SRM INSTITUTE OF SCIENCE AND TECHNOLOGY

SCHOOL OF BIOENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

20BTC601J - MOLECULAR IMMUNOLOGY LAB MANUAL

M.TECH BIOTECHNOLOGY

II SEMESTER

SRM
INSTITUTE OF SCIENCE & TECHNOLOGY
(Deemed to be University u/s 3 of UGC Act, 1956)

20BTC601J Molecular Immunology laboratory

List of experiments

1. Separation of serum and plasma
2. Isolation of monocytes/lymphocytes
3. Culturing of lymphocytes for activation assays
4. Immunoelectrophoresis
5. ELISA –SANDWICH
6. Dot ELISA
7. Assay of cytotoxicity
8. Western Blot
9. Detection of cell surface molecules by Flow cytometry
10. Detection of apoptotic proteins by florescence microscopy

EXPT NO: 1

SEPERATION OF SERUM AND PLASMA FROM BLOOD AND ESTIMATATION OF PROTEIN CONTENT

AIM:

To separate the serum and plasma from the given blood samples and estimate its protein content.

PRINCIPLE:

Blood plasma is the liquid component of blood in which the blood cells are suspended. It makes about 60% of the total blood volume. It is composed of mainly water (90% by volume), and contains dissolved proteins, glucose, clotting factors, mineral ions, hormones and carbon dioxide (plasma being the main component for excretory product transportation). Plasma is the supernatant fluid obtained when the anti-coagulated blood has been centrifuged. The blood is mixed with an appropriate amount of anti-coagulant like heparin, oxalate or EDTA. This preparation should be mixed immediately and thoroughly to avoid clotting. Blood serum is blood plasma without fibrinogen or other clotting factors.

REQUIREMENTS:

Glassware: Eppendorf tubes, syringe

Reagents: Blood sample, EDTA, Complex-forming Reagent, Folin Reagent, Bovine Serum Albumin, Distilled Water, Alcohol.

Other Requirements: Micropipette, Tips, Centrifuge, Spectrophotometer.

PROCEDURE:

Blood Plasma Preparation

1. Draw blood into centrifuge tube containing approximately 1.8mg of potassium EDTA/ml blood. Be sure to take the full volume to ensure the correct blood to anti-coagulant ratio.

2. Invert centrifuge tubes 10 times carefully to mix the blood and anti-coagulant and store at room temperature until centrifugation.

3. Samples should undergo centrifugation immediately. This should be carried out for a minimum of 10min at 1000-2000rcf (generally 1300rcf) at room temperature. Do not use breaks to stop the centrifuge.

4. This will give three layers from top to bottom plasma, leucocytes (Buffy coat), and erythrocytes.

5. Carefully aspirate the supernatant (plasma) at room temperature and pool in a centrifuge tube. Take care not to disturb the cell layer or transfer any cells.

6. Inspect plasma for turbidity. Turbid samples should be discarded and centrifuged and aspirated again to remove remaining insoluble matter.

Blood Serum Preparation-

1. Draw the whole blood into centrifuge tubes containing no anticoagulant. Draw approximately 2.5 times the volume needed for use.

2. Incubate in an upright position at room temperature (30-45 degrees) for approximately 60 mins to allow clotting.

3. Centrifuge at 1000-2000rcf.

4. Carefully aspirate the supernatant (serum) and pool into a centrifuge tube, taking care not to disturb the cell layer or transfer any cells. Use a clean pipette for each tube.

5. Inspect serum for turbidity. Turbid samples should be centrifuged and aspirated again to remove insoluble matter.

Estimation of protein content-

1. Add 0.2 ml of BSA working standard in 5 test tubes and make up to 1ml using distilled water.

2. The test tube with 1 ml distilled water serves as blank.

3. Add 4.5 ml of alkaline copper reagent and incubate for 10 minutes.

4. After incubation add 0.5 ml of Folin reagent and incubate for 30 minutes

5. Measure the absorbance at 660 nm and plot the standard graph.

6. Estimate the amount of protein present in the given sample from the standard graph.

OBSERVATIONS:

RESULT:

EXPT NO: 2

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

AIM:

To isolate peripheral blood mononuclear cells and also to calculate its viability percentage.

PRINCIPLE:

Isolation of mononuclear cells from a population of heterogeneous cells depends on the density gradient of different cell population. The most commonly used method for separation of lymphocytes is sedimentation through a high-density medium (1.077 g/ml of human cells). In the presence of high-density medium granulocytes and RBC sediment fast due to its high density, whereas mononuclear cells sediment slowly that can be seen as buffy coat between RBC pellet and aqueous layer.

REQUIREMENTS:

Glassware: Eppendorf tubes, syringe

Reagents: Blood sample, EDTA, Trypan blue, Ficoll, Distilled Water, Alcohol.

Other Requirements: Micropipette, Tips, Centrifuge, hemocytometer.

PROCEDURE:

1. Collect human peripheral blood (2ml) and dilute in 2ml PBS buffer. Total volume 4 ml.
2. Take equal volume of ficoll in a centrifuge tube.
3. Slowly add the diluted blood in layer over the ficoll and centrifuge at 1300 rpm for 15 min at 4°C.
4. Four layers will be formed-plasma, buffy coat, mononuclear cells and erythrocytes. Gently remove the buffy coat layer in the interphase containing the mononuclear cells.
5. Centrifuge interphase suspension for 10 min at 4°c.wash pellet in PBS.
6. Resuspend the cells in 100 µl of PBS.
7. Take 10 µl of suspension and 10 µl of trypan blue and mix in eppendorf tube.
8. Load on hemocytometer and calculate its viability percentage.

Cells per ml = The average count per square x Dilution factor x 10^4

Procedure for individual experiments with lesser volume

1. Add 0.5 mL of ficoll to 1 mL eppendorf tube.
2. Add 0.5 mL of freshly drawn blood over ficoll taking care that the both layers a not disturbed.
3. Centrifuge the tube at 15,000 rpm at 4 degrees.
4. You may find three clear layers formed,
    a. Upper plasma layer
    b. Hazy PBMC layer + ficoll
    c. RBC layer
5. Take the middle layer with PBMC + ficoll into a new eppendorf tube.
6. Spin it down at 5000 RPM at 4 degree for 5 minutes and discard the supernatant.
7. Resuspend the pellet with PBMC in 100 uL of ice cold PBMC and repeat step 6.
8. Repeat step 7 again and resuspend the pellet in 100uL of ice cold PBMC.
9. Take 10 µl of suspension and 10 µl of trypan blue and mix in eppendorf tube.
10. 8. Load on hemocytometer and calculate its viability percentage.

Cells per ml = The average count per square x Dilution factor x 10^4

OBSERVATION:

RESULT:

EXPT NO: 3

CULTURING OF LYMPHOCYTES FOR ACTIVATION ASSAY

AIM:

To culture lymphocytes and measuring lymphocyte activation.

PRINCIPLE:

A proliferation assay is a simple method of non- specifically measuring lymphocytes activation and proliferation ability. The proliferation of lymphocytes is measured by presence of various chemokines, which is measured upon harvesting cell cultures after mitogen stimulation. The lymphocytes can be artificially stimulated using the following mitogens:
Phytohemagglutinin (PHA): T- cell activation
Lipopolysaccharide (LPS): B- cell activation

The assay includes mitogenic stimulation and then measuring the presence of certain chemicals. This can be done by isolating the lymphocytes from blood culturing the cells in suitable medium and then stimulating it by using mitogens

REQUIREMENTS:

Reagents: Blood sample, tryphan blue, Ficoll hypaque, 1x PBS, RPMI medium, Distilled Water, Alcohol, Mitogens: LPS, PGN, anticoagulant: EDTA, Heparin, Sodiumcitrate

Other Requirements: Micropipette, Tips, Centrifuge, hemocytometer. Eppendorf tubes, syringe, Co2 incubator, ELISA plates, Biosaftey Chamber.

PROCEDURE:

1. Isolate the monocytes or lymphocytes as described in previous experiment.
2. Count the number of viable cells using haemocytometer.
3. Now resuspend the cells in culture medium containing 10% FCS.
4. After culturing the cells, cells are activated by mitogens.
5. These activated cells are then kept for overnight incubation in a CO_2 incubator
6. After incubation the cells are centrifuged and the supernatant is collected for estimation of any proteins.

Note: This should be done in highly septic condition to avoid contamination.

OBSERVATION:

RESULT:

EXPT NO: 4

COUNTER CURRENT IMMUNOELECTROPHORESIS

AIM:

To identify the antigen specific antibody present in the antisera by counter current immunoelectrophoresis.

PRINCIPLE:

Counter current immunoelectrophoresis is a rapid version if Ouchterlony double diffusion technique. It is primarily a qualitative test, although from the thickness of the precipitin line relative measure of quantity can be obtained.

The antigen is placed in a well at the cathode end and antibody is placed at the anode side. During electrophoresis molecules placed in an electric field acquire a charge depending on their PI (Isoelectric point). Hence they move towards the appropriate electrode. The antigen, if it is negatively charged moves towards the anode.

Antibody (immunoglobulin) at pH 7.6 has a charge nearing zero. During electrophoresis, the agarose matrix absorbs hydroxyl ions on the surface resulting in a net increase in positive ions at a distance from matrix. These positive ions migrate towards the negative pole with a solvent shield, resulting in a net solvent flow called endosmosis. Hence antibody molecules which have no charge move towards cathode along the solvent shields due to this phenomenon. Thus the antigen and antibody travel towards each other and at a point of optimal concentration of both, a line of precipitin is formed.

REQUIREMENTS:

Glassware **:** conical flask, measuring cylinder, glass plates

Reagents **:** Antigen, Test antiserum, Agarose, Distilled Water, Alcohol, Electrophoresis buffer.

Other Requirements : Micropipette, Tips, Positive control gel puncture.

PROCEDURE:

1. Dilute electrophoretic buffer to 1X concentration from the 5X concentration using double distilled water.

2. Prepare 1.5% agarose using buffer for 10 ml
3. Clean the immunoelectrophoresis plate and to this add agarose with a uniform flow.
4. Allow it to solidify completely. Care should be taken that the thickness is uniform and thin enough for the diffusion to take place.
5. Punch 3mm well at one side of the gel in the centre using gel cutter and cut out the gel corresponding to the markings on the template.
6. Place the slides in the electrophoresis tank and fill the tank with 1X electrophoresis buffer till the buffer just covers the gel surface. Do not add excess of buffer.
7. Add 10 µl of antigen in each of the four wells towards cathode and 10 µl of positive control antiserum and three test antisera in wells towards anode.
8. Connect the cord to the electrophoretic power supply.
9. Apply 50V -100V and allow the electrophoresis to continue for about 45 minutes.
10. Once the dye has run for 3-4cm length, stop the electrophoresis and remove the gel, allow to air dry to remove the buffer.
11. Observe for precipitin line between the antigen and antisera wells.

OBSERVATION:

RESULT:

EXPT NO: 5

SANDWICH ELISA

AIM:

To determine the concentration of antigen by sandwich ELISA

PRINCIPLE:

ELISA or enzyme linked immunosorbent assay is a sensitive laboratory method used to detect the presence of antigens (Ag) or antibodies (Ab) of interest in a wide variety of biological samples. This assay requires an immunosorbent, i.e., antigen or antibody immobilized on solid surface such as the wells of micro titre plates or membranes.

In this method, two antibodies that can bind to two different epitopes on the same antigen are required. One of the antibodies is immobilized on a microtitre well and is referred to as capture antibody and the other antibody is labelled with a suitable enzyme [e.g., horse radish peroxidise (HRP), alkaline phosphatase (ALP) etc.] and is referred to as labelled antibody. Sample (standard and test) containing the antigen is allowed to react with the immobilized antibody. After the well is washed, labelled antibody is added and allowed to react with the bound antigen. Unreacted labelled antibody is washed out and the enzyme bound to solid support is estimated by adding a chromogenic substrate. The colour developed is measured spectrophotometrically.

REQUIREMENTS:

Glassware	: Eppendorf tubes, syringe.
Reagents	: 1X assay buffer, Test serum sample, Dot Elisa strip, Antibody- HRP, Substrate- TMB H_2O_2.
Other Requirements	: Micropipette, Tips, Centrifuge.

PROCEDURE:

I Coating of wells with Capture antibody

1. Dilute 60 µl of reconstituted capture antibody with5.94 ml of coating buffer. Concentration of the diluted capture antibody is 10 µg/ml.

2. Pipette 200 µl of diluted (1X) capture antibody into each of the micro titre well (24 wells). Tap or shake the plate to ensure that the capture antibody solution is evenly distributed over the bottom of each well.
3. Incubate the micro titre wells overnight at 4°C.

II Blocking the residual binding sites on the wells.

4. Discard the well contents. Rinse the wells with distilled water three times draining out the water after each rinse.
5. Fill each well with 200 µl of blocking buffer and incubate at room temperature for 1 hour.
6. Rinse the plate three times (as in step 4) with distilled water. Drain out the water completely by tapping the plate on a blotting paper. Addition of antigen to wells.
7. Prepare standard and test antigen dilutions.
8. Add 200 µl of standard antigen, diluted test samples and PBST to the coated wells as indicated in fig 1(in duplicates).
9. Incubate at room temperature for 30 minutes.
10. Discard the well contents, fill the wells with 1X PBST, and allow it to stand for 3 minutes, discard the contents. Repeat this step two more times. Addition of HRP labeled antibody.
11. Dilute 5 µl of 1000X Antibody-HRP conjugate with 5 ml of sample diluents to get 1X concentration.
12. Add 200 µl of 1X HRP labelled antibody to all the wells.
13. Incubate at room temperature for 30 minutes.
14. Discard the well contents and rinse the wells 3 times with 1X PBST. (As in step 10).

Addition of substrate and measurement of absorbance.

15. Dilute required amount of 10X TMB/H2O2 (substrate) solution to 1X using distilled water.
16. Add 200 µl of 1X substrate to each well.
17. Incubate at room temperature for 10 minutes.
18. Add 100 µl of 1X stop solution to each well.
19. Transfer the contents of each well to individual tubes containing 2 ml of 1X stop solution.20.Prepare substrate blank by adding 200 µl of 1X substrate solution to 2.1 ml of 1X stop solution.
21. Read the absorbance at 450 nm after blanking the spectrophotometer with substrate blank, record your readings as follows:

Calculation of antigen concentration in test sample:

22. Calculate the average A450 for each of the samples (standard and test)
23. Plot A450 of standards on Y axis (linear scale) versus the concentration of antigen in ng/ml on X axis (log scale) on a semi-log graph sheet. (Refer Fig 2).
24. From the standard curve, determine the concentration of antigen in the test samples.
25. Calculate the concentration of antigen in mg/ml, in each of the test samples as follows:

Concentration of antigen in the sample=

Concentration in ng/ml (from the graph) X Dilution factor

106

= _____ mg/ml

OBSERVATION:

RESULT

EXPT NO: 6

DOT ELISA

AIM:

To perform sandwich dot ELISA test for the detection of antigen.

PRINCIPLE:

Dot ELISA is an extensively used immunological tool in research, as well as in analytical and diagnostic laboratory. In this the antigen is sandwiched directly between the antibodies which react with two different epitopes on the same antigen. Here one of the antibodies is immobilized on to a solid support and the second antibody is linked to an enzyme. Antigen in the test sample first reacts with the immobilized antibody and then the second enzyme linked antibody. Among the enzyme linked antibody bound this assay by incubating the strip with an appropriate chromogenic substrate which is converted into a coloured insoluble product. The later precipitates on the strip in the area of enzyme activity hence the name Dot ELISA. The enzyme activity is indicated by the intensity of the spot which is directly proportional to the antigen concentration.

REQUIREMENTS:

Glassware **:** Eppendorf tubes, syringe.

Reagent **:**1X assay buffer, Test serum sample, Dot Elisa strip, Antibody-HRP, Substrate- TMB H_2O_2.

Other Requirements **:** Micropipette, Tips, Centrifuge.

PROCEDURE:

1. In a vial take 1ml of 1X assay buffer and 50 µl of test serum sample. Mix thoroughly and insert a Dot ELISA strip.
2. Allow the reaction to occur at room temperature for 20 minutes.
3. Wash the strip by dripping it in 1ml of 1X assay buffer for about 5 minutes. Repeat it thrice and replace the buffer each time.
4. Take 1ml of 1X assay buffer in a fresh vial and add 10 µl antibody HRP conjugate. Mix thoroughly.

5. Dip the strip and allow the reaction to take place for 20 minutes.
6. Wash the strip by dipping it in 1ml of 1X assay buffer for about 5 minutes. Repeat it thrice and replace the buffer each time.
7. In a fresh vial take 0.1ml of 10X TMB or H_2O_2 and 0.9ml of distilled water.
8. Dip the strip in the substrate solution.
9. Observe the strip after 10 to 20 minutes for the appearance of blue colour.
10. Rinse the strip with distilled water to stop the reaction.

OBSERVATION:

RESULT

EXPT NO: 7

MTT ASSAY FOR CELL CYTOTOXICITY

AIM:

To determine the cytotoxicity of cells based on their viability.

PRINCIPLE:

MTT (3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide) assay, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tertrazolium rings of the pale yellow MTT and form a dark blue colored formazan crystals which is largely impermeable to cell membrane, thus resulting in its accumulation within healthy cells. Solubilization of cells by the addition of detergents results in the liberation of crystals which are solubilized. The number of surviving cells is directly proportional to the level of formazan product created. The color can be quantified using a multi-well plate reader.

MATERIALS REQUIRED:

Glassware **:** Culture plates, wash beaker.

Reagents **:** Cells in a growth medium, DMSO (Dimethyl sulfoxide), Triton X-100, MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide)(5 mg/ml).

Other Requirements : Micropipette, Tips, Cuvette, UV-Spectrophotometer

PROCEDURE:

1. Take 200µl of cells/well (20,000-50,000 cells) in 3 wells and name the wells as 1, 2 and 3 respectively.
2. Add 20 µl of MTT (5mg/ml) to well 2 and well 3.
3. Add 10 µl of Triton X-100 to well 3.
4. Incubate the above at 37^0C for 2-3 hours.
5. Add 100 µl of DMSO to all the wells to solubilize the crystals.
6. Centrifuge the contents in each well at 1500 rpm for 5 minutes.
7. Take the supernatant and measure the absorbance at 570nm.

Negative control : cells (well 1)

Test : cells + MTT (well 2)

Positive control : Cells + MTT + Triton X-100 (well 3)

Formula:

Control OD - Test OD/Control OD X 100

OBSERVATION:

RESULT:

EXPT NO: 8

WESTERN BLOTTING

AIM:

To learn the technique of western blotting which involves the following experiments:
Electrophoresis of the protein (SDS PAGE)
Electro transfer of protein onto nitrocellulose membrane (Western blotting)
Immunodetection of the transferred protein (Blot development)

PRINCIPLE:

Western blotting is a rapid and sensitive assay for detection and characterization of proteins. Western blotting technique exploits the inherent specificity of antigen - antibody interaction to identify the specific antigens by polyclonal or monoclonal antibodies with the intergrative experiment of SDS-PAGE, western blotting and immunodetection.

SDS PAGE:

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS – PAGE) is carried out in a discontinued buffer system wherein the reservoir buffer is of a different pH and ionic strength from the buffer used to cast the gel. The SDS – polypeptide complexes in the sample applied to the gel are swept along by a moving boundary created when an electric current is passed between the electrodes. After migrating through the stacking gel of high porosity, complexes get deposited in a very thin zone on the surface of the resolving gel. On further electrophoresis, polypeptides get resolved based on their size in the resolving gel.

Western blotting:

Blotting is transfer of resolved proteins from the gel onto a surface of a suitable membrane, done commonly by electrophoresis and referred to as electroblotting. The gel is place in contact with nitrocellulose membrane which is then sandwiched between filter paper, two porous pads and two plastic supports. The entire set up is then placed in an electrophoretic tank containing blotting buffer. The proteins get transferred to the corresponding position on the membrane as resolved on the polyacrylamide gel, forming a

mirror image of the gel. Protein of interest on the membrane is further located by immunodetection.

Immunodetection:

The transferred proteins bound to the surface of nitrocellulose membrane are detected using immunological reagents. This process is known as immunodetection. All the unoccupied sites on the membrane are first blocked with either an inert protein, a detergent or any other suitable blocking agent. The membrane id then probed with a primary antibody specific to the protein of interest. The Ag- Ab complex formed on the membrane is then identified using an enzyme – labelled secondary antibody and a suitable substrate to the enzyme, which results in a coloured band on the nitrocellulose membrane, referred to as blot development.

MATERIALS REQUIRED:

Equipment : Gel rocker, SDS – PAGE apparatus , transfer setup,

Glassware : conical flask, measuring cylinder, petridish, staining tray

Reagent : protein sample, protein marker, acrylamide and bis-acrylamide, APS, TEMED, Tris buffer, primary antibody, secondary antibody, wash buffer, HRP, substrate, distilled water

Other Requirements: Micropippettes, Tips, Water bath, nitro cellulose memberane.

PROCEDURE:

SDS – PAGE:

1. Assemble the plates for casting the gel.
2. Clamp the assembly of plates to fix it in a gel casting apparatus. Ensure the assembly is leak proof by filling water between the plates.
3. Add 50ul of APS solution to 5ml of SDS separating gel mix and pour the gel solution between the plates till the level is 2cm below the top edge of the notched plate.
4. Add 200 to 250ul of water to make the surface even.
5. After the gel is set, wash the top of the separating gel with distilled water and drain off the water completely.
6. Add 20ul of APS solution to 2ml of stacking gel mix and pour directly onto the polymerized separating gel.
7. Insert the comb onto the gel solution carefully.

8. Add 25ul of sample loading buffer to the protein sample and also the protein marker.
9. After the stacking gel has set, carefully remove the comb and the bottom spacer.
10. Load 30ul protein marker in well 1 and 30ul of all protein samples. (this gel to be used for SDS staining), while the second gel to be transferred to NC membrane , 10ul of the marker and all protein samples were added.
11. Connect the cords to the power supply
12. Set voltage at 100V and switch on the power supply.
13. When the dye front comes to 0.5cm above the bottom of the gel, turn of the power.
14. Remove the gel plates and gently pry the plates apart using a spatula.
15. Transfer the gel to a tray containing water; wash the gel for 1-2 minutes at room temperature.
16. Decant the water,
17. To the gel add 20ml of stainer, stain at room temperature for 1-2 hours.
18. Decant the staining solution add minimum quantity of water to cover the gel.

Electroblotting:
19. Assemble the blotting sandwich within the blotting cassette. Take care to avoid air bubbles between the gel and NC membrane.
20. Insert the cassette into the apparatus filled with blotting buffer and connect blotting unit to power supply.
21. Electrophorese the sample at 50V for 2 hours for blotting to occur.
22. Remove the NC membrane gently from the cassette and place the membrane in 10ml of freshly prepared blocking buffer taken in a petridish. Leave it overnight at 4 degree celcius.

Immunodetection:
23. Discard blocking buffer.
24. Immerse blot in 10ml of primary antibody solution and mix gently for 30minutes. Discard the primary antibody solution.
25. Wash the blot by immersing in 10ml wash buffer for 3-5 minutes. Discard buffer each time.
26. Immerse the blot in 10ml of 1X HRP labelled antibody, mix gently for 30minutes. Discard the HRP labelled antibody.
27. Wash the blot by immersing in 10ml wash buffer for 3-5 minutes. Repeat the wash process four to five times, discarding the buffer each time.

28. Remove the blot, wash with distilled water, discard and dry.
29. Compare the SDS-PAGE gel with the developed NC membrane.

OBSERVATION:

RESULT:

EXPT NO: 9

DETECTION OF CELL SURFACE MOLECULES BY FLOW CYTOMETRY

AIM:

To detect CD4+ T cells by flowcytometry

PRINCIPLE:

CD4+ T helper cells are white blood cells that are an essential part of the human immune system. They are often referred to as CD4 cells, T-helper cells or T4 cells. They are called helper T cells because one of their main roles is to send signals to other types of immune cells, including CD8 killer cells. CD4 cells send the signal and CD8 cells destroy and kill the infection or virus. If CD4 cells become depleted, for example in untreated HIV infection, or following immune suppression prior to a transplant, the body is left vulnerable to a wide range of infections that it would otherwise have been able to fight.

Flow cytometry can be used to analyze various intracellular and extracellular molecules including phosphorylated signalling proteins and cytokines. There are many variables that must be optimized for individual flow cytometry experiments such as antibody incubation time and temperature. Furthermore, to stain intracellular molecules, the cells need to be fixed in suspension and then permeabilized before the detection antibody is added. This fixation/permeabilization treatment allows the antibody to pass through the plasma membrane into the cell interior, while keeping the morphological characteristics used to sort the cells intact. Alcohols, such as methanol or ethanol are commonly used to permeabilize the cell. Cold methanol is typically used as a permeabilization agent when using flow cytometry to detect phosphorylated proteins and transcription factors, because it can increase the reactivity of antibodies to certain nuclear antigens.

MATERIALS REQUIRED:

Equipment : Centrifuge, Vortexer.

Glassware : FACS™ Tubes (5 mL round-bottom polystyrene tubes)

Reagent : Blood sample, Isotype Control Antibodies, 1X RBC Lysis Buffer 200 mL, PBS (1X): 0.137 M NaCl, 0.05 M NaH_2PO_4, pH 7.4 or Hank's Balanced Salt Solution (HBSS; 1X), Flow Cytometry Fixation Buffer

or an equivalent solution containing 1 - 4% paraformaldehyde), -20 °C Methanol, Detection Antibodies.

Other Requirements: Micropippettes, Tips, eppendorf tubes.

PROCEDURE:

1. Take 1-200 µl of blood into heparin microfuge tube.
2. Add 0.1ml 10x phosphate buffered saline (PBS), mix well and spin at 1200rpm for 5 minutes (RT).
3. Carefully remove supernatant and RBC leaving WBC pellet
5. Suspend in 200 µl of FACs buffer (0.1% BSA in PBS)
7. Split 2 above into:

a) 100 µl control (in PBS)

b) 100 µl test

8. Add 10 µl of fluorochrome- conjugated antibody for 1 hour at 4 °C.
9. Wash (2 times) with 100 µl FACs buffer and spin at 1200 rpm for 5 minutes.
14. Add 500 µl 4% formaldehyde in PBS, mix, cover in foil and store at 4° C until ready to run on flow cytometry.

OBSERVATION:

RESULT:

EXPT NO: 10

DETECTION OF APOPTOTIC PROTEINS BY FLORESCENCE MICROSCOPY

AIM:

To detect the apoptotic proteins by annexins v staining method

PRINCIPLE:

Annexins are a family of calcium dependent phospholipid binding proteins that preferentially bind phosphatidylserine (PS). Under normal physiologic conditions, PS is predominantly located in the inner leaflet of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution across the phospholipid bilayer and is translocated to the extracellular membrane leaflet marking cells as targets of phagocytosis. Once on the outer surface of the membrane, PS can be detected by fluorescently labelled Annexin V in a calcium dependent manner. In early stage apoptosis, the plasma membrane excludes viability dyes such as propidium iodide (PI). These cells will stain with Annexin V but not a viability dye, thus distinguishing cells in early apoptosis. However, in late stage apoptosis, the cell membrane loses integrity thereby allowing Annexin V to also access PS in the interior of the cell. A viability dye can be used to resolve these late stage apoptotic and necrotic cells (Annexin V, viability dyepositive) from the early stage apoptotic cells (Annexin V positive, viability dye negative)

MATERIALS REQUIRED:

Equipment : Centrifuge, Vortexer.

Reagent : Blood sample, Annexin V-FITC Apoptosis Detection Kit, 10X Annexin V Binding Buffer, Make 15mL of fresh 1X buffer with H2O, FITC Annexin V, Propidium Iodide Staining Solution (PI), PBS (phosphate buffered saline)

Other Requirements : Micropipettes, eppendorf tubes, Tips, Water bath.

PROCEDURE:

1. Transfer $1x10^6$ cells to a eppendorf tube containing 1mL media.
2. Pellet cells at 1,500 rcf for 4 minutes.
3. Aspirate supernatant and resuspend the cell pellet in 1mL cold PBS.
4. Pellet cells at 1,500 rcf for 4 minutes.
5. Repeat steps 3 and 4 one more time.
6. Aspirate supernatant, and resuspend cells in 1mL 1X buffer.
7. Place the cell suspension on a glass slide.
8. Cover the cells with a glass coverslip.
9. For analyzing adherent cells, grow cells directly on a coverslip. Following incubation (A.5), invert coverslip on a glass slide and visualize cells.
10. The cells can also be washed and fixed in 2% formaldehyde before visualization. (Cells must be incubated with Annexin V-FITC before fixation since any cell membrane disruption can cause nonspecific binding of Annexin V to PS on the inner surface of the cell membrane.)
11. Observe the cells under a fluorescence microscope using a dual filter set for FITC. Cells that have bound Annexin V-FITC will show green staining in the plasma membrane.
12. Cells that have lost membrane integrity will show red staining (PI) throughout the nucleus and a halo of green staining (FITC) on the cell surface (plasma membrane).

Note:

a. Cells negative for Annexin V and negative for propidium iodide are nonapoptotic.
b. Cells positive for Annexin V and negative for propidium iodide are viable, but undergoing apoptosis.
c. Cells positive for Annexin V and positive for propidium iodide are dead.

OBSERVATION:

RESULT: