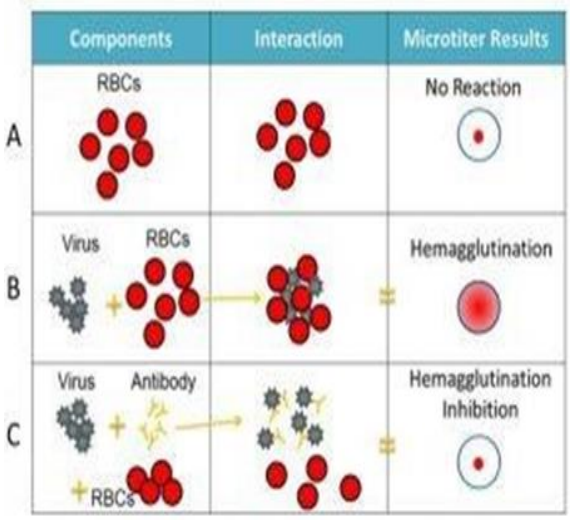
Exp No:4 Hemagglutination Inhibition Test (HAI)

AIM: To perform hemoagglutination assay

Principle: The nucleic acids of various viruses encode surface proteins that agglutinate the red blood cells (RBC) of a variety of species. For example; Influenza virus particles have an envelope protein called the hemagglutinin, or HA, which binds to erythrocytes, causing the formation of a lattice. This property is called hemagglutination. Reaction of viral hemagglutinins with red blood cells results in a lattice of agglutinated cells which settle irregularly in a tube or microtiter well. Unagglutinated cells settle in a compact button.



Hemagglutination and Hemagglutination Inhibition Test

Hemagglutination phenomenon is almost commonly used for diagnosis of infection produced by Orthomyxoviruses, paramyxoviruses, and the abroviruses-togaviruses (including rubella), flaviviruses, and bunyaviruses. The presence of virus in infected cell cultures can be detected by hemagglutination; the identity of the virus or of antibodies in a patient's serum can be determined by specific inhibition of that hemagglutination. Although influenza viruses can be detected by hemadsorption test, typing of the isolate is done most efficiently by hemagglutination inhibition (HAI). Reagents and conditions for the test vary by virus. The basis

of the HAI assay is that antibodies to that particular virus (for example-influenza virus) will prevent attachment of the virus to RBC. Therefore hemagglutination is inhibited when antibodies are present.

HAI Titer:

The highest dilution of serum (Ab) that prevents hemagglutination is called the HAI titer of the serum.

1. If the serum contains no antibodies that react with influenza virus, then hemagglutination will be observed in all wells.
2. Likewise, if antibodies to the virus are present, hemagglutination will not be observed until the antibodies are sufficiently diluted.

The HAI test may be complicated by the presence of non-specific inhibitors of viral haemagglutination and naturally occurring agglutinins of the erthrocytes. Therefore, the sera should be treated before use or false positive or negative results may arise.

Materials and Reagents:

1. Red cells from an appropriate species (Chicken, goose, guinea pig, trypsinized human O) collected in Alsever's solution or heparin
2. Diluent (e.g. Bovine albumin veronal buffer) at appropriate pH
3. Solutions to remove nonspecific hemagglutinins from serum
4. Infected cultural fluid or standard antigen (e.g preparation of influenza virus) for serology

Procedure

1. Obtain a preparation of virus (e.g. influenza viruses) with known HA titer or determine its HA titer
2. Prepare two-fold dilutions of patient/test serum to be tested e.g. from 1:4 to 1:1024.
3. Add a fixed amount of virus to every well of a 96-well plate, equivalent to 4 HA units

(varies according to virus), except for the serum control wells.

4. The plate is then allowed to stand at room temperature for 60 minutes (time varies according to specific requirements).
5. Add red blood cells (RBC) and incubate at 4^{o}C for 30 minutes.
6. Read the wells.

Results/interpretation

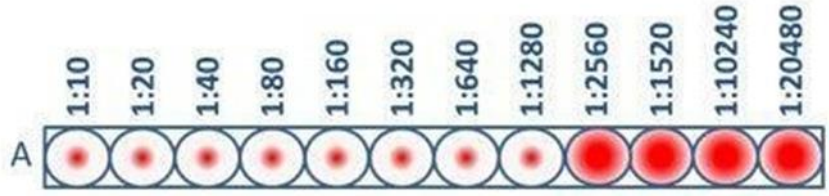
The highest dilution of serum (Ab) that prevents hemagglutination is called the HAI titer of the serum. A smooth or jagged shield of cells or an irregular button indicates agglutination.

Observation of movement of the button of red cells when the plate is tilted may help to clarify the end point.

This virus sample has an HAI titer of 1280, which means that the greatest dilution of antibody that still blocked hemagglutination from occurring was at 1280 dilution. At this dilution, the antibodies were still capable of recognizing and binding to the antigens on the virus.

Quality Control

- Known positive serum
- Known negative serum
- Serum and cells without antigen (to detect nonspecific agglutination)
- Back titration of hemagglutination activity of the antigen (to ensure that hemagglutinating virus (HAU) were tested).



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Exp No: 5 Separation of serum from whole blood

Aim: Effective Separation of blood products

Purpose: To standardize separating procedures so that research samples will be uniform in quality The decision to collect anticoagulated (plasma, buffy coat, RBC) or coagulated (serum, clot) blood samples must be made prior to collection so that proper blood draw tubes will be used.

Serum (needs clot time)

A serum separator tube (SST, tiger top tube). Let the blood sit for 30 minutes to one hour at room temperature to clot before spinning and separating. A delay in centrifugation may have a detrimental effect on the sample quality and may result inaccurate results. Avoid hemolysis.

Separating plasma (time sensitive)

Tube with an anti coagulant eg: Edta (lavender top)sodium heparin (green top), sodium citrate (blue top) are used for separating Plasma You need to spin and separate within one hour of receiving the specimen (time sensitive)

Note:

Universal Precautions must be used when working with blood. Use of personnel protective equipment is mandatory. Use of eye protection is mandatory unless blood tubes are being opened and serum/plasma/whole blood are being aliquoted inside a BL2 safety cabinet.

***Keep blood on wet ice and process within one hour of blood draw Separation of plasma**

1) Blood will be collected into purple top EDTA tubes and centrifuged (2000 rpm) at 4 degrees centigrade for 20 minutes.

2) After centrifugation using clean pipette technique place 1.0ml of plasma into 1.5ml eppendorf tube labeled with tracking number and “plasma”.

3) Freeze immediately at –80 degree freezer

Separation of Serum

- A 10 ml tube of whole blood will be collected following standard procedures using a serum separator tube (SST, tiger top tube) from each patient.
- Allow samples to clot for one hour at room temperature
- Centrifuge for 10 minutes at approximately 1000g
- Using clean pipette technique Aliquot 210ul of serum into labeled cryovials.
- Immediately freeze vials of serum at –80-degree freezer

Aliquoting whole blood

Whole blood will be aliquoted into sterile tubes upon receipt by carefully inverting the blood tube so that it is gently mixed before pipetting appropriate amounts (protocol specific) of whole blood into appropriate storage tubes using clean pipette tipps between each patient.

- Gently invert the tube of blood to mix contents
- Carefully open blood tube (universal precautions; gloves, eye protection)
- With clean pipette tipp aliquot appropriate amount of whole blood into clean/labeled storage tubes.
- Freeze in –80 freezer

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Exp No: 6 Double Immunodiffusion Ouchterlony

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: 1. Single Immunodiffusion 2. 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. A band 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.

Procedure:

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

2. Cool the solution to 55-60^{o}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.

3. Place the glass plate on the template provided.

4. 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.

5. Add 10 µl each of the antiserum and the corresponding antigens to the wells as shown in fig 2.

6. Keep the glass plate in a moist chamber overnight at 37^{o}C.

7. After incubation, observe for opaque precipitin lines between the antigen and antiserum wells.
X Y Z

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

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Exp No:7 Enzyme linked immunosorbent assay or 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.

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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.

9. Ensure that all the three zones of the strip are immersed in solution.

10. Assay buffer: Phosphate buffered saline – Tween (PBST).

Observation