18BTC106J- IMMUNOLOGY LAB MANUAL

B.TECH BIOTECHNOLOGY

IV SEMESTER

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIOENGINEERING
SRM INSTITUTE OF SCIENCE AND TECHNOLOGY
KATTANKUALTHUR -603202

Immunology laboratory

List of experiments

1. **Blood Grouping**
2. **Differential Leukocyte Count**
3. **Total Leukocyte Count**
4. **Widal Test**
5. **Rapid Plasma Reagin (RPR) Test**
6. **Single Radial Immunodiffusion (SRID)**
7. **Ouchterlony Double Diffusion**
8. **Rocket Immuno Electrophoresis**
9. **Counter Current Immunoelectrophoresis**
10. **Enzyme Linked Immunosorbent Assay (ELISA) – DOT**
11. **Enzyme Linked Immunosorbent Assay (ELISA) – Plate**
12. **Immunoprecipitation**
13. **Western Blotting**

1. ABO BLOOD GROUPING

Exp. No:
Date:

Blood group antigens

ABO system consists of three allelic genes A, B & O. The A & B genes control the synthesis of specific enzymes responsible for the production of the basic antigenic glycoprotein known as the H substance. The O gene is an amorph and cannot transform the H substance. There is no known specific anti O hence only four phenotypes are recognized.

Rhesus system

The Rh factor Ab was identified by Leving and Stetson. This is a complex system coded by allelic genes at three closely related loci, alternative antigen Cc, Ee, together with D or no D (recessive D) genes. So a person may inherit CDE from father and cde from mother and have a genotype CDE/cde. Genotype cde/cde is Rh negative and all other genotypes are Rh positive.

Blood group antibodies

Naturally occurring antibodies

They are present in the serum of individuals who last the corresponding antigen and who have not been transfused. Anti A and Anti B are most important, they are IgM immunoglobulins reacting optionally at room temperature (cold antibodies).

Immune Antibodies

They are produced in response to introduction of red cells processing Ag which person lacks, e.g. by transfusion or due to transplacental access. These are usually IgG Antibodies and react best at body temperature (warm Ab). Rh Ab, anti D is a very important immune antibody.

Antiserum of group A agglutinates A_1 cells powerfully but A_2 cells only weakly.

Agglutinins

These are two types i.e., anti A & anti B is also known as natural antibody (saline agglutinating). While anti B is called immune antibody (albumin agglutinating).

H antigens

RBCs of all ABO groups possess a common antigen, the H antigen which is the precursor for the formation of A and B antigen.

In 1900, Landsteiner first reported the presence of two antigen called A and B on the surface of human red blood cells. Based on this discovery, he divided human blood cells into three groups like A, B and O. Later in 1902, Decastell and Sturil recognized the existence of a

fourth group called AB. Anti A and Anti B monoclonal IgM agglutinating sera are produced by murine hybridoma cell lines grown in tissue culture. Each hybridoma produces a single antibody with well defined characteristics and these antibodies are used either singly or blended together with other monoclonal antibody to produce potent and useful reagents. Anti A and Anti B are monoclonal IgM antibodies directed against human red blood cell antigens A and B respectively.

About 95% individuals amongst Indian population and 85% of the individuals of Caucasian origin possess D (Rh) antigen on their erythrocytes. Human red blood cells are classified as "Rh-Positive" or "Rh-Negative" depending upon the presence or absence of this antigen on their surface. Anti D (Rh) Monoclonal is used for the detection of presence of Rh antigen on the red blood cells.

Anti D (Rh) specific Monoclonal antibody is produced by *in vitro* culture of a human lymphoblastoid cell line obtained after transformation of human B lympbocytes by the Epstein - Barr virus (EBV).

Principle

Human red blood cells possessing A and/or B antigen will agglutinate in the presence of antibody directed towards the respective antigen. Agglutination of red blood cells with Anti A Monoclonal, Anti B Monoclonal is a positive test result and indicates the presence of the corresponding antigen. Absence of agglutination red blood cell with Anti A Monoclonal, Anti B Monoclonal is a negative test result and indicates the absence of the corresponding antigen.

Human red blood cells possessing D antigen are agglutinated by the antibody, directed against D (Rh) antigen.

Sample

Blood collected with or without anticoagulant may be used. Carry out the tests as soon as possible after collection. Store the samples at 2-8^{o}C in case of any delay. Blood obtained by finger puncture may be tested directly by the slide method, but to avoid clotting, blood collected in this manner should be missed with the reagent quickly.

Precautions

1. Although the reagent contains a preservative, care should be taken to avoid extraneous microbial contamination.
2. The optimal temperature for carrying out blood grouping reaction is 25^{o}C ± 5^{o}C and the tests should not be carried out at 37^{o}C.

Procedure

A. Modified Tube Test (Don't write this in observation book)

1. Prepare 2-5% suspension of red blood cells in normal saline.

2. To a small test tube add one drop of Anti A Monoclonal.
3. To the second test tube add one drop of Anti B Monoclonal.
4. To the third test tube add one drop of Anti D (Rh) Monoclonal.
5. To each of the above test tubes add one drop of the prepared 2-5% cell suspension and mix well.
6. Centrifuge both tubes at 1000 rpm for 1 minute otherwise the test may be allowed to stand at room temperature of 25ºC ± 5ºC for 15-60 minutes.
7. Observe the tubes for the evidence of macroscopic or microscopic agglutination.

B. Rapid Slide Test. (Write this in observation book)

1. Prepare approximately 10%suspension of RBCs in normal saline. Alternatively, oxalated whole blood can be used.
2. Mark out three areas on a glass slide and label them as A, B and D.
3. Add one drop of corresponding reagents to the labeled areas.
4. Add one drop of the prepared cell suspension or oxalated blood to each of the three labeled areas.
5. With separate applicator sticks, mix each cell reagent mixture well.
6. Tilt the slide back and forth and observe the macroscopic or microscopic agglutination. Tests that fail to show agglutination within two minutes are considered negative.

Note

In both modified tube and rapid slide test, it is advisable to include samples of known A, B and D cells as controls with every batch to tests. Observe the control before reading the test samples.

Caution

Do not interpret peripheral drying or fibrin strands as agglutination.

Interpretation

1. Agglutination of red blood cells by the reagent indicates a positive test result.
2. Absence of agglutination of red blood cells indicates a negative test result.

Anti A	Anti B	Blood Group
+	-	A
-	+	B
+	+	AB
-	-	O

Anti D (Rh)	RH
+	+
-	-

Result:
The given blood sample is found to be

2. DIFFERENTIAL LEUKOCYTE COUNT

Exp. No:
Date:

Introduction

Differential leukocyte count is counting at least 100 cells (WBCs) and representing them in percentage of the cells seen.

Peripheral Blood film (PBF)

The peripheral blood films are of two types

1. Thin blood film (Write this in observation book)

It can be prepared from anti-coagulated blood obtained by vein puncture or from the free flowing blood by finger prick by a slide method.

Qualities of a good blood film

(1) It should not cover the entire surface of the slide.
(2) It should have smooth and even appearance
(3) It should be free from waves and holes
(4) It should not have irregular tail

Parts of a thin blood film

A peripheral blood film consists of 3 parts

(i) Head : It is the portion of blood film near the drop of blood.
(ii) Body : It is the main part if the bloody film
(iii) Tail : It is the main tapering end of the blood film

2. Thick Blood Film (Don't write this in observation book)

This is prepared for detecting blood parasites such as malaria and microfilaria

Various stains for PBF

Romanawsky stains are universally employed for staining of blood films. All Romonawsky combinations have essential ingredients that are methylene blue and eosin or azure.

Mehtylene blue is a basic dye and has affinity for acidic components of the cell like the nucleus.

Eosin/azure is the acidic dye and has affinity for basic component of the cell like the cytoplasm.

Most Ramonawsky stains are prepared in methyl alcohol so that thy combine fixation and staining.

Various stains included under Romanawsky stains are as under

(i) Leischman stain
(ii) Giemsa stain
(iii) Wright stain
(iv) Field stain
(v) Jenner stain
(vi) JSB stain

Normal values of leucocytes in healthy adults

S. No.	Leukocytes	Normal value	Absolute range
1	Polymorphs (P) (or) Neutrophils (N)	40-75%	2000-7500/µl
2	Lymphocytes (L)	20-50%	1500-4000/µl
3	Monocytes (M)	2-10%	200-800/µl
4	Eosinophils (E)	1-6%	40-400/µl
5	Basophils (B)	0-1%	10-100/µl

Materials required

Glass slide, blood sample, leischman stain, distilled water, microscope and etc.

Protocol

Preparation of PBF (slide method)

1. A drop of blood was placed in the centre of a clean glass slide, 1 to 2 cm from one end.
2. Another slide with a smooth edge (spreader) was placed at an angle of 30-35^{o} near the drop of blood.
3. The spreader was moved backward so that it made contact with the blood.
4. Then the spreader was moved. Rapidly over the slide.
5. A thin peripheral blood film was prepared.
6. It was then dried and stained.

Preparation of Leischman stain

1. 0.2g of powdered Leischmans dye was dissolved in 100ml of acetone free methyl alcohol in a conical flask.
2. It was warmed to 50^{o}C for half-an-hour with occasional shaking.
3. It was then cooled and filtered.

Staining

1. Leischmans stain was poured dropwise (counting the drops) on the slide and was kept for 2 minutes. This allows the fixing of PBF in methanol.
2. Double the quantity of buffered water was added dropwise on the slide.
3. It was mixed by rocking for 8 minutes.

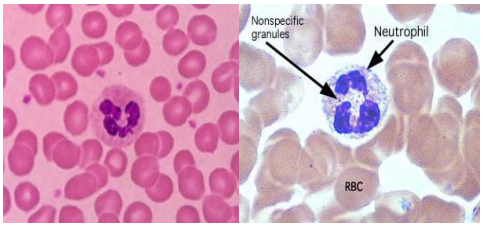
4. The slide was washed in water for 1 to 2 minutes.
5. It was air dried and examined under oil immersion.

Examination of PBF for DLC

An area was chosen near the junction of body with the tail of the smear where there is slight overlapping of RBC i.e., neither there was rouleaux formation which occurred in head and body nor there were totally scattered RBC as that occurred at the tail. By the movement of the slide in horizontal directions under oil immersion the counting of the types of WBC was done. The WBC were entered as P (Polymorphs), L (lymphocytes), M (Monocytes), E (Eosinophils) and B (Basophils) in a box having hundred cubes expressed in the sequence of P,L,M,E,B in percentage.

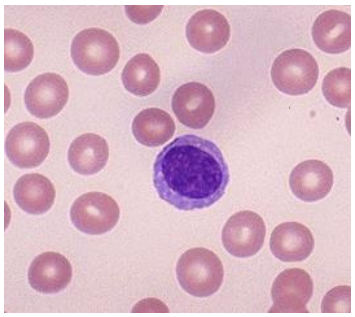
Morphological identification of Mature Leukocytes

Polymorphs (Neutrophils)



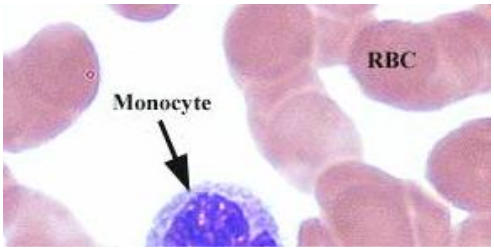
A PMN, commonly called polymorph or neutrophil is 12-15µm in diameter. It consists of a characteristic dense nucleus, having 2-5 lobes and pale cytoplasm containing numerous fine violet-pink granules.

Lymphocyte



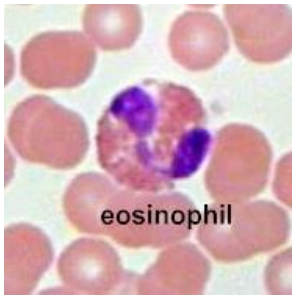
Majority of lymphocytes in the peripheral blood are small (9-12µm in diameter) called microlymphocyte, but large lymphocytes (12-16µm in diameter) called macrolymphocyte are also found. Both small and large lymphocytes have round or slightly indented with nucleus with coarsely clumped chromatin and a scanty basophilic and agranular cytoplasm.

Monocyte



The monocyte is the largest mature leukocyte in the peripheral blood measuring 12-20µm in diameter. It posses a large, central oval notched or indented or kidney shaped nucleus which has a characteristically fine reticulated chromatin network. The cytoplasm is abundant, pale blue and contains many fine granules and vacuoles.

Eosinophils



Eosinophils are similar to segmented neutrophil in size (12-15µm) in diameter, but have coarse, deep red staining granules in the cytoplasm and have usually two nuclear lobes in the form of a spectacle.

Basophil

Basophil resembles the other mature granulocytes but is distinguished by coarse, intensely basophilic granules which usually fills the cytoplasm and often overlaps the nucleus.

Feature	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Cell diameter	12-15µm	Small 9-12µm Large 12-16µm	12-20µm	12-15µm	15-15µm
Nucleus	2-5 lobed clumped chromatin	Large nucleus round to fill the cells clumped chromatin	Large, lobulated, undivided with fine chromatin	Bilobed, clumped, chromatin	Bilobed, clumped chromatin
Cytoplasm	Pink or violet Organization	Peripheral organisation of basophilic cytoplasm, no granules	Light, basophilic may contain fine granules or Vacuoles	Coarse crimson red granules	Large to purplish granules observed in the nucleus
Normal %	40-75	20-50	2-10	1-6	0-1
Absolute count per µl	2000-7500	1500-1400	200-800	40-400	10-100

Result

The given blood sample has

3. TOTAL LEUKOCYTE COUNT

Exp. No:
Date:

Principle

A sample of whole blood is mixed with a weak acid solution that lyses nonnucleated red blood cells. Following adequate mixing, the specimen is introduced into a counting chamber where the white blood cells (leukocytes) in a diluted volume are counted

Procedure

(1) Draw well-mixed capillary or venous blood exactly to the 0.5 mark in a white blood cell diluting pipet. This blood column must be free of air bubbles.
(2) Wipe the excess blood from the outside of the pipet to avoid transfer of cells to the diluting fluid. Take care not to touch the tip of the pipet with the gauze.
(3) Immediately draw diluting fluid (3% acetic acid solution) to the "11" mark while rotating the pipet between the thumb and forefinger to mix the specimen and diluent. Hold the pipet upright to prevent air bubbles in the bulb.
(4) Mix the contents of the pipet for 3-5 minutes to ensure even distribution of cells. Expel unmixed and relatively cell-free fluid from the capillary portion of the pipet (usually 4 drops).
(5) Place the forefinger over the top (short end) of the pipet, hold the pipet at a 450 angle, and touch the pipet tip to the junction of the cover glass and the counting chamber.
(6) Allow the mixture to flow under the cover glass until the chamber is completely charged. Similarly, fill the opposite chamber of the hemacytometer.
NOTE: If the mixture overflows into the moat or air bubbles occur, clean and dry the chambers, remix the contents of the pipet, and refill both chambers.
(7) Allow the cells to settle for about 3 minutes. Under low-power magnification and reduced light, focus on the ruled area and observe for even distribution of cells.
(8) Count the white cells in the four 1 sq mm corner areas corresponding to those marked A, B, C, and D of Figure 1 in each of two chambers.
(9) Count all the white cells lying within the square and those touching the upper and right-hand center lines. The white cells that touch the left-hand and bottom lines are not to be counted. In each of the four areas, conduct the count as indicated by the "snake-like" line in figure 5-1. A variation of more than 10 cells between any of the four areas counted or a variation of more than 20 cells between sides of the hemacytometer indicate uneven distribution and require that the procedure be repeated.

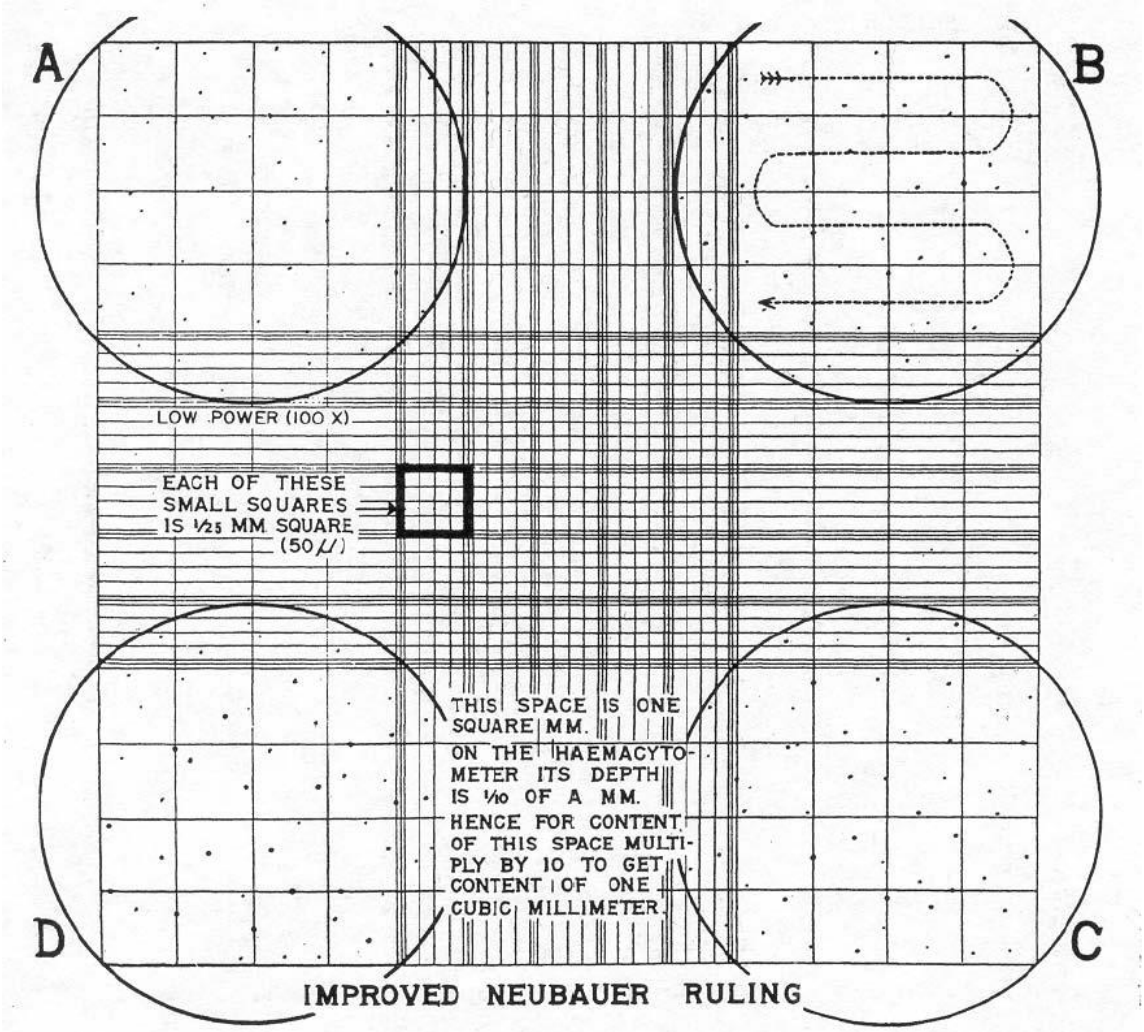


Figure 1. Hemacytometer counting chamber (WBCs). Areas marked A, B, C, and D are used to count white blood cells.

Calculation

(1) Routinely, blood is drawn to the 0.5 mark and diluted to the 11 mark with WBC diluting fluid. All the blood is washed into the bulb of the pipet (which has a volume of 10). Therefore, 0.5 volumes of blood are contained in 10 volumes of diluting fluid. The resulting dilution is 1:20. (These figures are arbitrary and refer strictly to dilution and not to specific volumetric measurements.)

(2) The depth of the counting chamber is 0.1 mm and the area counted is 4 sq mm (4 squares are counted, each with an area of 1.0 sq mm therefore, 4 x 1.0 sq mm = a total of 4 sq mm). The volume counted is: area x depth = volume. Four sq mm x 0.1 mm = 0.4 cu mm.

(3) The formula is as follows:

$$\text{WBCs per cu mm} = \frac{\text{Average number of chambers (2) WBCs counted x dilution (20)}}{\text{Volume (0.4)}}$$

(4) For example:

First Chamber Cells counted in each square	Second Chamber Cells counted in each square

35	45
40	37
44	36
39	44
158 WBCs counted	162 WBCs counted

Calculate the average number of WBCs per chamber:

$$\text{Ave num WBCs} = \frac{158+162}{2} = 160$$

Calculate the number of WBCs per cubic mm:

$$\text{WBCs per cu mm} = \frac{160 \times 20}{.4} = 8000$$

Result:
The given blood sample has …….. WBCs per cu mm

Normal Values
(1) Adults (both sexes): 4,500-11,500 WBCs per cu mm.
(2) Childhood: 6,000-14,000 WBCs per cu mm.
(3) Birth: 9,000-30,000 WBCs per cu mm.

Discussion

4. WIDAL Test

Exp. No:
Date:

Introduction

The WIDAL test is a serological technique which tests for the presence of Salmonella antibodies in the patients serum when facilities for culturing of antigen testing are not available, the WIDAL test is performed for reliability and interpreted with care. It can be of value in diagnosing typhoid and paratyphoid in endemic areas. It is of no value in the investigation of Salmonella in food poisoning when the investigation is carried out for typhoid, the patients serum is tested for 'O' (somatic) and 'H' (flagella) antibodies (agglutinins) against the following antigen for O and H suspensions (stained suspensions)

Salmonella typhi 'O' antigen
Salmonella tyhpi 'H' antigen
Salmonella paratyphi 'AH' antigen
Salmonella paratyphi 'BH' antigen

Testing for Paratyphoid

The following antigen suspensions are required

Salmonella paratyphi 'AO' antigen suspension
Salmonella paratyhpi 'AH' antigen suspension
Salmonella paratyphi 'BO' antigen suspension
Salmonella paratyphi 'BH' antigen suspension
Salmonella paratyphi 'CO' antigen
Salmonella paratyphi 'CH' antigen

In enteric fever, specific agglutinins are usually detectable in the patient's blood after 6 days of fever. Serum from individuals vaccinated with TAB may also show moderately elevated titre of all three H agglutinins.

Stained Salmonella Antigens are used to detect, identify and quantitate specific antibodies in serum sample from patients suffering from Pyrexia of Udermined Origin (PUO). These antigens are standardized, smooth suspensions of killed bacilli which have been stained to facilitate reading of agglutination. They are suitable to be used in the standard WIDAL tube test as well as in the rapid slide test. They may also be used as control antigens while carrying out serological identification of bacterial isolates from clinical specimens.

Principle

Antibodies in serum, produced in response to exposure to Salmonella organisms will agglutinate bacterial suspension which carries homologous Antigens.

Sample

Fresh serum, store at 2-8^{o}C in case of any delay in testing. Serum should be clear and should not be heated or inactivated.

Precautions

1. Bring all the reagents to room temperature before use.
2. Use clean and dry glassware.
3. For greater proficiency in the interpretation of test result, always include positive and negative control sera.
4. Serum samples to be tested should be clear and free from any bacterial contamination.
5. Do not inactivate sera prior to testing.
6. Shake antigen vials well before sue, to make the suspension homogenous.
7. Very rarely reagent may show fibric structure (appearance like fungus), which will not interfere in the specificity and sensitivity of the test performance.

Procedure

A. Rapid slide (Screening) Test. (Write this in observation book)

1. Clean the glass slide and wipe it free of water.
2. Place one drop of undiluted test serum in each of the 1st four circles (1-4) and one drop of positive control serum in each in each of the last two circles (5&6).
3. Place one drop of antigens O, H, A (H) and B (H) in circle 1, 2, 3 and 4 respectively and O antigen in circle 5 and any one of the H antigens H, A (H) or B(H) in circle 6.
4. Mix the contents of each circle with separate wooden applicator stick and spread to fill the whole area of the individual circle.
5. Rock the slide for one minute and observe for agglutination. If agglutination is visible within one minute, proceed for quantitative slide or tube test or for the quantitative estimation of the titre of the appropriate antibody.

B. Quantitative Slide Test. (Don't write this in observation book)

1. Clean the glass slide and wipe it free of water.
2. Place 0.005 ml, 0.01ml, 0.02ml, 0.04ml & 0.08ml of undiluted serum in 1st, 2nd, 3rd, 4th and 5th circles respectively on the slide.
3. Add one drop of the appropriate antigen suspension which showed agglutination in screening slide test, to each of the above circles.
4. Mix the contents of each circle with separate applicator stick.
5. Rotate the slide slowly for one minute and observe for agglutination
6. The titer of the antibody is the highest dilution of serum up to which there is clear agglutination.
7. Repeat steps 1 through 6 with all the antigens, which showed agglutination in screening slide test.

The serum volumes in the quantitative slide test correspond approximately to the tube test titre as:

Serum Volume in ml	Approximate Tube Test Titre
0.08	1:20
0.04	1:40

0.02	1:80
0.01	1:160
0.005	1:320

Quality Control Procedure

The use of positive control serum, negative control serum and saline control in parallel with unknown test serum is recommended to assure the laboratory worker that the antigens used in the test area sensitive as well as specific and to show what results are to be expected in positive and negative specimens.

Result

The given ……. sample is found to be positive / negative for

Interpretation

Agglutinin titre greater than 1:80 is considered significant and usually suggestive of infection, whereas low titres are often found in normal individuals. A single positive result has less significance as a definite evidence of infection. A moderate simultaneous rise in the titre of all three H agglutinins is suggestive of recent TAB vaccination.

5. RPR TEST

Introduction

Syphilis is a chronic, contagious, veneral disease caused by *Treponema pallidum*. After infection, two main types of antibodies are produced in the host. (First antibody – Reagin antibody, second antibody –Treponemal antibody).

Reagin antibodies are produced more rapidly than Treponemal antibodies. Serological testing for diagnosis of Syphilis is based upon the detection of reagin type of antibody using Cardiolipin antigen.

Syphilis is usually transmitted through genital organs, placenta and blood transfusions. Hence, it is very essential to test every pregnant woman and blood donor, besides routine suspected cases of Syphilis. Antibiotic therapy inhibits the production of regain antibody and hence, the test also has prognostic importance during medication. RPR test is the modification of VDRL test (Venereal Disease Research Laboratory).

The RPR (Rapid Plasma Reagin) Test is a non-treponemal test for the serological diagnosis of syphilis. Specificity, reactivity and sensitivity are similar to that of classical VDRL Test. It is a reliable, economical and rapid test which is easy to read without the help of microscope. It is highly reproducible and hence it is recommended for the use as a screening test.

Principle

RPR antigen suspension is a carbon-containing cardiolipin antigen, which detects 'reagin' antibody present in serum of syphilitic individuals. 'Reagin' is occasionally present in serum of persons with other acute or chronic conditions. When a specimen contains antibody, flocculation occurs due to co-agglutination of the carbon particles of the RPR antigen, which appear as black clumps against the white background of the card. This co-agglutination is read macroscopically. Non-reactive specimens show an even light grey colour.

Sample

Serum or plasma 50-100microliters EDTA may be used as anticoagulants. While excess of EDTA or Heparin does not normally interfere with the test, it is advisable to avoid excess of oxalates during blood collection.

Reagent/accessories

RPR antigen suspension, Positive Control Serum and Negative Control Serum, Disposable Plastic Cards, Disposable Plastic Droppers, Disposable Applicator Sticks, Antigen Delivery Dropper (for delivering a drop of approximately 15-20 microliters) and rubber teats.

Precautions

1. Allow all the reagents to attain room temperature before use.
2. Improper mixing of the sample with the reagent may lead to erroneous result.

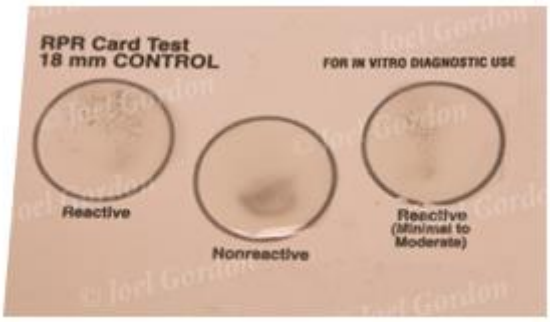
3. The card must be rocked gently, since vigorous rocking may impair agglutination.
4. Drying of reagents on the card may also lead to erroneous results.
5. Discard hemolyzed or contaminated samples.
6. Do not use an excess of anticoagulants, such as potassium oxalate or sodium fluoride which can lead to unreliable results.
7. RPR Test results should be read immediately after rotation of the card under a high intensity lamp or strong day light.

Protocol

1. Place a drop of serum or plasma (50 microliter or 0.05ml) on the card with the help of disposable serum dropper on to RPR test card. Spread to cover the entire circle.
2. Using the dispensing bottle/needle assembly allow one free falling drop (16ul) of antigen to drop on to the test specimen (Do not restir).
3. Rotate the test card at 100rpm for 8 minutes.
4. Immediately after the 8 minutes, inspect the result visually in a good light.

Interpretation of test results

Read the result under a strong source of light with a hand lens. Regardless of the degree of reactivity and test result showing slight but definite clumping is reported as reactive or positive.



Positive result

Black aggregates (Carbon) which may be deposited at the periphery of the liquid appearing before the 4 minutes of rotation.

Negative result

Complete absence of black aggregates with a uniform grayish background at the end of 4th minute rotation.

Limitations

1. The test described here is primarily valid as a screening procedure. It is advisable to confirm positive samples with other methods employing specific treponemal antigen e.g. TPHA, FTA-ABS, etc.

2. This method may give false positive results in the presence of diseases such as leprosy, malaria, toxoplasmosis, infectious mononucleosis or lupus erythematosus or if the sample is having high degree of bacterial contamination.

6. SINGLE RADIAL IMMUNODIFFUSION

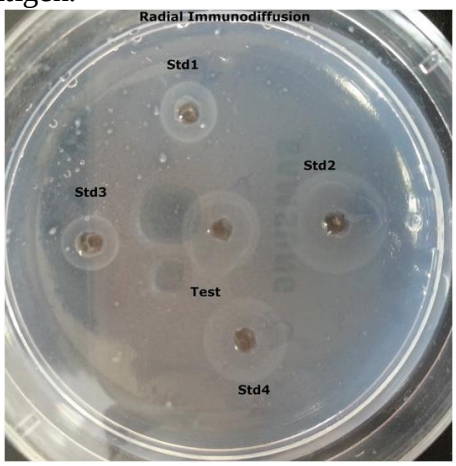
Introduction

In Single immunediffusion two dimensions (radial immunodiffusion) involves incorporation of one reactant, an antibody, into gel that is subsequently poured into a flat plate. Addition of corresponding antigen (reactant), by placing it in a circular well cut in the gel medium, produces an advancing circular concentration gradient. As the molecule move outward from the point of application, precipitate stabilizes in the form of a precipitation ring, which forms at the site of approximate equivalence.

The single radial immuno diffusion (SRID) is a simple but sensitive method for quantitating the antigens. It has been employed for estimation of immunoglobulin classes in the serum. SRID test uses antibody incorporated in to the agarose and placing the antigen in the wells cut the agarose. This is comparatively more sensitive than agar gel immuno diffusion tests.

PRINCIPLE

The test is based on the principle of antibody incorporated into the agarose and placing the antigen in the wells. Antigen diffuses radially, forming a ring of precipitation around the well and moves outwards eventually becoming stationary at equilence. At equivalence, the diameter of the ring is related to the antigen concentration in the well. Using standard antigen concentrations, a calibration curve may be constructed to determine the unknown concentrations of the same antigen.



MATERIALS REQUIRED

S.No	Materials	Storage
1	Antigen	-20°C
2	Antibody	-20°C
3	Test antigen	-20°C
4	Agarose(Low EEO)	RT
5	Normal Saline	RT
6	Glass Slide(75mmX50mm)	RT
7	Gel punch	RT
8	Template	RT

1. Glassware's (conical flask, Measuring cylinder, Pipette)
2. Micropipette,Tips
3. Distilled water
4. Glass slide

PRECAUTIONS

- Use agarose having Low Electro Endosmosis (EEO)
- Do not add antiserum while the molten agarose is hot since the antibodies may denature under excessive heat.
- Use separate microtips for different reagents.
- Avoid overflowing or bubbling while filling up the wells.
- Reagents contain 0.01% sodium azide as preservative
- Discard all used glassware property.

PROTOCOL

NOTE: All the micropipettes should be well calibrated and the pipetting should accurate to get optimum results.

A. PREPARATION OF AGAROSE GEL PLATES

1. Prepare1.0% agarose solution with the saline provided. Heat the solution in a boiling water bath until agarose dissolve completely and no obvious particles of agarose should remain in suspension.
2. Cool the molten agarose to bearable warm and mix 100µl of the antiserum with around 10ml of molten agarose and pour 9ml onto a microscopic slide provided that kept on lavelled surface. The agarose would be about 3-4 mm thick. Avoid spillage, even a little spillage cause the whole agarose get drawn from the slide due to capillary action.
3. The agarose slides can be stored 4°C until it is used immediately.

B. CUTTING WELLS IN THE AGAROSE

1. Using the template provided, cut six wells three on top and three below to that with the gel punch. The well would be 3mm in diameter and 14mm from the edge of the slide.
2. Cut the well in agarose after it has hardened sufficiently. Before cutting the wells keep the slides at 2-8°C for a short period, So that the cut edges of the wells do not break down when the agarose plugs are removed.
3. The agarose plugs can be removed from the wells using a fine gauge glass or metal cannula attached to a vacuum line. Alternatively 20-gauge hypodermic needle can be inserted in to the plug to lift it free of the agar.

C. FILLING WELLS

1. Add 10µl of neat antigen (undiluted) to the first well using a micropipette and mark it as 1.
2. The antigen can be diluted two fold and add 10 µl to the remaining 4 wells respectively. Take 50µl of normal saline in 4 wells of a 96 well microtitre plate. Add

50µl of antigen to the first well of the microtitre plate, mix thoroughly then serially diluted into another wells. Now you have antigen diluted to 1:2, 1:4, 1:8 and 1:16 in four wells of the 96-well microtiter plate.50µl of diluted antigen from the last (fourth) well of the microtiter plate can be discarded.

3. Now add 10 µl of the four diluted antigen to the 2nd, 3rd, 4th and 5th wells respectively using a micropipette. This amount will fill the well without allowing it to overflow into the adjacent well.
4. Add 10 µl of test antigen in the 6th (last) well.
5. After the wells are filled keep the plate in a flat-bottomed container such as a plastic breadbox with a tight fitting cover. The interior of the container should be kept moist by keeping a damp paper towel or wet cotton.
6. The slides can be held at room temperature or at 37°C in an incubator. Lower temperatures may show the formation of precipitin lines and prolong the test. Allow the plate to stand undisturbed in the humid box at room temperature for 8-12 hours.

D. READING THE SINGLE RADIAL IMMUNO DIFFUSION TEST

1. Observe the slides after 24 hours. In most of the cases the reaction would have reached a point where the results can be recorded by this time. In some cases, when weak positive samples are involved it is recommended that the slides can be held for an additional 24 hours to observe the result.
2. A high intensity light source or narrow beam of light is used to examine the precipitin reaction. Also it is useful to view the reaction against a dark background.
3. The diameter of the precipitin ring will vary with the concentration of antigen in the standard or sample.

E. DETERMINATION OF ANTIGEN CONCENTRATIONS

1. Ring diameter of standards (concentration of undiluted and diluted antigen) and samples are measured using a clearly marked scale. For the purposes of calculation, each ring diameter is measured twice. The resulting average value is used as the basis for calculating antigen concentration.
2. Using the measured ring diameter of standard solutions, draw a standard curve against antigen concentration

F. RESULT

The concentration of given (T_1 or T_2) test sample is ……..

G. INTERPRETATION

The quantity of the antigen can be established form the standard curve or software provided if standard antigens with known concentrations are used in the test along with samples with unknown antigen concentrations. This test is widely used to assess concentration of immunoglobulin classes in serum using known antibody against each of the immunoglobulin classes incorporated in the agarose gels.

7. OUCHTERLONY DOUBLE DIFFUSION - PATTERN

Introduction

Ouchterlony Double Diffusion technique is widely used for characterization and quantitation of antigens or antibodies. This technique is based on the ability of antibody to from complexes with specific antigen. This is a highly versatile technique, useful for identifying and characterizing precipitating antigen-antibody systems. In this method, arranging wells equidistant around a centre well allows the placement of decreasing concentrations of one component (e.g. antibody) around the radially diffusing central well and makes tittering or determining a relative strength for antibody solution possible.

This method is also employed to compare different antigens and antisera (antibody) directly. If the antigens in adjacent wells are identical, the line of precipitation formed will fuse smoothly at the junction. This is called 'Line of Identity' or line of fusion. If the antigens in adjacent wells are unrelated, the lines will cross each other. This is called 'Line of non-identity' or 'line of intersection'. If the antigens in adjacent wells are partially related, there will be a spur formation i.e., when one antigen and antibody form a homologous pair, and the other antigen cross reacts fusion of two lines still occurs but a spur forms pointing towards the cross reacting antigen, representing the reaction between homologous antigen and those antibodies that do not combine with cross reacting antigen. This pattern is known as 'line of partial identity'. The AGPT requires 10-12 hours for yielding a result.

Principle

In Ouchterlony Double Diffusion technique a free diffusion of antigen and antibody takes place in the gel, resulting in the formation of precipitin band. This can be visualized by the naked eye. While immunodifussion tests are among the least difficult serological tests to perform, it is still important that all the steps of the procedure be done with precise and careful techniques.

Materials required

Antigen A, B & C, Antibody I, II & III, Agarose (low EEO), Glass Slide, Gel Punch, Template, Normal saline, Distilled water, Conical Flask, Measuring cylinder, pipette, Micropipette, tips. Etc.

Precautions

- Use agarose having low electro endosmosis
- Use separate microtips for different reagents.
- Avoid overflowing or bubbling while filling up the wells.
- Reagents contain 0.01% sodium azide as preservative.
- Discard all used glassware properly.

Protocol

Preparation of agarose gel plates

1. Prepare a 1.0% solution of agarose in the normal saline provided. Heat the solution in a boiling water bath until the agarose is completely dissolved and no obvious particles or agarose remain in suspension. Cool the agarose solution to around 50^{o}C.
2. By using a glass pipette, add about 4.5 ml of molten agarose onto a glass slide kept on a leveled surface. The agarose would be about 3-4mm thick. Avoid spillage, even alittle spillage cause the whole agarose get drawn from the slide due to capillary action.
3. The agaorse slides can be stored at 4^{o}C unless it is used immediately.

Cutting wells in the agarose

1. Using the template provided cut three sets of wells in agarose gel with gel punch, after the agarose has hardened sufficiently.
2. Before cutting the wells keep the slides at 2-8^{o}C for a short period, so that the cut edges of the wells do not break when the agarose plugs are removed.
3. Label the wells (first set) with marker as antibody I top well Antigen A and Antigen A in the bottom two wells. In the second set Antibody II in the top, Antigen A and Antigen B in the bottom wells. In third set Antibody III in the tip and Antigen A and Antigen C in the bottom wells.
4. The agar plugs can be removed from the wells using a fine gauge glass or metal cannula attached to a vacuum line. Alternatively 20–gauge hypodermic needle can be inserted in to the plug to lift it free of the agar.
5. Optimal results are obtained if reagents are added to the wells just after the plugs are removed.

Filling wells and incubation of agarose slides.

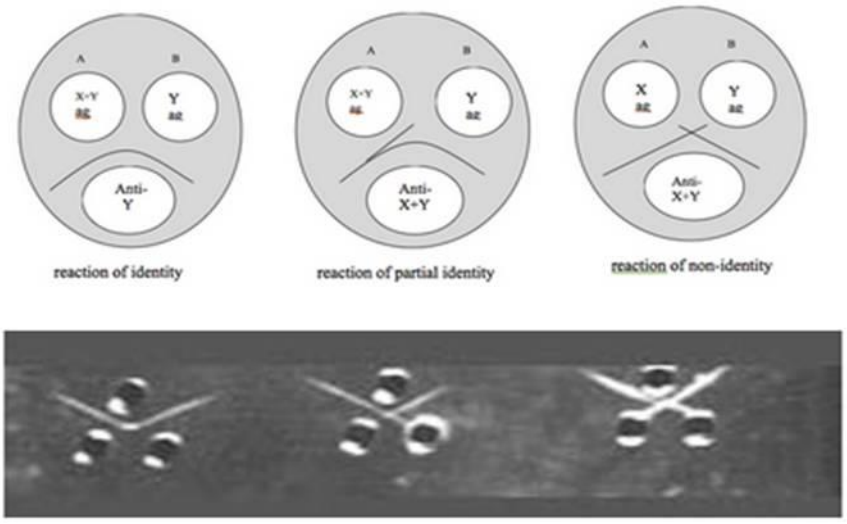
1. Add 10 microliters of Antibody I and 10 microliters of Antigen A to their respective wells in the first set.
2. Add 10 microliters of Antibody II and 10 microliters of Antigen A and 10 microliters of Antigen B in to their respective wells in the second set.
3. Add 10 microliters of Antibody III and 10 microliters of Antigen A and 10 microliters of Antigen C in to their respective wells in the third set.
4. Each well should be filled with 10microliters of reagent. This amount will fill the well without overflowing into the adjacent well.
5. After the well are filld kep the slide in a flat bottomd container such as a plastic bread box with a tight fitting cover. The interior of the container should be kept moist by keeping a damp paper towel.
6. The slides can be held at room temperature for 18 hours. Lower temperatures may slow the formation of precipitation lines and prolong the test.

Reading the immuno diffusion test

1. The slides can be examined after 24 hours. In most of the cases the reaction would have reached a point where the results can be recorded by this time. In some cases, when weak positive samples are involved it is recommended that the slides can be held for an additional 24 hours and reexamined especially.
2. A high intensity light source or narrow beam of light is used to examine the precipitin reaction. Also it is useful to view the reaction against a dark background.

3. The type of reaction (precipitin line) will vary with the concentration of antibody added.

Results



1. Reaction of Identity: This occurs between identical antigenic determinants, th lines fuse to give one continuous arc.
2. Reaction of Partial Identity: This has two components. Those antigenic determinants which are common to both antigens give a continuous line of identity.
   The unique determinants are recognized on one of the antigens given. In addition, a line of non-identity as like a spur is formed.
3. Reaction of Non Identity: In this two antigens do not contain any common antigenic determinants. The two lines are formed independently and cross without any interaction.

8. ROCKET IMMUNO ELECTROPHORESIS

Introduction

Rocket immuno electrophoresis (RIE) has the need for antigen to be negatively charged for electrophoretic movement within the agar matrix. Some proteins (immunoglobulins) are not sufficiently charged to be quantitatively analyzed by RIE nor is it possible to measure the amounts of several antigens in a mixture at the same time. In RIE, antigen migrates in an electric field from a well into layer of agarose that contains antibody. A series of wells for antigen samples is cut along one side of a glass plate that is coated with agarose containing specific antibodies.

The concentration of antigen in a mixture of antibodies can be determined if purified specified antibody for that antigen under study is available, its concentration can be evaluated with antiserum of mixed specificities. As the antigen migrates and encounters antibody molecules, antigen-antibody complexes form and precipitate. At leading boundary of antigen migration, antigen is in excess. As the excess migrates into area of precipitation, complex is dissolved to reform farther along the path of antigen migration. Along the outside edges of the migration path, excess antibody is present and lines of precipitation do not dissolve but persists. Eventually, all the excess at moving front is complexed with antibody and lines of precipitation converge to form a rocket like shape. After the point is reached, lines of precipitation remain stationary regardless of how electrophoresis is continued. Height or area of rocket is more accurately directly proportional to concentration of the antigen.

This technique is basically similar to single radial immunodiffusion test except that there is considerable increase in sensitivity by passing the current. Quantitative estimation of antigen is possible. However, in contrast to agarose gel immunodiffusion or counterimmunoelectrophoresis, this test is mostly used to quantify the antigen concentration rather than qualitative detection of antigen or antibody in the sample.

Principle

The antibody is incorporated in the agarose gel. The antigen is allowed to migrate towards anode, while antibody migrates towards cathode under the influence of electric current. Soluble complexes are formed at first, excess antigen migrates further, eventually equivalence is reached and an insoluble precipitate is formed. A stable arc is formed when no more antigens remains to either the precipitation, which becomes stationary. Rocket shapes of precipitation are formed, the rocket in proportional to the concentration of antigen. For quantitative reproaching measurements, rocket heights of 1 to 5cm are recommended.

Materials

Barbitol buffer (Veronal buffer) pH 8.6

- Tris 17.7g
- Barbitone sodium 9.9g

- Sodium acyl 0.3g
- Distilled water 2000ml
- pH adjusted to 8.6

Antigens
Antibodies
Test antigens
Agarose (EEO-high)
Glass slide – 40mmx40mm
Gel punch
Template
Electrophoresis apparatus with power pack
Glass wares
Micropipette with tips

Protocol

Note: All the micropipettes should be well calibrated and the pipetting should be accurate to get optimum results.

A. Preparation of agarose gel plates

1. Prepare 1.0% solution of agarose with the buffer provided (Running Buffer 1x). Heat the solution in a boiling water bath until the agarose is completely dissolved and no obvious particles of agarose remain in suspension.
2. Cool the molten agarose to bearable warmth and mix 75 microliters of the antiserum with around 5ml of molten agarose and pour 4.5ml onto a glass microscopic slide that kept on a leveled surface. The agarose would be about 3-4 mm thick. Avoid spillage, even a little spillage cause the whole agarose get drawn from the slide due to capillary action.

3. The agarose slides can be stored at 4^{o}C unless it is used immediately.

B. CUTTING WELLS IN THE AGAROSE

1. Using the template provided, cut five wells at one end of the slide (1.5cm away from the edge) with athe gel punch. The wells are 3mm in diameter and 5mm interspace between the wells.
2. Cut the well in agarose after it has hardened sufficiently. Before cutting the wells keep the slides at 2-8 C for a short period. This gives a clean cut edge of the wells which won't break down when the agarose plugs are removed.
3. The agarose plugs can be removed from the wells using a fine gauge glass or metal cannula attached to a vacuum line. Alternatively 20-gauge hypodermic needle can be inserted in to the plug to lift it free of the agarose.
4. If plates are not used immediately after they are cut, it will be necessary to remove the moisture that tends to accumulate in the bottom of the wells.
5. Optimal results are obtained if reagents are added to the wells just after the plugs are removed.

C. FILLING WELLS AND ELECTROPHORESIS

1. Add 10µl of neat antigen (undiluted) to the first well using a micropipette.
2. The antigen can be diluted two fold and loaded to the other wells. Take 50µl of normal saline in 4 wells of a 96 well micortitre plate. Add 50µl of antigen to the first well of the microtitre plate, mix thoroughly then serially diluted into another wells. Now you have antigen diluted to 1:2, 1:4 and 1:8 in the three wells of the 96-well microtitre plate. 50µl of diluted antigen from the last well of the microtiter plate can be discarded.
3. Now add 10µl of the four diluted antigen to the 2^{nd}, 3^{rd} and 4^{th} wells respectively using a micropipette. This amount will fill the well without allowing it to overflow into adjacent well.
4. Add 10µl of test antigen to the 5^{th} well.
5. After the wells are filled keep the slide on the Electrophoresis Bridge of the electrophoresis tank that has been filled with the running buffer provided (use 1x Running Buffer). The antigen containing wells should be placed on the cathode (negative side) of the electrophoresis tank (Black terminal).
6. Connect the slide with filter paper strips (wicks) with the respective chambers of the tank.
7. Electrophoresis at 8-9 mA or 50-100 Volts for 45 minutes to one hour. Wet the wicks with the buffer in the tank frequently after every 10 minutes.

D. READING THE COUNTER IMMUNOELECTROPHORESIS TEST

1. A high intensively light source or narrow beam of light is used to examine the precipitin lines. Also it is useful to view the reaction against a dark background.
2. The slides can be examined immediately after the run. A precipitin line in the shape of a rocket will be seen at some height over the wells loaded with the antigen.
3. The height of the rocket will vary with the concentration of antigen present in the sample.

Optional: If the rockets are not clearly visible, dip the electrophoresed slide in 4% enhancer solution. Add about 30ml of the enhancer solution into a 100mm petridish and keep the slide for 5-10 minutes to observe the rockets more clearly. After enhancer treatment, in addition to rockets, other uncomplexed antigen or antibody may also be seen as a thick ‘smudgy’ white precipitate near the wells. This enhancer treatment is only temporary and is unable to store the slides.

E. **Results and Interpretation**

1. The presence of a rocket-shaped precipitin line indicates that the antigen added in the wells has reacted with (specific to) the antibody incorporated in the gel. This test is mostly used to quantify the antigen concentration in the sample.
2. The antigen concentration is proportional to the height of the rockets observed. The height of the rocket in the diluted antigen-loaded well would be lower than the height seen in undiluted (neat) antigen well.
3. To measure the heights of the rocket, to determine the test antigen concentration compare with standard antigen rocket heights.

Note: Standard Antigen Concentration (undiluted) is 20mg/ml.

9. Counter Current Immunoelectrophoresis

Introduction

Counter Current Immunoelectrophoresis is a simple and sensitive method. It takes less than one hour for a complete run. It is otherwise called as one-dimensional / double electro immunodiffusion, which are used frequently in clinical laboratory.

In developing countries, this technique is extensively used for detecting the presence of hepatitis associated antigen (HAA) in the donor's blood collected for transfusion.

It is the same as immunodiffusion technique but the rates of migration of the antigen and the antibody are expedited by placing the antigen (test specimen containing viral antigen) and the antibody opposite the poles towards which they are attached (antigens migrates towards anode and antibody towards cathode). Counter immunoelectrophoresis is a modification of the Ouchterlony method (passive immunodiffusion) that speeds up migration of an antigen and antibody by applying an electric current (active immunodiffusion). Most bacterial antigens are negatively charged in a slightly alkaline environment, whereas antibodies are neutral. This feature of bacterial antigens is exploited by counter current immuno electrophoretic assays in which solution of antibody and sample fluid to be tested are placed in small wells cut into slab of agarose on a glass surface. A paper or a fibre wick is used to connect the two opposite ends of agarose to troughs of slightly alkaline buffer, formulated for each Antibody-Antigen system. When an electric current is applied through the buffer, the negative antigen moves towards positive electrode and therefore towards the well filled with antibody. The neutrally charged antibodies move towards the negative electrode by the flow of buffer. The entire procedure takes about an hour.

Any antigen for which antisera are available can be tested for countercurrent immunoelectrophoresis. The sensitivity appears to be less than that of particle agglutination, detecting approximately 0.01 to 0.05 mg/ml antigen which transforms to about 10^3 microorganism/ml of fluid. This technique is costly, large quantities of antigen and antibody needs to be used and is more expensive than agglutination test.

Immunoelectrophoresis is used to study the antigen-antibody reactants, has an advantage over gel diffusion technique in that the diffraction of electrophoresed proteins are separated in an electric field and gives better precipitation reaction. There is migration of charged particles in an electric field. The rate of migration is dependent on the magnitude of electric charges on the particles, voltage of current and pH of the buffer.

Principle

Counter Current Immunoelectrophoresis (CCIE) utilizes the migrating property of antigens to anode as most of the antigens are negatively charged at the high pH of the buffer used. Passing current brings about the precipitation, which enhances the movement of antigens towards antibody where as antibody moves because of endosmosis. This technique has gained more practical utility than double diffusion test due to the sensitivity and quickness.

Materials

1. Antigen
2. Antibody
3. Agarose (high EEO)
4. Running buffer 5x (make it to 1x with distilled water)
5. Glass slide
6. Gel punch
7. Electrophoresis with Power Pack, distilled water
8. Conical Flask
9. Measuring Cylinder
10. Pipette
11. Wicks (filter papers)
12. Micropipette and Tips

Note: The running buffer can be reused.

Precautions

- Use agarose having high electro endosmosis (EEO)
- Occasionally wet the wicks connecting the slide and running buffer during electrophoresis
- Use separate micro tips for different reagents
- Avoid overflowing or bubbling while filling up of the wells
- Reagents contain 0.01% sodium azide as preservative
- Discard all used glassware properly

Protocol

Preparation of agarose gel plates

1. Prepare 1.0% solution of agarose in the buffer provided (Running Buffer 1x). Heat the solution in a boiling water bath until the agarose is completely dissolved and no obvious particles of agarose remain in suspension. Cool the agarose solution to around 50^0C.
2. By using Glass Pipette, add about 4.5ml of molten agarose onto a glass microscopic slide that kept on a leveled surface. The agarose would be about 3-4 mm thick. Avoid spillage, even a little spillage cause the whole agarose get drawn from the slide due to capillary.

3. The agarose slides can be stored at 4^0C unless it I used immediately.

CUTTING WELLS IN THE AGAROSE

4. Two sets (positive & negative control) of wells are punched on the slide with the gel punch using the template provided. The well would be 3 mm in diameter and 5 mm interspace. The interspace between the wells is of utmost importance for efficient formation of precipitin lines within 45 minutes.
5. Cut the well in agarose after it has hardened sufficiently. Before cutting the wells keep the slides at 2-8 C for a short period. This gives a clean cut edge of the wells which won't break down when the agarose plugs are removed.

6. The agarose plugs can be removed from the wells using a fine gauge glass or metal cannula attached to a vacuum line. Alternatively 20-gauge hypodermic needle can be inserted in to the plug to lift it free of the agarose.
7. If plates are not used immediately after they are cut, it will be necessary to remove the moisture that tends to accumulate in the bottom of the wells.
8. Optimal results are obtained if reagents are added to the wells just after the plugs are removed.

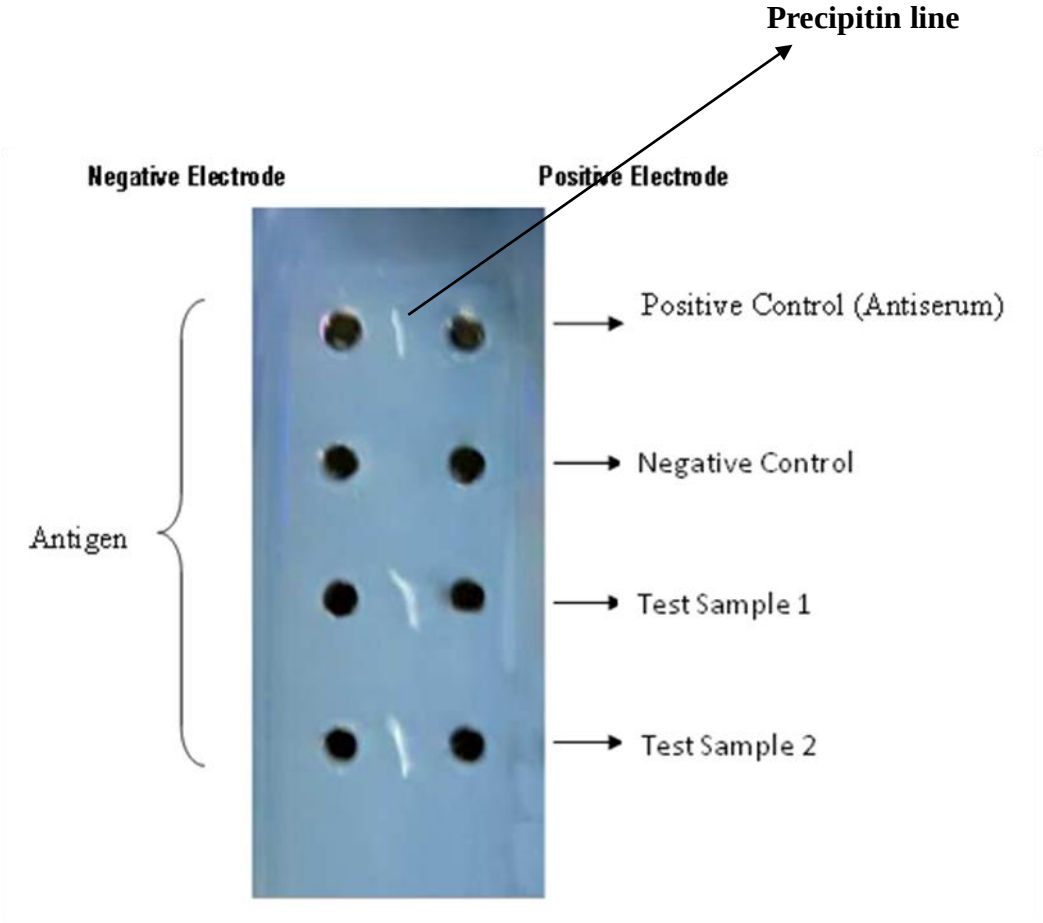
FILLING WELLS AND ELECTROPHORESIS

9. Mark the antigen and antibody well, and add 15µl of antigen and antibody to the respective wells in the first set (positive control).
10. In the other set of well (negative control) add 15µl of antigen and normal saline respectively after marking them.
11. While filling the well, avoid overflow into the adjacent well.
12. After the wells are filled keep the slide on the Electrophoresis Bridge of the electrophoresis tank that has been filled with the running buffer provided (use 1x Running Buffer). The antigen containing wells should be placed on the cathode (negative side) of the electrophoresis tank (Black terminal).
13. Connect the slide with filter paper strips (wicks) with the respective chambers of the tank.
14. Electrophoresis at 8-9 mAh or 50-100 Volts for 45 minutes to one hour. Wet the wicks with the buffer in the tank frequently after every 10 minutes.

READING THE COUNTER IMMUNOELECTROPHORESIS TEST

15. Disconnect the power supply and remove the slides and keep at room temperature for 2-5 minutes.
16. A high intensively light source or narrow beam of light is used to examine the precipitin lines. Also it is useful to view the reaction against a dark background.
17. A precipitin line will be formed between the antigen and antibody wells (positive control). The position of the line is determined by the relative amounts of antigen and antibody.

RESULTS AND INTERPRETATION



18. The presence of precipitin line (positive control) indicates that the antigen added in the wells has reacted with (specific to) the antibody added adjacent to it. This test is mostly used for the qualitative detection of antigen or antibody in the sample.
19. In the second set (negative control) precipitation line doesn't appear since no antibody is added & hence no reaction happened.
20. The position of the lines may also vary depending on the relative concentrations of antigen and antibody. As the serum dilutions increases the position of the precipitin lines would get closer to the antiserum wells indicating relative excess of antigen over antibody.

10. DOT ELISA (ENZYME LINKED IMMUNO SORBENT ASSAY)

INTRODUCTION:

Enzyme immunoassays (EIA) were developed in the mid sixties for identification of antigens in histological preparations analogous to immunofluroscence methods, identification of precipitation lines obtained by immuno diffusion and immuno electrophoresis.

The observation that antigen or antibody can be immobilized on solid phases made it possible to go in for quantification of immuno reactants in test tubes. An antigen is specifically detected due to the discriminatory power of antibodies and a marker attached to these antibodies indicates that such a reaction takes place. Enzymes are used as markers in EIA since they have in contrast to fluorescent labels, have capacity of amplification.

Enzymes used are Horse radish peroxidase (HRP), alkaline phosphotase, microperoxidase. ELISA takes advantage of biological properties of two important macromolecules; enzyme and antibodies. Enzymes are 'biological catalysts' which accelerate specific chemical reaction enormously and can thus be detected by adding substrate. The strategy in ELISA is to conjugate an appropriate enzyme to one of immuno reactants (antigen or antibody).

Let the immunological reaction takes place, add substrate and determine production conversion by enzymes. The amount of product indicates how much enzyme labeled immuno reactant is incorporated for the complex. The visible colour reaction is indicator of final product of test.

ELISA, also called enzyme linked immuno sorbent assay, employs antigens or antibodies conjugated to enzymes in such a way that the immunological and enzymatic activity of each component is maintained. These assays are very sensitive and give accurate results. The estimation of results can be made either visually or spectrophotometrically.

In DOT ELISA, the antigen is coated on a membrane (instead of an ELISA plate) and the end colour reaction is read visually. Thus it is a qualitative test indicating the presence or absence of the antigen or antibody under test.

PRINCIPLE

ELISA is extremely sensitive and used to detect antigen or antibody. A variety of direct, indirect and reversed assays have been described, most involve a solid phase ELISA. These systems depend upon capacity of antigen/antibody to bind to inert carrier surfaces which form an immuno adsorbent for subsequent attachment of antigen/antibody as the case may be. The most successful and simple technique for measurement of antigen in serum and is also called as sandwitch ELISA. The main components of ELISA are absorption of antibody to solid face and washing off excess, unattached antibody, blocking of unabsorbed antibody with BSA, addition of serum followed by incubation and then washing of excess unattached antigen. Addition of enzyme labeled anti immunoglobulin conjugate again followed by incubation and washing.

Addition of enzyme-specific soluble substrate which produces a colour change which is measured spectrophotometrically.

In most cases, enzyme used is HRP. Several soluble substrates have been used for peroxidase enzyme (orthophenylin diammine dihydrochloride –ODP) or (Tetra methyl benzidene, TMP) or [2, 2'-azyrodi (3-ethyl benzothiazoline -6 sulphonic acid –ABTS)]. Different substrates give different colour after enzyme reaction.

For detection of antibodies, the known antigen is coated on the solid phase surface, (96 wells plate.) and then test serum samples, properly diluted are added. After incubation, the surface is washed and conjugate enzyme labeled species specific antibody is added which binds with antibodies present in the test serum and can be detected by adding substrate which gives a coloured reaction that can be seen by naked eyes or read in spectrophotometer.

For detection of antigen, the sandwich assay is preferred. In sandwich assay, antibody is bound to solid phase. The antibody is known as capture antibody then the specimen suspected for presence of antigen is added. After appropriate incubation, the surface is washed and second antibody specific to the antigen is applied which is known as indicator antibody. In indirect method, the secondary antibody should be raised in different animal species with that of capture antibody. The reaction is detected by applying substrate (enzyme which gives a coloured reaction).

Variants of the test

The enzyme linked immuno sorbent assay had a series of changes, enhancing its efficiency and reducing its cost, in a regular manner, over a period of time.

The few variants are

(i) Plate ELISA
(ii) Dipstick ELISA
(iii) Dot ELISA
(iv) Avidin-Biotin assay
(v) Antigen capture ELISA

The various methods involving their variation are:

Antigen detection

Direct ELISA

Antigen is coated on the solid surface and the test serum is added (Antibody along with enzyme conjugate).

Indirect ELISA

Antigen is coated with the solid surface and specific antibody is added. Antibodies raised for specific antibody is added on top. If this triplet is specific, there is a positive result.

Antibody detection

Direct ELISA (Antigen capture ELISA)

Antibody is coated for the specific suspected antibody and then the test material is added. Detector antibody is added along with the enzyme.

Indirect ELISA

Antibody is coated on the surface and test antigen is added. Detector antibody is added and antibody with enzyme is added.

MATERIALS

S.No.	Materials	Storage
1	Antigen	-20^{o}C
2	Antibody	-20^{o}C
3	Nitrocellulose strips (0.45µm thick)	RT
4	Wash Buffer	RT
5	Blocker (dissolve 100 mg in 5 ml x PBS)	4^{o}C
6	Substrate (Always prepare Substrate freshly before each test)	4^{o}C
7	Conjugate	4^{o}C
8	Hydrogen peroxide	4^{o}C
9	1 x PBS	RT
10	Distilled water	-
11	Glassware's (Conical Flask, Measuring cylinder, Pipette)	-
12	Micropipette, Tips	-

C .WORKING SOLUTION PREPARATION

i) BLOCKING SOLUTION (BOVINE SERUM ALBUMIN)

To prepare 2% blocking solution, take 5ml of 1xPBS and add 100mg of blocker provided and mix well.

Note: Prepare freshly every time before each experiment.

ii) SUBSTRATE

With the given substrate quantity, add 1ml of triple distilled water and mix well by repeat pipetting. To this 1ml again add 29ml of distilled water. Aliquot these 30ml stock solutions into 3 separate 10ml storage tubes and wrap it with aluminium foil and store at -20^{o}C for subsequent usage. This will avoid the loss of effectiveness of the substrate stock solution at the time of thawing for the subsequent usage of each test.

Now before each test prepare this following step freshly. Take 10ml of the substrate stock solution and mix with 10µl of hydrogen peroxide.

D. PROTOCOL

Note: All the micropipettes should be well calibrated and the pipetting should be accurate to get optimum results. Any small variations would lead to major differences in the optical density values.

Coating

Coating buffer is prepared using carbonate-bicarbonate mixture. 0.05ml of carbonate bicarbonate buffer of pH 9.6 is used for immobilization of antigen or antibody on the polystyrene plate of pH 2.5 containing 0.1M Nacl for 10 minutes.

Coating of the antigen on the nitrocellulose membrane involves 1µl of the antigen placement on the strips and keeping it for passive adsorption at 37^{o}C in incubator for at least 45 minutes. By this time the antigen gets adsorbed onto the membrane. Leave one strip empty without adding antigen for negative control.

Washing

PBST – Phosphate buffer Saline Tween 0.2M PBS (pH7.2) containing 0.2 Tween 20

The concept of ELISA involves separation of bound and free reagents with the washing step. The unadsorbed antigens need to be removed by washing thrice. Washing is done by dipping the strips in the washing buffer and shaking it vigorously. The wash buffer provided can either be poured in to a petri dish or a 96-well plate and the strips immersed in it. Repeat this washing thrice in between every step of reagent addition and incubation.

Blocking

After coating and removal of unbound antigens, the remaining sites on the membrane have to be blocked to avoid direct binding of antibody or conjugate which would lead to false colour reactions. Hence add 300µl of the blocker solution provided in the required number of wells of a 96-well plate (depending on the number of strips tested) and dip the strips in it. Incubate the strips at 37^{o}C for 45 minutes.

Washing

The concept of ELISA involves separation of bound and free reagents with the washing step. The unadsorbed antigens need to be removed by washing thrice. Washing is done by dipping the strips in the washing buffer and shaking it vigorously. The wash buffer provided can either be poured in to a petri dish or a 96-well plate and the strips immersed in it. Repeat this washing thrice in between every step of reagent addition and incubation.

Serum incubation

After washing the strips, add 200µl of the diluted serum (1:50 in phosphate buffered saline). For example add 12µl of serum in 588µl of 1x PBS this gives 1:50 dilution) in to the required

number of wells of the 96-well plate. Dip the strips in the serum in wells and incubate at 37ºC for 45 minutes. Use phosphate buffered saline as negative control and dip one of the strip in the buffer instead of serum. If the serum has antibodies specific to the antigen adsorbed on to the membranes, they would bind. If not, the unreacted antibodies would be removed by washing.

Washing

The concept of ELISA involves separation of bound and free reagents with the washing step. The unadsorbed antigens need to be removed by washing thrice. Washing is done by dipping the strips in the washing buffer and shaking it vigorously. The wash buffer provided can either be poured in to a petri dish or a 96-well plate and the strips immersed in it. Repeat this washing thrice in between every step of reagent addition and incubation.

Conjugate Incubation

After washing the strips, add 200µl of the diluted conjugate provided (1:1000 in phosphate buffered saline), to the required number of wells of a 96-well plate. Dip the strips in to the conjugate containing wells and incubate at 37ºC for 45 minutes. The conjugate would bind to the antibody in the serum, only if they had bound to the antigen. If there were no antibodies in the serum, then no binding would have occurred and hence no binding of the conjugate would take place. All the unreacted conjugate would be removed by the washing.

Washing

The concept of ELISA involves separation of bound and free reagents with the washing step. The unadsorbed antigens need to be removed by washing thrice. Washing is done by dipping the strips in the washing buffer and shaking it vigorously. The wash buffer provided can either be poured in to a petri dish or a 96-well plate and the strips immersed in it. Repeat this washing thrice in between every step of reagent addition and incubation.

Substrate addition

For peroxide conjugate

- diamminobenzidine
- orthopheny hydrochloride
- tetramethyl benzidene
- ABTS- Azinobis ethyl (benzthiozoline sulphonic acid)
- Amino salicylic acid

For alkaline phosphatase conjugate

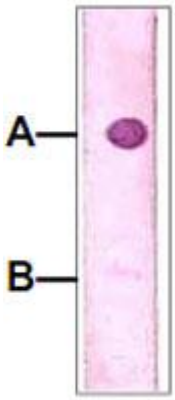
- p-nitrophenyl phosphate (PNP

For β-D-galactosidase

- p-nitrophenyl phosphate (PNP

Add 200µl of the substrate solution into the wells of 96-well plate and incubate the strips in the solution at 37ºC for 10 minutes. After washing the strips in tap water leave it air dry for few minutes.

E. RESULT AND INTERPRETATION

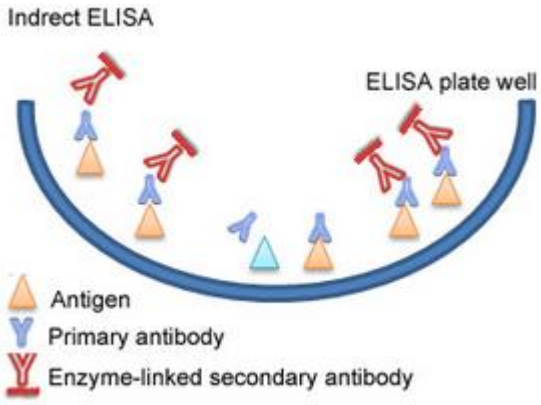


The appearance of brown/purple dot indicates the presence of antibody to the antigen in the serum tested. The absence of a brown dot indicates negative reaction. No colour should be seen in the membrane where in phosphate buffer saline was added instead of serum.

11. INDIRECT ELISA

Introduction

Indirect ELISA is a two-step ELISA which involves two binding process of primary antibody and labeled secondary antibody. The primary antibody is incubated with the antigen followed by the incubation with the secondary antibody. However, this may lead to nonspecific signals because of cross-reaction that the secondary antibody may bring about.



Reagents and Equipment

1.Phosphate buffered saline (10 mM PBS): NaH2PO4.H20 - 0.35gm, Na2HPO4.2H20 –
1.34gm, NaCl – 8.47gm make up the volume with Milli Q water to 1 liter and 0.1% sodium azide.
2.Washing buffer (PBS-T): 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20 (Product No. P3563).
3. Standard antigen (recombinant smad2) and test sample (HT-29 cell treated with TGF
beta at 5ng/ml)
4.Monoclonal primary antibody (Smad2).
5.Antibody controls: species- and isotype-matched, non-specific immunoglobulin (e.g.,
mouse myeloma proteins as a control for a mouse monoclonal primary antibody)
6.Peroxidase-conjugated secondary antibody
7. Substrate for peroxidase conjugated secondary antibody (TMB/H_2O_2).
8.Stopping reagent for peroxidase: 3 M HCl or 3 M H2SO4 (optional).
9.Microtiter plates
10.Microtiter plate reader equipped with a 600-650nm.

.

Procedure

Antigen Coating

Prepare an standard antigen and test sample in PBS.
Pipette 0.1 ml of the above solution to each well of the microtiter plate.
Incubate at 37 °C for 30 min., or incubate (covered) overnight at 4 °C.
Remove the coating solution. Wash three times with PBS-T.
Note: If problems with non-specific binding occur, an additional blocking step (30 min. 5% BSA-PBS) may be required. For further information see: Vogt, R.F., et al., J. Immunol. Meth., 101, 43 (1987).

Primary Antibody Reaction

Dilute the monoclonal primary antibody in PBS-T. The optimal dilution should be determined using a titration assay.

Add 0.1 ml of the diluted monoclonal antibody to each well. The negative control should be species- and isotype-matched, non-specific immunoglobulin diluted in PBS-T.

Incubate at room temperature for 30mins.

Wash as in step 4 of Antigen Coating.

Application of Secondary Antibody

Dilute the enzyme-conjugated secondary antibody in PBS-T. Add 0.1 ml of this solution to each well. The optimal dilution should be determined using a titration assay.

Incubate at room temperature for 30 mins.

Wash as in step 4 of Antigen Coating.

Substrate Preparation

During the last incubation and immediately before use, prepare the enzyme substrate.

Development

Add 0.1 ml of the freshly prepared substrate to each well.

Color should develop in positive wells after 30 minutes (blue color).

Absorbance may be read directly in a microplate reader (630-650 nm. Recommended wavelength is 650 nm) or the reaction may be stopped with 50 µl per well of the appropriate stopping reagent.

Results:

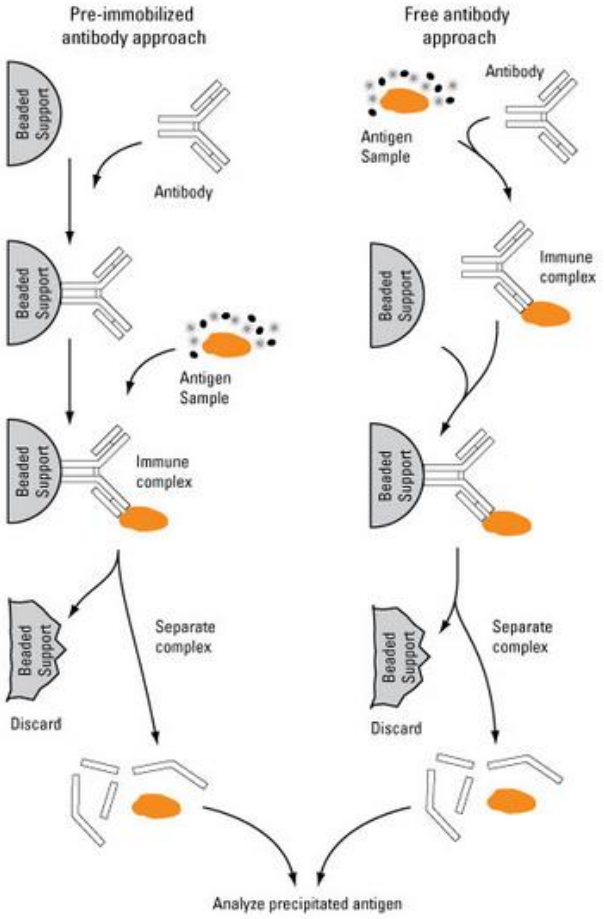
Inference:

12. IMMUNOPRECIPITATION

Introduction

Immunoprecipitation (IP) is one of the most widely used immunochemical techniques. Immunoprecipitation followed by SDS-PAGE and immunoblotting, is routinely used in a variety of applications: to determine the molecular weights of protein antigens, to study protein/protein interactions, to determine specific enzymatic activity, to monitor protein post-translational modifications and to determine the presence and quantity of proteins. The IP technique also enables the detection of rare proteins which otherwise would be difficult to detect since they can be concentrated up to 10,000-fold by immunoprecipitation.

In the IP method, the protein from the cell or tissue homogenate is precipitated in an appropriate lysis buffer by means of an immune complex which includes the antigen (protein), primary antibody and Protein A-, G-, or L-agarose conjugate or a secondary antibody-agarose conjugate. The choice of agarose conjugate depends on the species origin and isotype of the primary antibody. The methods described are comparable and the choice of method depends on the specific antigen-antibody system.



Reagents and Equipment

1.Phosphate buffered saline (10 mM PBS): NaH2PO4.H20 - 0.35gm, Na2HPO4.2H20 –

1.34gm, NaCl – 8.47gm make up the volume with Milli Q water to 1 liter.

2.Washing buffer (PBS-T): 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05%

3. Anti-TGF beta R2 primary antibody .

4.Cell lysate (HT-29 cell line treated with TGF beta)

5. Protein G Agarose Beads

6.Eppendorf tubes
7.Table top centrifuge

Procedure

A. Preparing Agarose beads

- Wash agarose conjugate twice with washing buffer, centrifuge for 10 sec. at 12,000xg at room temperature. Discard supernatant.

Note: If agarose conjugate is a powder, reconstitute it with deionized H_2O and allow it to swell for 5 minutes.

- Resuspend agarose conjugate in washing buffer (50% suspension). - See more at:

B. Preparing Cell Lysates

- Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
- To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice cold PBS.
- Remove PBS and add 0.5 ml ice-cold 1X cell lysis buffer to each plate (10 cm) and incubate the plates on ice for 5 minutes.
- Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
- Sonicate samples on ice three times for 5 seconds each.
- Microcentrifuge for 10 minutes at 14,000 X g, 4°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at –80°C.

C. Immunoprecipitation

- Pipette our agarose conjugate of 50-100 µl in microcentrifuge tubes.
- Add to each tube 10 µl of primary antibody at appropriate dilution (1:10).
- Incubate for 15-60 min at room temperature, gently mixing the sample on a suitable shaker.
- Centrifuge at *3,000xg* for 2 min. at 4 °C. Discard supernatant.
- Wash samples each with 1 ml washing buffer, centrifuge at *3,000xg* for 2 min. at 4 °C. Repeat this step at least twice.
- Add to each tube 0.1-0.5 ml of cell lysate (protein concentration 100-1000ug).
- Incubate for 90 min. to overnight at 4 °C, gently mixing the sample on a suitable shaker.
- Collect immunoprecipitated complexes by centrifugation at *3,000xg* for 2 min. at 4 °C. Discard supernatant.
- Wash pellet with 1 ml washing buffer, centrifuge at *3,000xg* for 2 min. at 4 °C. Repeat this step at least 3 times.

D. Preparation for SDS-PAGE

- Resuspend each pellet in 25-100 µl Laemmli sample buffer to a final concentration of 1x sample buffer. Heat samples at 95 °C for 5 min.
- Centrifuge for 30 sec. at 12,000xg at room temperature. Collect supernatant (IP sample). If required, (and where protein stability permits) IP samples can be stored in sample buffer at -70 °C.
- Run samples and MW standards with known concentrations on SDS-PAGE (appropriate percentage of polyacrylamide gel is according to the molecular size of the protein).

- Transfer to nitrocellulose and perform immunoblotting

Results:

Inference:

13. WESTERN BLOTTING

Introduction

Western blotting also known as protein or immunoblotting is a rapid and a sensitive assay for detection and characterization of proteins. It works by exploiting the specificity inherent in antigen-antibody recognition. It is used to identify specific antigens recognized by polyclonal of monoclonal antibodies.

Western blotting is carried out along with protein (antigen) separation in gel by electrophoresis and the blot development.

It is essentially a combination of three techniques viz. Electrophoresis (PAGE), western blotting (protein blotting) and immunochemical detection (blot development).

Principle

Identification of proteins separated by gel electrophoresis is limited by the small pore size of the gel, as macromolecular probes for protein analysis cannot permeate the gel. This limitation is overcome by blotting the proteins onto an adsorbent porous membrane (usually nitrocellulose). The apparatus consists of a tank containing buffer, in which is located a cassette, clamping the gel and the membrane tightly together. A current is applied from electrodes located on either side of the cassette. The buffer is often cooled during transfer to avoid heating effects. The proteins are separated according to their electrophoretic mobility and blotted onto the membrane and identified using suitable immunochemicals to locate the protein of interest. The individual techniques are explained below.

SDS PAGE

Stage I

Prepare a PAGE gel slab and fix to a vertical electrophoresis apparatus. Treat sample with suitable buffer and load on to the gel slots.

Stage II

Apply electric current. After a few minutes, proteins in the sample migrate according to their electrophoretic mobility in the stacking gel. The stcking gel has a lower polyacrylamide concentration resulting in higher pore size and a lower pH (<7). This enables the protein to concentrate into a sharp band due to isotachophoresis or band sharpening effect. At the end of the stacking gel it meets the separating gel, which has a higher polyacrylamide concentration and higher pH. IN the separating gel the proteins travel according to their size.

Stage III

When the dye front reaches the bottom of the separating gel, the proteins in sample are resolved depending on their size. However the protein cannot be visualized directly. The gel needs to be stained with suitable stainer to visualize all the proteins. Th identification of protein of interest can be done using a suitable probe and a developing system.

Western Blotting

Blotting is the transfer of resolved proteins from the gel to surface of a suitable membrane. This is done commonly by electrophoresis (known as electroblotting). IN this method, the transfer buffer has a low ionic strength, which allows electrotransfer of proteins. Methanol in the buffer increases binding of proteins to nitrcellulose and reduces swelling of the gel during transfer. The use of membranes as a support for protein enables the ease of manipulation, efficient washing and faster reaction during the immunodetection as proteins are more accessible for reaction.

(a) Membrane is in close contract with PAG gel containing proteins. The proteins are elctrotransfered to NC membrane.

(b) At the end of electrotransfer, all proteins have migatd to NC membtrane.

The protein is transferred to the corresponding position on the membrane as on the gel. A mirror image of the gel is formed. However the protein location and detection can only be assessed after immunodetection.

Immunodetection

The transferred proteins are bound to surface of NC membtrane and are accessible for reaction with immunochemical reagents. All the unoccupied sites on the membrane are blocked with inert proteins, detergents or other suitable blocking agents. The membrane is then probed with a primary antibody to the protein of interest. An enzyme labeled secondary antibody and a suitable substrate to the enzyme identifies the antigen antibody complex formed on the membrane.

Precautions

Wear gloves while handling the gel and membrane.

Preparation of reagents.

- Blotting buffer: Add 25ml of blotting buffer component A and 25ml component B to 450ml distilled water.
- Other buffers: Dilute the required amount of buffer concentrate to 1X concentration with water.
- Antibody: Diluted primary antibody and labled second antibody HRP conjugate in assay buffer.
- Substrate: Dilute TMB/H_2O_2**,** 10X concentrate ten times with water just before use.
- Protein standard: Mix 25µl of standard with 25 µl of sample loading buffer and incubate at 80°C for 10min..
- Blocking buffer: Add 300 mg of blocking agent to 10ml of 1X diluent and mix.

Procedure

1 SDS-Polyacrylamide Gel Electrophoresis

1.1 Assemble the plates for casting gel by placing the spacers onto the base plate and the notched plate onto the top of spacers. Now the spacers will be between the base plate and the notched plate. Apply silicon grease to the spacer for a good water-tight seal. Clean the comps with water and ethanol.

Transfer the assembled plate into a gel casting apparatus. Ensure that the assembly is leak proof by filling water between the plates. The plates should be clean and free of detergents.

Add 1ml of water to the APS vial.(stable for 1month)

Mix 50 µl of APs with 5ml of separating gel mix.

Pour separating gel between the plates till the level is below 3-4 cm from the top of notched plate. Add 200 to 250 µl of water to make the surface even.

Allow the separating gel to set (about 20-30min.). Wash the top of the separating gel with distilled water and drain water completely.

Mix 20 µl of APS solution with 2ml of stacking gel mix and pour directly onto the polymerized separating gel. Insert an appropriate comb into the gel solution without trapping any bubbles about 1cm above the separating gel. Allow the stacking gel to set about 10min.).

Add 30 µl of distilled water and 30 µl of sample loading buffer to protein sample.

Add 25 µl of sample loading buffer to 25 µl protein standard.

Incubate protein standard and protein sample in water bath at 85-95°C for10 min.

Once the stacking gel has set, remove the comb carefully. Wash the wells immediately with distilled water to remove non-polymerized acrylamide. Straighten the teeth of the stacking gel, if necessary, and fix to a appropriate PAGE apparatus with Trisglycine buffer in the bottom reservoir. Remove any air bubbles trapped between the plates at the bottom of the gel by squirting Tris-glycine buffer through a syringe with a bent needle. Add Tris-glycine buffer to top reservoir.

Load 30 µl-processed protein standards in well # 1 and 50 µl of processed protein sample in well # 2 &10 µl of protein sample to well # 4. The samples can be conveniently loaded using either a microliter syringe (wash by pipetting the buffer in the bottom reservoir before loading each sample) or a micropipetter fitted with a long narrow tip.

Start electrophoresis at 50-150 V.

When the dye front is about to reach the bottom of the gel, turn off the power.

Remove the glass plates from apparatus and gently separate the plate-using spatula. Cut a corner from the bottom of the gel that is closest to the well # 1 for identification. Cut the gel along lane 3 and keep the lane 4 (protein sample) in blotting buffer and proceed for electroblotting immediately.

Transfer the gel containing protein standard and sample (Lane 1 & 2) to a glass or plastic tray containing a minimum of 20 ml of ezee blue stain. Stain at room temperature for overnight. (no distaining required)

View the gel against a bright background.

2 Electroblotting

2.1 Assemble the blotting sandwich within the blotting cassette as shown in the figure. Take care to avoid air bubbles between the gel and NC membrane.

2.2 Insert the cassette into the apparatus filled with blotting buffer such that the gel faces the cathode.

2.3 Connect the power supply. Set voltage to 35 V overnight or 50 V for 5 hours for blotting.

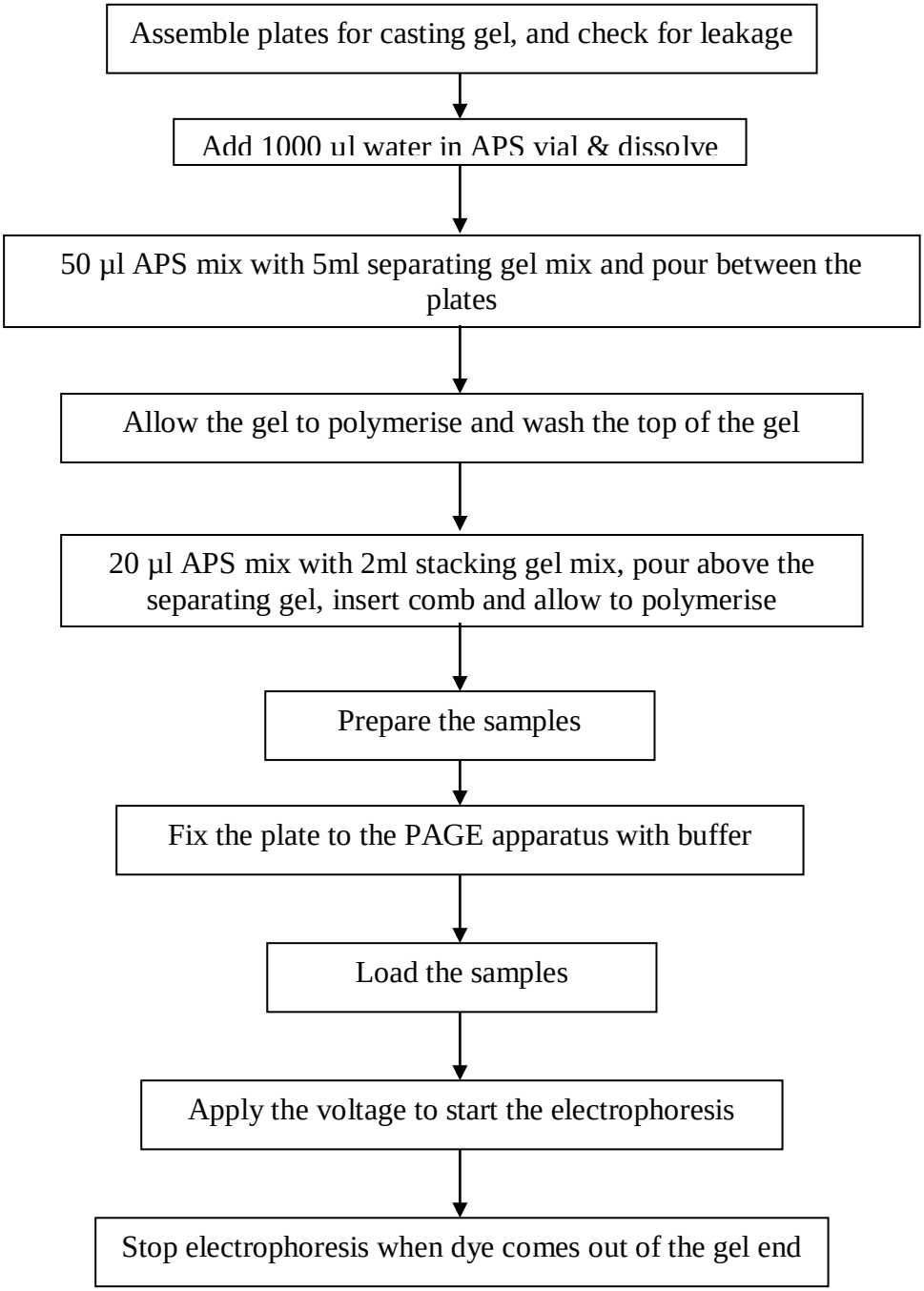
3 Immunodetection

3.1 Remove the NC membrane gently from the cassette and place the membrane in freshly prepared blocking buffer for 2 hours at room temperature or overnight in the cold.
3.2 Suspend primary antibody in 10 ml with the assay buffer using suitable tube.
3.3 Immerse blot in the primary antibody solution and agitate gently for 30 min.
3.4 Wash the blot by immersing in wash buffer for 3-5 minutes. Repeat two more times.
3.5 Prepare a 1:1000 dilution of labeled antibody in assay buffer. Prepare a sufficient (10ml) volume of diluted antibody to cover the blot.
3.6 Immerse the blot in the labeled second antibody solution and aditate gently for 30 min.
3.7 Wash the blot by immersing in wash buffer for 3-5 minutes. Repeat four times.
3.8 Immerse the washed blot in 10ml of substrate solution with gentle shaking. Bands should develop sufficient color within 5-10 minutes.
3.9 Remove the blot, wash with distilled water and dry.
3.10 Although the colored bands fade with time, the rate of color loss can retarded if the blots are kept in the dark.

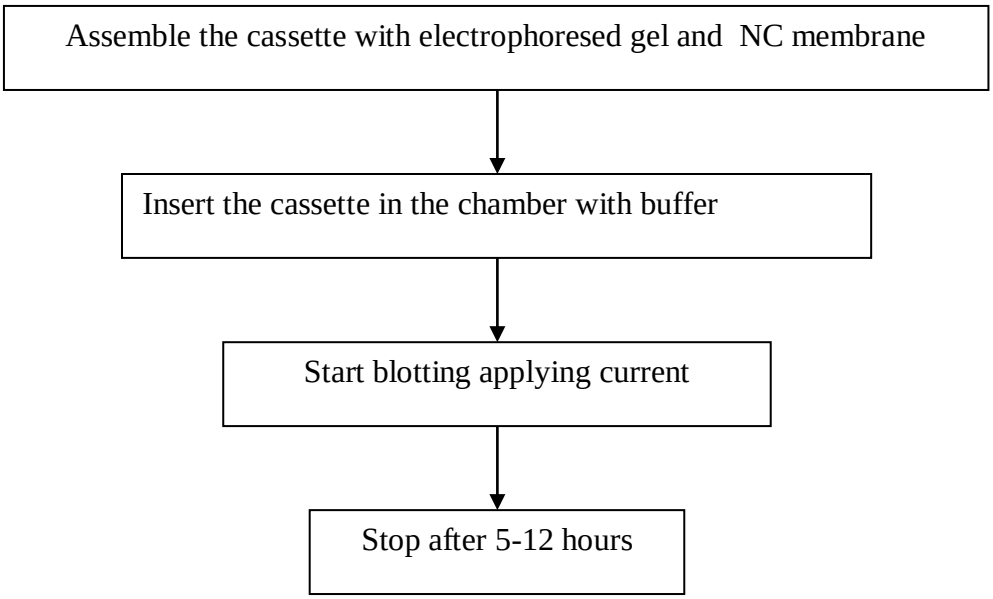
Interpretation

The band on the nitrocellulose membrane indicates the proteins identified by antibody (anti TGF beta R2). The position of the bands on the membrane indicates its electrophoretic mobility during electrophoresis.

FLOW CHART


Assemble plates for casting gel, and check for leakage
Add 1000 µl water in APS vial & dissolve
50 µl APS mix with 5ml separating gel mix and pour between the plates
Allow the gel to polymerise and wash the top of the gel
20 µl APS mix with 2ml stacking gel mix, pour above the separating gel, insert comb and allow to polymerise
Prepare the samples
Fix the plate to the PAGE apparatus with buffer
Load the samples
Apply the voltage to start the electrophoresis
Stop electrophoresis when dye comes out of the gel end

ELECTROBLOTTING



IMMUNODETECTION