AARUPADAI VEEDU
INSTITUTE OF TECHNOLOGY
OMR, PAIYANOOR, CHENNAI - 603104

VINAYAKA MISSION'S
RESEARCH FOUNDATION
(Deemed to be University under section 3 of the UGC Act 1956)

## B.Tech - Biotechnology

# IMMUNOLOGY LABORATORY MANUAL

### 17BTCC89

### DEPARTMENT OF BIOTECHNOLOGY

### AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY

### VINAYAKA MISSION'S RESEARCH FOUNDATION

**(Deemed to be University)**

**Paiyanoor, Chennai**

# CERTIFICATE

| | | |
|---|---|---|
| Name of the laboratory | : | IMMUNOLOGY LAB |
| Manual Subject Code | : | **17BTCC89** |
| Prepared by | : | **Dr.B.PRABASHEELA**<br>(Associate Professor ) |
| Verified by | : | Dr.R.Balachandar<br>(Assistant professor GII) |
| Approved by | : | **Dr. R. Devika**<br>Professor and Head<br>Department of Biotechnology |

**HOD**

## LIST OF EXPERIMENTS

1. Handling of animals, immunization and raising antisera.
2. Identification of cells in a blood smear.
3. Identification of blood groups.
4. Immuno diffusion
5. Immuno electrophoresis.
6. Testing for Typhoid antigens by Widal test.
7. Enzyme Linked ImmunoSorbent Assay (ELISA).
8. Isolation of pheripheral blood mononuclear cells.
9. Isolation of monocytes from blood.
10. Immuno fluorescence

# HANDLING OF ANIMALS, IMMUNIZATION AND RAISING ANTISERA

1. Introduction •Healthy and well being of lab. animal entirely depend almost entirely on a. the care b. humanity c. watchfulness of the staff of the animal house.
2. 1. Fluid 2. Diet 3. Cleanliness 4. Litter 5. Cages 6. Labelling of the cages 7. Ventilation 8. Temperature and Humidity 9. Handling 10. Breeding 11. Marking animals 12. The detection of signs of disease in animals
3. 1. Taking an animal's temperature 2. Prevention of disease 3. Insect pests 4. General anesthesia
4. RABBIT Rectal temperature:- 38.7 C-39.1 C Normal respiratory rate:- 55 per minute Pulse rate:- 135 per min Gestation period:- 28-31 days Weaning age:- 6-8 weeks Mating age:- 6-9 months Litters:- 4 yearly ; average 4 litter Room temp:- 15.5-18.5 Humidity:- 40 -45 percent Weight – adult:- 0.9-6.75 kg
5. Cages(Rabbit) •Cages are best made of galvanized iron. •The minimum size for a medium sized rabbit is 2×2×1(1/2)ft., •Young rabbits up to 3 months of age may be housed together but after that time sex should be separated. •From 8 -10 young rabbits may be kept together in pen similar to that used for guinea pigs.
6. Feeding(Rabbit) • Pelleted diet 18 of Bruce and Parkers(1947) or commercial breeders pellets are suitable • Daily supply of 2.5 0z(72 gm) of a mixture of one part oats and three parts bran may be fed as a slightly moist mash • Green stuffs or root vegetables • Clean drinking water
7. Handling(Rabbit) • Smooth ear of the rabbit back • pick up the ears and loose skin at the back of the neck with one hand in a firm grip • place the other hand under the hind quarter to support the weight and lift gently. • Never be lifted by ear alone • Should be placed on a non-slippery surface • If restraint is required during anesthesia or inoculation , should be wrapped in a roller towel or placed in a special box.

8. Common diseases(Rabbit) • Coccidioisis (hepatic and intestinal) • Pseudo tuberculosis • Respiratory infections( Snuffles) • Pneumococci • Streptococci • Intestinal infections( mucoid enteritis, Diarrhoea) • Rabbit syphilis (Treponema cuniculi) • Worms (cysticercus stage of dog tape worm, Taenia pisiformis)
9. Experimental procedure(Rabbit) • Antisera • Anesthesia • Scarification • Subcutaneous inoculation • Intra-venous inoculation • Intra-peritoneal inoculation • Intra-cerebral inoculation • Intra-testicular • Ophthalmic • Collection of blood
10. Guinea pig • Rectal temp.:- 37.6-38.9 • Normal respiration rate:- 80 per minute • Pulse rate:- 150 per minute • Gestation period:- 59-72 days( avg. 63 days) • Weaning age:- 14-21 days • Mating age:- 12-30 weeks • Litters:- 3 yearly ;average litter,3 • Room temp:- 18.5- 21 • Humidity:- 45% • Weight –weaning :- 120g, adult:- 200-1000g
11. Cage (Guinea pig) • Stock runs should be abt. 4×6 ft. and 1 ft. 8 in high • One square foot of space should be allowed for each animal • not more than 25 animals should not be kept in any one pen. • For expt. Animals galvanized iron cages are recommended and sterilized. • A convenient size of 14×9×8 in fitting in a tray 1.5 in deep
12. Feeding(Guinea pig) • A diet in pelleted form is recommended in preference to mashes. • Diet of Bruce and Parks (1947) contains balanced proportions of protein, fats and carbohydrate with added vitamins salt and trace element. • Crushed oats 2 part+ Broken bran 1 part • Supplemented with cabbage and hay • Necessary to add fish or meat meal.
13. Handling • Place one hand across the back of the animal with thumb behind the shoulder and the other fingers well forward on the opposite side • Lift the animal gently and support its weight with other hand placed palm uppermost under the hind quarters.
14. Common diseases(Guinea pig) • Pseudo tuberculosis (acute or chronic) • Abscesses in lymphatic glands • Respiratory tract infections • Intestinal infections • Protozoan disease( Coccidioisis, Toxoplasmosis) • Viral disease
15. Experimental procedures(Guinea pig) • Anasthesia( Pentobarbitone sodium 28mg/kg-body weight) • Subcutaneous inoculation • Intracutaneous inoculation • Intraperitoneal inoculation • Collection of blood
16. Mice • Normal temperature:- 37.4 • Pulse rate:- 120 • Estrous cycle:- 4-5 days • Gestation period:- 19-21 days • Weaning age:- 19-21 days • Mating age:- 6-8 weeks • Litters:- 8-12

yearly; average litter, 7-8 • Room temp.:- 20-21 • Humidity:- 50-60 % • Weight- weaning : 7g ,adult:- 25-28 g

17. 18. Cage(Mouse) • Many different designs of mouse and no one pattern is the standard • Aluminium box approx. 6×12×6 in deep with tapering side to facilitate stacking . • The lids are made of steel sheet or of strong wire mesh and are designed so that hopper is built into them and accommodation provided to hold the drinking bottle. • The cages are light, durable and easily sterilized by dry or moist heat.
18. Feeding(Mouse) • Pelleted diets such as diet 86 of Howie(1952) or diet 41 of Bruce (1950) are satisfactory • Fresh water in drinking bottles must be provided
19. Handling(Mouse) • An assistant takes a grip on the middle of tail of the animal with the left hand and gently raises the hind limbs floor of the cage • A mouse held in the position cannot turn around the bite. • Then with the right finger and thumb a fold of skin is taken up as close as possible to the head. • The animal can now be lifted into convenient position for the operator to carry out simple inoculation procedures. • Place the animal on a rough surface and hold it by its tail with right hand, • then pick up loose skin at the base of neck with the left forefinger and thumb, • lift and turn the left hand palm uppermost at the same time catching the tail and pressing it against the palm with the left little finger. • The right hand is free to pick up the syringe
20. Common diseases(Mouse) • Salmonellosis • Ectromelia ( mouse pox) • Streptobacillus moniliformis infection • Miscellaneous virus infection • Worms( Taenia taenia- formis)
21. Experimental procedures(Mouse) • Anasthesia short acting : Ether Long acting: Pentobarbitone sodium • Subcutaneous inoculation • Intraperitoneal inoculation • Intracerebral inoculation • Intravenous inoculation • Intranasal inoculation • Collection of blood

## IDENTIFICATION OF CELLS IN BLOOD SMEAR

**EXPT NO. 2**

**DATE :**

**AIM**

To identify different WBC cells present in the blood smear

**MATERIALS REQUIRED**

Blood sample, microscopic slides, leishmann's stain, light microscope and double distilled water.

**PROCEDURE**

- A drop of blood is placed over the clean glass slide at the centre and spread the blood to form a smear.
- Air dry the blood smear and label properly
- Apply leischmann's over the smear and allow for couple of minutes, then add equal volumes of double distilled water and mix properly.
- Keep the slide aside for 10-20 minutes and wash the slide in running water and air dry the smear.
- After air drying, observe the blood smear under light microscope.

**RESULT**

The following cells were identified in the blood smear using light microscope

## IDENTIFICATION OF BLOOD GROUPS

**EXPT NO. 3**

**DATE :**

**AIM**

To demonstrate antigen-antibody reaction by Hemeagglutination reaction.

**PRINCIPLE**

Blood group classification have extreme importance in medical field. ABO blood groups were discovered by Carl Landsteiner. The system classifies human blood into four main groups based on the presence or absence of the cell surface antigens.

- If the red cells have only antigen A at its surface, the blood type is 'A' where the plasma contains Anti-B antibodies which clumps the cells.
- If the red cells have only Antigen B, the blood is type 'B' where the plasma contains Anti-A antibodies, which clumps cells having antigen A.
- If the cells have both antigen A and antigen B, the plasma contains neither Anti-A or Anti-B.
- Rh blood types form the second major blood group system. Rh factor agglutinate with Anti-Rh antibody. This reaction can produce serious illness or death. When the blood agglutinates with Anti-Rh antibody, it indicates the person is Rh positive and if there is no agglutination, it indicates the person is Rh negative.

ABO blood grouping has tremendous application in the field of blood transfusion, tissue typing, graft transfer and other medical ailments.

**MATERIALS REQUIRED**

Blood sample, antiserum (anti-A, anti-B, anti-D), glass slide, lancet or needle, rectified spirit and cotton.

**PROCEDURE**

- Label three glass slides as A,B, and D .
- Add one drop of blood to each labeled areas.
- Add a drop of antisera-A to labeled A, a drop of antisera-B to labeled B and a drop of antisera D to labeled D.
- Mix the antisera with blood slowly and properly using the edges
- After mixing, observe for agglutination if any.

**OBSERVATION**

- Aggregates of blood sample is observed in the labeled glass slide.

**INTERPRETATION**

- Agglutination of blood with antisera occurs. If agglutination occurs at glass slide A, it is A blood group.
- If agglutination occurs at glass slide B, it is B blood group.
- If there is no agglutination in glass slide A and B, it indicates O blood group.
- Agglutination at glass slide D indicates the presence of Rh factor.

**RESULT**

The blood sample was analyzed and the blood group is found to be_____

## SINGLE RADIAL IMMUNO DIFFUSION (SRID)

**EXPT NO. 4**
**DATE :**
**AIM**

To quantify the amount of antigen present in test sample by single radial immuno diffusion.

**PRINCIPLE**

Radial immuno-diffusion is a method for determining the concentration of an antigen. The antigen sample is placed in a well and allowed to diffuse into agar containing a suitable dilution of an anti-serum. The area of the precipitin ring that forms around the well in the region of equivalence is proportional to the concentration of the antigen.

The antigen-antibody precipitation is made more sensitive than in double immuno diffusion by the incorporation of the antiserum in the agarose solution before the gel is made. Thus the antibody remains uniformly distributed throughout the gel. Antigen is then allowed to diffuse from wells cut in the gel. This is an example of single immuno diffusion.

Initially as the antigen diffuses out of the well, the antigen concentration is relatively high and soluble antigen-antibody adducts are formed. However, as antigen diffuses farther from the well, the antigen-antibody complex reacts with more amount of antibody resulting in a lattice that precipitates to form a precipitin ring. The concentration of antigen is directly proportional to the diameter of the precipitin ring.

**REQUIREMENTS**

Antigen, antibody, test antigen. Agarose , gel punch, class slide, saline, template. Titer plates, micropipette, glasswares etc.,

**PROCEDURE**

- 1% agarose was prepared in saline and heated to dissolve the agarose.
- At optimum temperature (~40C), antibody of desired volume is added to agarose 3-4mm of agarose gel is prepared on microscopic slide.
- The slides were stored at 4C for a short time.
- After agarose has hardened sufficiently, two sets of wells, 3 on top and3 on bottom was cut using gel punch with the help of template.
- The antigen was diluted two fold in micro titer plate.
- Antigen is serially diluted in normal saline to obtain 1:2, 1:4,1:8,1:16 dilutions.
- 10ul of undiluted antigen was added to the first well of the agarose gel.
- 10ul of serially diluted antigen was added to $2^{nd}$, $3^{rd}$, $4^{th}$ and $5^{th}$ wells.

- 10ul of sample antigen was added to the $6^{th}$ well.
- After filling the wells, the slide was kept in a flat bottom container whose interior was kept moist by using damp cotton.
- The slides were placed at room temperature for 18-24 hours.
- Diameter of precipitin ring was measured and calibration curve was plotted.

**RESULT**

The antibody titration at various dilution was carried out and the concentration of the test antigen was found to be

## DOUBLE DIFFUSION (OUTCHERLONY METHOD)

**EXPT NO. 5**

**DATE :**

**AIM**

To quantitate and characterize antigen and antibodies by ouchterlony double diffusion

**PRINCIPLE**

Immunodiffusion in gel encompasses a variety of techniques, which are useful for the analysis of antigen and antibodies. One such method is ouchterlony double diffusion. Both qualitative and quantitative information can be obtained from double diffusion assays. This techniques is used for comparing different Ag preparations.

In the ouchterlony method, both antigen and antibody diffuse radially from wells towards each other, thereby establishing a concentration gradient. As equivalence is reached, a visible live of precipitation is formed. This simple technique is an effective quatitative tool for determining the relationship between antigens and the number of different antigen-antibody systems present. The pattern of the precipitin lines that form, where two different antigen preparations are placed in adjacent wells indicates whether they share epitopes. The pattern of lines that form, can be interpreted to determine whether, the antigens are same or different.

**REQUIREMENTS**

Microscopic slide, template, gel punch, saline, agarose, antigens and antibodies, test serum, glasswares etc.,

**PROCEDURE**

- 1% agarose was prepared in normal saline and heated to polymerize the agarose
- 3-4mm thick agarose gel was prepared on glass slide
- The slides were stored at 4C for a short time.
- After agarose has hardened sufficiently, two sets of wells were cut using gel punch with the help of template.
- Antibody and the test serum was diluted to two fold and used.
- In the first set well, antigen was added in central well and diluted antibody was added in surrounding wells (1:2 to1:16 dilutions)
- In second set, antigen was added to central well and diluted test serum was added in surrounding wells.
- After filling the wells, the slide was kept in a flat bottom container whose interior was kept moist by using damp cotton.
- The slides were placed at room temperature for 18-24 hours.

**RESULT**

The antibody titration at various dilution was carried out and the variation in the precipitin arc was observed.

## IMMUNO ELECTROPHORESIS

**EXPT NO. 6**
**DATE :**
**PRINCIPLE:**

Immunoelectrophoresis consists of two methods that are combined, namely agar-gel electrophoresis and immunodiffusion. The agar plate has a longitudinal trench cut which accommodates the antiserum after electrophoresis and a central well or wells to accommodate test material. The serum sample is placed in the well or wells and electrophoresis is carried out for 1-2h at a field strength of 5-9 V/cm. The protein components separate according to their charge, molecular weight and shape but unlike normal electrophoresis, the bands are not stained for proteins. Instead antiserum is pipetted into the longitudinal trough and the plate is incubated in humid chamber for overnight. Diffusion of the antiserum and protein occurs and, where they meet, precipitin arcs are formed that give rise to a characteristic pattern.

**MATERIALS:**

1. 0.07M Baribiturate buffer; pH 8.6 (Dissolve 2.58g of diethylbarbituric acid and 14.42g of sodium diethylbarbiturate in water and make up to 1litre).
2. Agarose (medium EEO) solution (1% w/v in the barbiturate buffer: heat the agarose at $90^0$C until all the lumps are dissolved and clear solution is obtained and pour on to a slide when it is cooled to say $55^0$C. Allow the agarose to solidify.)
3. Glass slides (7.5 cm x 5cm).
4. Incubator at $37^0$C
5. Horizontal electrophoresis apparatus
6. Power supply
7. Cords
8. Gel puncher
9. Shandon cutting device
10. Bromophenol blue
11. Saline (0.9%w/v NaCl; 0.1w/v Na azide)
12. Antigen
13. Rabbit antiserum or peak fraction of the first peak in the previous experiment.
14. 0.25% coomassiee brilliant blue$R_{250}$ prepared in 4:1:5 (v/v/v) in ethanol: acetic acid: distilled water.
15. Destain solution (4:1:5 (v/v/v) in ethanol: acetic acid: distilled water).

**METHOD:**

- Heat glass plates in an oven at $100^0$C for about 30 minutes then remove them and place on a level surface.
- Carefully pipette about 8 ml of the hot agarose solution on to the surface of the slides.
- The molten agarose will set in a uniform layer and the surface tension will hold the agarose at the edges. Allow the agarose to solidify.
- Punch the gel with one well of relatively bigger size and with the help of Shandon cutting device give 4.5 cm long trough of 1mm diameter. With the help a of sharp needle remove the agarose from the well as well as from the trough.
- The antigen is mixed with 0.025% bromophenol blue, the tracking dye. Fill the electrophoresis chamber with 0.07M barbiturate buffer and place the slide in the chamber and connect the slide with wigs prepared from Whatman No 3 filter papers on either side and the wigs must dip into the buffer solution.
- Apply the potential difference of 5-9 V/cm for about 1-2h or until the tracking dye reaches the 2.0 -2.5 cm from the well.
- Remove the slide from the electrophoresis chamber and add of the antiserum or purified IgG place the slide in the moist chamber and incubate for overnight at $37^0$C and observe the precipitin line formation on the following day.

**OBSERVATION**

- The appearance of the precipitin line is noticed in the presence of specific antibody.

**INTERPRETATION**

- Immunoelectrophoresis is a diagnostic tool for qualitative and quantitative determination of biological samples.
- The specificity of the antigen can be ascertained to the specific antibody based on the pattern of precipitin arc.

**RESULT**

Thus the specific of the antigen towards the specific antibody is ascertained.

## TESTING FOR TYPHOID ANTIGENS BY WIDAL TEST

**EXPT.NO. 7**

**DATE.**

**AIM**

To diagnose the suspected case of Enteric fever serologically

**PRINCIPLE**

Widal test is serological method used in the diagnosis of Enteric fever which is caused by the organisms of the genus salmonella. They are *salmonella typhi, Salmonella Paratyphi* A,B,C,D, etc.

Enteric fever due to *S.typhi* and *S.Paratyphi* A,B,C and D are common in India. Antigens specifically prepared fromthis orgaism are used in the agglutination test to detect the presence of antibodies in patient sera which are elucidated in response to infection by these bacteria.

The organisms causing enteric fever possess two major antigens namely somatic 'O' antigen and flagellar "H' antigen along with another 'VI" surface antigen. During infection, antibodies are produced in patients sera against these 'O', 'H' *S.typhi* , '*A(H)'* and '*B(H)' S.paratyphi* antigens.

**MATERIALS REQUIRED**

Six circle glass slides, disposable mixing sticks, positive patient serum, micropipette.

**PROCEDURE (Rapid Slide Test-Screening test)**

- Take a properly cleaned glass slide
- Add a drop of undiluted test serum in the corresponding reaction circles and a drop of positive and negative control serum in the circles marked positive control and negative control.
- Add a drop of antigen O,H, A(H) amd B(H) in respective circles.
- Add any one of ht 'H' antigen [H,A(H), or B(H)] in the positive and negative control.
- Mix the contents of each circle with separate disposable mixing sticks.
- Keep the slide for one minute and then observe the visible agglutinins.

**RESULT**

## ELISA (SANDWICH)

**EXPT.NO. 08**

**DATE.**

**AIM**

To perform quantification of antigen using ELISA

**MATERIALS REQUIRED**

Titer plate, micropipette, glasswares, PBS, saline, primary antibody, antigen, conjugate , substrate, blocking buffer, washing buffer, substrate buffer, coating buffer, distilled water.

1. **Coating Buffer**

Solution A - 1.0 M $NaHCO_3$ 8.4 g / 100ml
Solution B - 1.0 M $Na_2CO$ 10.6 g / 100ml.
Mix 45.3 ml Sol A
18.2 ml Sol B
Adjust the pH to 9.6

2. **10 X Phosphate Buffered Saline (PBS).**

| | |
|---|---|
| KCl | 2.0 g |
| NaCl | 80.0 g |
| $Na_2HPO_4$. 7 $H_2O$ | 21.7 g |
| $KH_2PO_4$ | 20.0 g |

Add distilled water upto 1 litre and adjust the pH to 7.4 with 10 N NaOH.

3. **Blocking solution**

| | |
|---|---|
| 10 X PBS pH 7.4 | 50 ml |
| 0.05% Tween -20 | 0.25 ml |
| 5% BSA | 25 g. |

Add distilled water up to 500 ml, filter

4. **ELISA Diluent**

| | |
|---|---|
| 10 X PBS pH 7.4 | 100.0 ml |
| 0.05% Tween - 20 | 0.5 ml |
| 1% BSA | 10.0 g. |

Add distilled water up to 1 liter, filter.

5. **Washing Buffer**

| | |
|---|---|
| 10 X PBS pH 7.4 | 400 ml |
| 0.05% Tween-20 | 2 ml. |

Add distilled water and make up to 4 liters.

6. *Substrate Diluent*

| | | |
|---|---|---|
| $NaHCO_3$ | 1.69 g | |
| $Na_2CO_3$ | | 2.51 g |
| Mg $Cl_2$ | 0.41 g. | |

Add distilled water and make up to 2 liter, pH 8.6.

**PROCEDURE**

**ANTIBODY COATING**

- The antibody was diluted in coating buffer provided at 1:100 dilutions and 100ul of antibody was added to each well of ELISA plate
- The plate was left at 4C overnight for passive absorption of antibody to the ELISA plate

**WASHING**

- Unabsorbed antibody need to be removed after overnight incubation
- Washing is done using ELISA washer or manually by adding 300ul of washing buffer to each well and discarding it.
- Manual washing is repeated thrice after which the plate is flipped to completely drain the washing buffer.

**BLOCKING**

- Blocking buffer was then added to the wells (300µl/well). Blocking buffer was added to prevent cross reactivity.
- Proteins in blocking buffer (BSA) bind to unbound sites in microtiter plate, so that these sites are not exposed during the course of the reaction process.
- The plate was incubated at 37°C for 1 hours.
- After 1 hour manual washing was repeated thrice.

**ANTIGEN DILUTION**

- The antigens that need to be quantified is serially diluted in PBS.
- Serially double dilution from 1:100 to 1:1600 was performed.
- Similarly the sample antigen was diluted in 1:100 in PBS.
- Diluted standard and sample antigen was added in duplicated. A set of well was left empty which is the blank.
- The plates were incubated at 37C for 1 hour.

**CONJUGATE ADDITION**

- After incubation, the plate is washed thrice manually and 100ul of diluted conjugate (detection antibody/ enzyme tagged) was added to all wells and incubated at 37C for 1 hour.
- Washing step was repeated following incubation

**SUBSTRATE ADDITION**

- 100ul of diluted substrate was added to all wells of ELISA plate and the plate was incubated at 37°C dark for 10 min's.
- The reaction was then stopped by addition of 25ul of stopping solution.

**RESULT:**

DOT ELISA (ENZYME LINKED IMMUNO SORBENT ASSAY)

**EXPT.NO.9**

**DATE.**

INTRODUCTION:

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. 34 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. 35 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°C |
| 2 | Antibody | -20°C |
| 3 | Nitrocellulose strips (0.45µm thick) | RT |
| 4 | Wash Buffer | RT |
| 5 | Blocker (dissolve 100 mg in 5 ml x PBS) | 4°C |
| 6 | Substrate (Always prepare Substrate freshly before each test) | 4°C |
| 7 | Conjugate | 4°C |
| 8 | Hydrogen peroxide | 4°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) 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 - 20oC 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. 36 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 37oC 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 37oC 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 37 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:

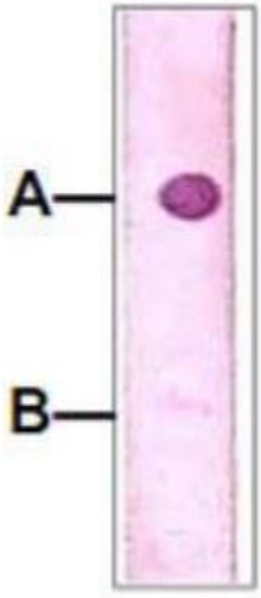
The concept of ELISA involves separation of bound and free reagents with the washing step. The unadsorbed antigens need to be removed by washing thrice. Washing is done by dipping

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 38 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.

**SEPARATION OF T AND B LYMPHOCYTES FROM HUMAN PERIPHERAL BLOOD(T-Cell Rosseting method)**

**EXPT.NO.10**

**DATE.**

**AIM:**

To separate T & B lymphocytes from peripheral blood by E-rosette technique.

**PRINCIPLE:**

Human T cells have specific surface antigen CD-2 on this surface. This has a natural affinity for a sheep erythrocyte on incubating isolated lymphocytes with sheep blood. If will get attached to the receptor on T cell and under microscope it will look like floral structure called rosette to three or mare sheep RBC.

**MATERIALS REQUIRED:**

- Isolated sample lymphocytes
- Sheep blood collected in alsever's solution
- Centrifuged tubes
- Haemocytometers.

**PROCEDURE:**

1. Human and sheep blood was taken in 1:1 ratio and incubated for 10 minutes at 37° C.
2. A drop of suspension was taken under microscope for rosette.
3. Centrifuge the suspension at 1000 rpm for 10 minutes to sediment the rosette.
4. Collect the supernatant containing B-cells and counted in haemocytometer.
5. Lyse the sheep RBC's and count the 'T' cells in haemocytometer.

**OBSERVATION:**

Flower like rosette were seen on. Incubation of isolated lymphocytes with sheep RBC.

**RESULT:**

Human T-cell and B-cell lymphocytes were separated by E-rosette technique.

## IMMUNOFLUORESCENCE PROCEDURE

**EXPT.NO. 11**

**DATE.**

**PRINCIPLE**

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on microbiological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. The specific region an antibody recognizes on an antigen is called an epitope. There have been efforts in epitope mapping since many antibodies can bind the same epitope and levels of binding between antibodies that recognize the same epitope can vary. Additionally, the binding of the fluorophore to the antibody itself cannot interfere with the immunological specificity of the antibody or the binding capacity of its antigen. Immunofluorescence is a widely used example of immunostaining (using antibodies to stain proteins) and is a specific example of immunohistochemistry (the use of the antibody-antigen relationship in tissues). This technique primarily makes use of fluorophores to visualise the location of the antibodies.

**Samples:**

1. Cultured cells: Cells may be grown on a 12mm round coverslips and stained in the wells of a 24-well plate. Alternatively cells can be grown in a petri dish, in which a hole has been made and a coverslip is glued in (Mat-Tek Corp. 200 Homer Ave, Ashland MA 01721, (800) 834-9018). Finally, now that we have an upright confocal microscope cells maybe grown on plastic and viewed live with the Zeiss LSM 510 Meta using dipping objecives.
2. Sections of fixed tissues: Sections up to 200 µm thick made with a Vibrotome can be viewed on a 2-photon microscope. For standard confocal (single photon), 50-100 µm should be max.
3. Thin sections: These should be made as usual for histological staining. After attaching to glass slide (coated with poly-L-lysine or purchased charged slides: Vector Labs), they must be fixed.

**Fixation:**

Since different fixatives can have various effects on cytoskeletal structures or on proteins by cross-linking, several methods of fixation should be tested for each antibody developed:

1. 3.7% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature followed by 0.2% Triton X100 in PBS (5 min) or 100 µM digitonin (5-10 min) permeabilization. This is the fix of choice if it works with your antibodies, because the cytoskeleton is best preserved. (Permeabilization may not be necessary in very thin sections.)
2. Absolute methanol at -20°C for 6 minutes; flood with PBS-0.5% BSA to prevent drying when incubation is finished.
3. Absolute ethanol at room temperature for 30 seconds; flood with PBS-0.5% BSA to prevent drying when incubation is finished.

**Fluorescence staining:**

1. Wash the cells several times in PBS-0.5% BSA. (I just use a squirt bottle and thoroughly flush the slide several times or fill and dump in the case of cells grown on coverslips and incubated in 24-well plates.)
2. Incubate with primary antibodies in PBS-0.5% BSA for 1 hour at room temperature or a half hour at 37°C. I usually only make enough working dilution for each experiment and don't reuse it or save left over. The total amount I use for 24-well plates or for circles drawn on slides is 10µl. But I don't measure less than a microliter of stock; so if it should be diluted 1:200, I make 200 µl. If there are two antibodies I mix them first, then place the solution on the cells. Whether on a slide or on coverslips in wells this incubation should be done in a humid chamber. This can be accomplished by placing the slide on toothpicks on a damp paper towel in a plastic box of some sort, or placing the damp paper towel in the lid of the 24-well plate.
3. After incubation, wash again in PBS, and add fluorescent secondary antibodies (at this point I add the direct labeling reagents, eg DAPI or phalloidin, as well), followed again by washing. Only secondary antibody should be incubated on one sample to control for nonspecific reaction. Incubate and wash again as above.

**Mounting:**

They are embedded in an anti-fade media (available from Molecular Probes or Vector labs). There are of course many variations on this media. It is only important that the issue of fading be addressed somehow. I will often rinse my cells for preparation once in deionized water before mounting too. This simply rinses off any salt residue, but is not necessary for viewing. Thick sections of 60-200 µm may be stored in a PBS a solution containing 2% DABCO instead of mounting.

**RESULT:**