



A Path to Protein Quantitation: PBMCs to Western Blot

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ABSTRACT

Proteins are biopolymers made up of one or more long chains of amino acid residues. These proteins mediate thousands of metabolic pathways that determine how an organism functions. When proteins are thoroughly investigated, they can provide information about health and disease. A variety of methods have been developed to quantify both complex protein mixtures for total protein content as well as a single type of protein. This manuscript covers the methodologies standardized in our lab based on previously published protocols, beginning with the isolation of peripheral blood mononuclear cells (PBMCs) from human blood samples, cell viability testing, cell counting, total protein extraction methods, and protein quantification and estimation techniques, which enable a researcher to find the protocol from PBMCs to Western blot in a single manuscript.

Keywords: PBMCs, Western blot, Chemiluminescence.

1. Introduction

A protein is an amphoteric, complex biomolecule made up of long chains of amino acid residues connected by peptide bonds. There are a set of 20 amino acids used to build proteins in all living beings. However, two additional amino acids Selenocysteine and pyrrolysine, which were found in proteins in 2002 and 1986 in small amounts are now considered 21st and 22nd amino acids, respectively [1]. Proteins serve as the foundation for many critical processes in the body and regulate a wide variety of organism functions, including DNA replication, transporting molecules, catalyzing metabolic reactions, and providing structural support to cells. A protein can be identified based on each level of its structure [2].

Protein analysis is critical for understanding their function and the effects of their presence, absence, and alterations. The discovery of the biomarkers and the understanding of health and diseases is possible due to the advancement in the basic techniques of protein isolation, identification, and quantitation [3]. Thousands of metabolic pathways that determine how an organism works are mediated by proteins. Current understanding of how proteins change cellular phenotype is based on in vitro and in vivo functional assays. When investigating proteins, the most difficult obstacle to overcome is determining the best protein extraction method. However, before choosing an extraction method, consider the type of tissue/cells as well as the protein's subcellular location. Proteins can be extracted from a variety of sources, including a patient's cells or tissues for diagnostic purposes, as well as microbes or cell lines derived from insects, vertebrate animals, or plants for laboratory research [4].

Separation techniques rely on the differences in the chemical, structural, and functional properties of the target protein in comparison to other proteins in the crude mixture. These characteristics include size, shape, charge, isoelectric point, charge distribution, hydrophobicity, solubility, density, ligand-binding affinity, metal binding, reversible association, posttranslational changes, and unique sequences or structures, [5], [6]. A sample must first be solubilized, typically in a buffered aqueous solution, before being analyzed for total protein concentration. The sample will contain a variety of non-protein components depending on the source material and the methodologies used prior to performing the protein assay. These factors



must be understood in order to select an appropriate assay method.

Our manuscript combines few protein chemistry techniques standardized in our lab based on previously published protocols, beginning with the isolation of peripheral blood mononuclear cells (PBMCs) from human blood samples, cell viability testing, cell counting, total protein extraction methods, and protein quantification and estimation techniques, in a single article. Researchers in this field will benefit from having simple access to these workable standardized protocols of our lab.

2. Protein Chemistry Techniques

2.1 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) include lymphocytes and monocytes. Various cell separation methods are used to isolate them directly from whole blood. There are three methods for isolating PBMCs: I) density gradient centrifugation, II) fluorescence-activated cell sorting (FACS), and III) magnetic field-activated cell sorting (MACS). This paper deals with the most commonly used method, density gradient centrifugation.

2.1.1 PBMC Isolation by Density Gradient Centrifugation

PBMCs are extracted from whole blood using Ficoll Histopaque, a hydrophilic polysaccharide that separates blood layers, followed by gradient centrifugation, which separates cell populations based on physical properties such as size and density. Centrifugation of a standard whole blood sample separates the general layers of blood components, concentrating red blood cells (roughly 45% of total volume) at the bottom of the tube, PBMCs (roughly 1% of total volume) in the middle layer, and blood plasma as the top layer [7].

Procedure:

2.1.1a Sample Collection

EDTA Vacutainer tubes should be used to collect 5 ml venous blood sample. If EDTA tubes are not available, then use 1% fresh EDTA (100 μ l/ml blood) to the non-EDTA tubes before adding blood.

2.1.1b PBMCs Isolation:

Separating PBMCs from whole blood is done with Ficoll-Histopaque (**Figure 1**).

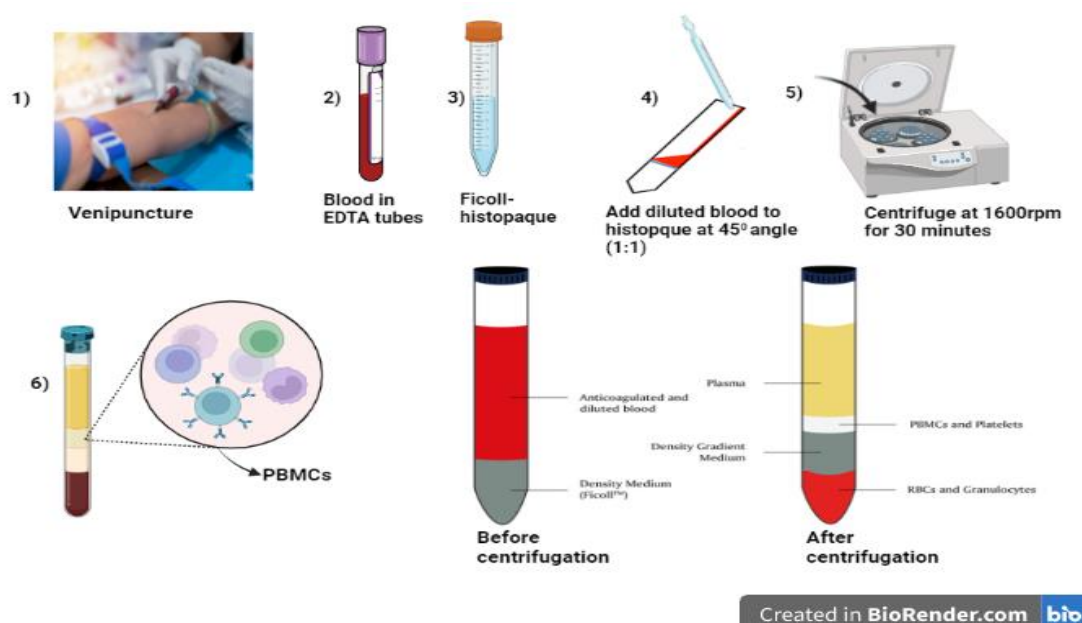


Figure 1: PBMC isolation from whole blood via Density gradient centrifugation.

- i. Dilute fresh blood with 0.9% NS (Normal saline) in a 15 ml falcon tube at 1:1 ratio.
- ii. Add Histopaque (RT) to a new falcon tube and gently layer an equivalent amount of blood along the side walls of the falcon tube. Maintain a 45° angle in the falcon tube before adding blood.
- iii. Then, without disrupting the falcon tubes, centrifuge them at 1600 rpm for 30 minutes.
- iv. Remove the tubes and collect the white buffy coat present in ring form in the middle, which contain only PBMCs, without disturbing the other layers.
- v. For cell counting, aliquot 10 µL of the PBMC cell suspension and keep the rest in -20 °C deep freezer for further use.

Note: Flowchart for PBMC isolation is given below (**Figure 2**).

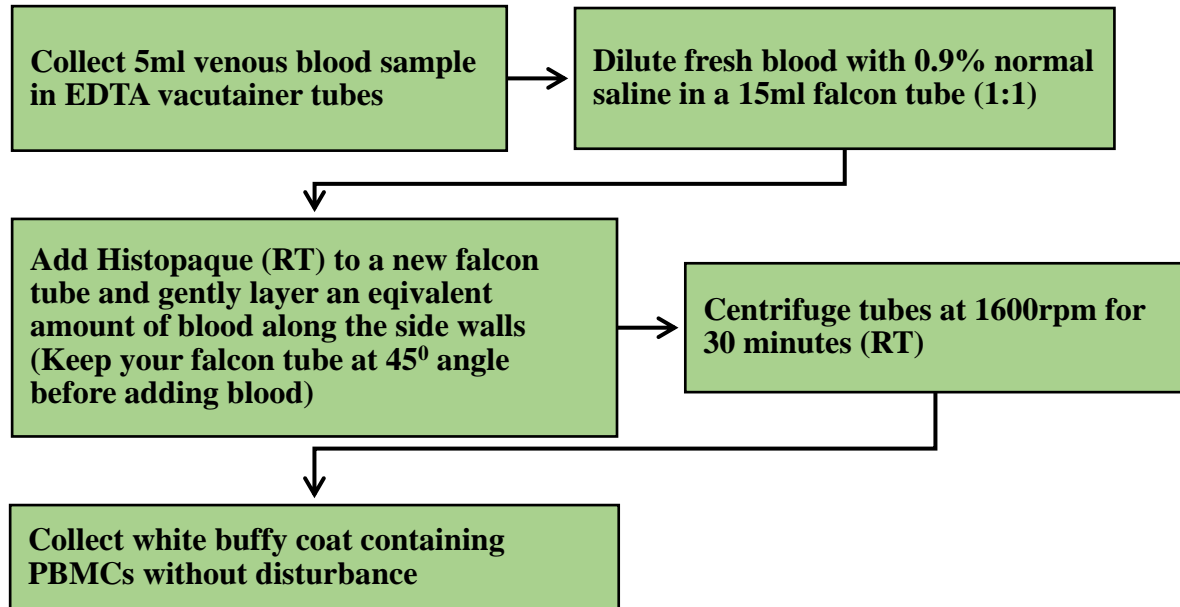


Figure 2: Flow chart -PBMC isolation from Human blood

2.2 Cell Counting and Viability Check

Cell counting is essential for determining concentrations, viability, and evaluating cell isolation results. A hemocytometer is a microscopic glass slide with a grid of perpendicular lines (**Figure 3**) in the center that counts cells. The grid has predefined dimensions that allow counting the number of cells in a given volume of solution by calculating the area covered by the lines [8].

Trypan blue dye is used for counting viable cells. It penetrates the cell membrane of dead cells and stains it blue whereas the live cells exclude the dye and remain colorless. The ability of live cells to exclude the blue dye distinguishes them from dead cells [9]. Four corner squares of hemocytometer are used for cell counting out of nine large squares. When counting cells, only the top and left borderline cells are counted and bottom and right borderline cells are ignored in order to prevent the duplicate counting of cells.

Procedure:

Prepare the material before examining the cells under the microscope.

2.2.1 Sample preparation

- i. Prepare 0.4 percent Trypan blue (TB) stock solution (0.04 g TB in 10 ml dH₂O).
- ii. Prepare 5x saline solution by dissolving 0.5 g NaCl in 10 ml dH₂O.
- iii. Take 2 ml TB from stock solution and add 0.5 ml (5x) saline solution to it in another tube and label the tube as working TB.

- iv. Take 10 μL of Trypan Blue working solution into a 1.5 ml fresh microfuge tube and then add 10 μL of PBMC cell suspension to it. Label the tube. The ratio of Trypan Blue to cell suspension should be 1:1.
- v. Vortex briefly (touch-spin if using 1.5 mL microfuge tubes to bring contents down from lid).

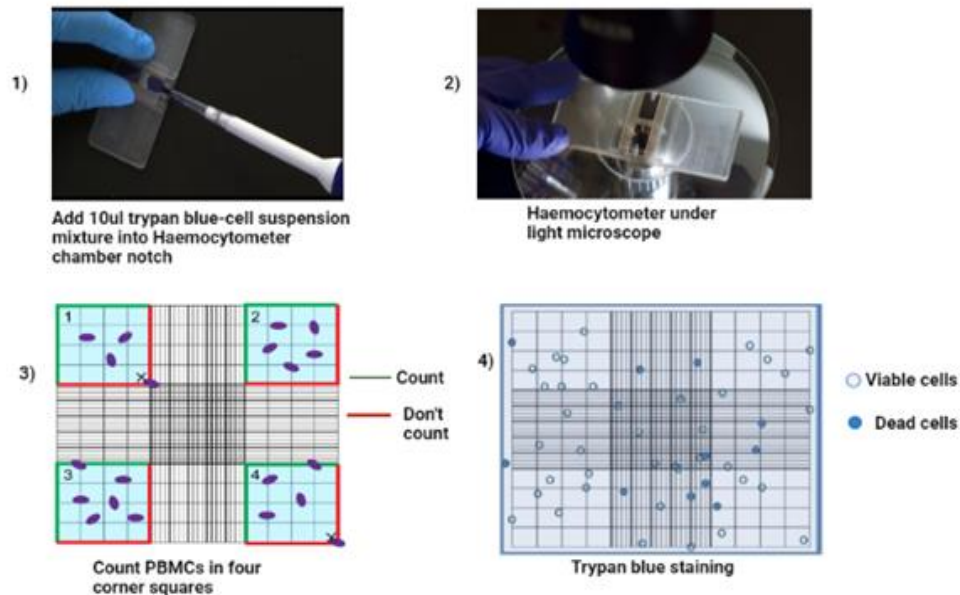


Figure 3: 1. Hemocytometer, 2. Visualization under microscope, 3. Hemocytometer grid lines, 4. Dead cells retain trypan blue dye and viable cells without dye.

2.2.2 Cell counting

- i. Pipette out 10 μL of Trypan Blue-cell suspension and load it into Hemocytometer chamber between chamber notch and glass coverslip (Fix cover slip on the hemocytometer by water drops).
- ii. Count cells in four large corner squares under a light microscope (20X) or phase contrast microscope. Count the top and left boundary cells and ignore right boundary and bottom line cells to prevent duplicate counting of the cells.

For example: Count large corner squares of Hemocytometer so the total squares counted = 4

Total viable cells in all counted squares = (9 + 7 + 10 + 9) = 35 cells.

Total non-viable/dead cells counted = (4 + 3 + 0 + 2) = 9 cells.

Percentage of Viable cells = no. of viable cells / total no. of cells x 100.

$$= 35 / 44 \times 100 = 79\%$$

Average no. of cells/square = no. of counted viable cells / no. of counted squares.

$$= 35 / 4 = 8.75$$

Dilution factor = Final volume (TB + cells) / volume of cells.

$$= (10 \mu\text{L} + 10 \mu\text{L}) / 10 \mu\text{L} = 2$$

Concentration (viable cells/ml) = Average no. of cells/square x Dilution factor x 10^4

*(10^4 is multiplied here to give the cell count per ml of blood)

$$\text{i.e., } 8.75 \times 2 \times 10^4 = 175000 \text{ cells/ml}$$

Scientific notation: 17.5 x 10^4 cells/ml

$$\text{Or } 1.75 \times 10^5 \text{ cells/ml}$$

Note: Flow chart for Cell viability check and counting is given below (**Figure 4**).

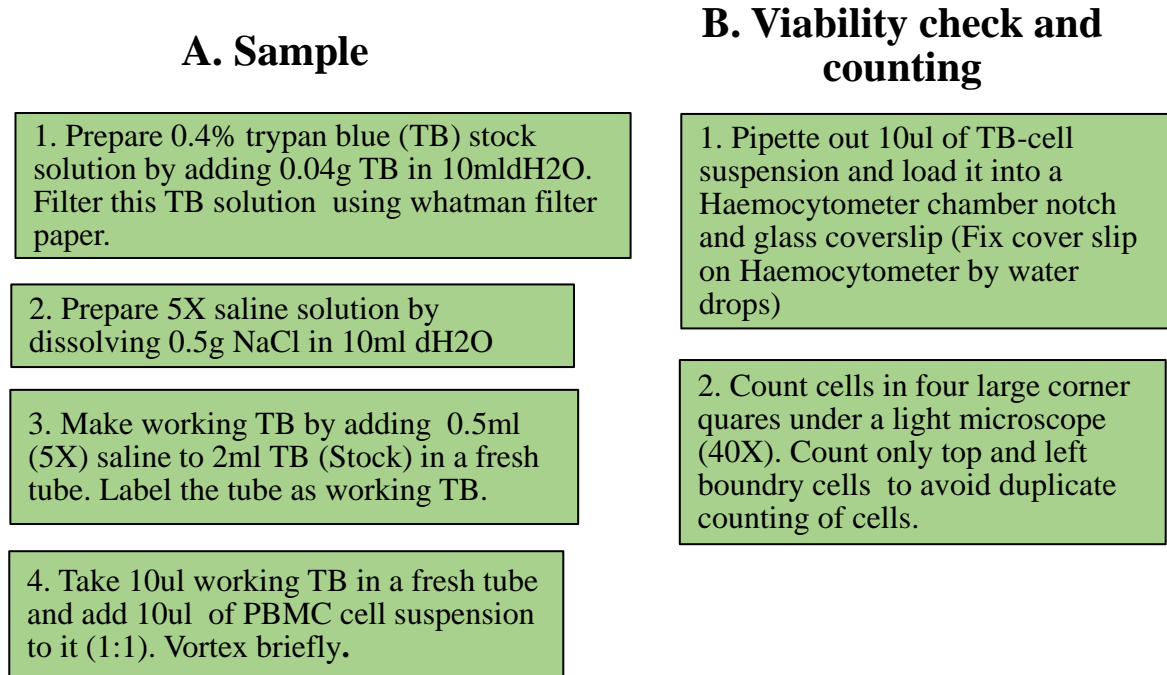


Figure 4: Flow Chart -Cell Counting and Viability.

2.3 Protein Extraction by RIPA (Radio Immunoprecipitation Assay) lysis buffer

RIPA buffer is one of the most reliable buffers for lysing mammalian cells from suspension cultures and plated cells. This solution can be used in a variety of applications such as reporter assays, protein assays, immunological assays, protein purification and is suitable for protein extraction from cytoplasmic, membrane, and nuclear proteins [10].

RIPA buffer lacks protease and phosphatase inhibitors, they must be added to RIPA buffer just before use. For every 1×10^6 cells, 100 μ l of RIPA buffer is used. To 1 ml of RIPA buffer 5 μ l of protease inhibitor need to be added before adding samples to RIPA buffer [11].

Procedure:

- i. Centrifuge the cell suspension at 5000 G for 5-7 minutes at 4 °C. Cells are collected at the bottom of the tube as white pellet (**Figure 5**). Discard the supernatant.
- ii. To the cell pellet, add ice-cold 1x PBS and wash the cells by centrifuging at 5000 G for 5-7 minutes at 4 °C (1.25 ml PBS for the PBMC cell suspension isolated from 5 ml blood sample). Repeat the same step one more time, it will remove all the contamination. Discard the supernatant.
- iii. Add ice-cold RIPA Buffer to the cell pellet. Agitate the mixture by pipetting up and down for few minutes. Shake mixture gently for 15 minutes on ice. Centrifuge mixture at 16000 G for 20 minutes at 4 °C.
- iv. Collect the supernatant containing proteins in autoclaved 1.5 ml tubes and place it on ice. Discard the pellet.
- v. Take an aliquot for quantification and further analysis if needed. Store protein extracts in -20 °C freezer.

Protein extraction protocol using RIPA lysis buffer

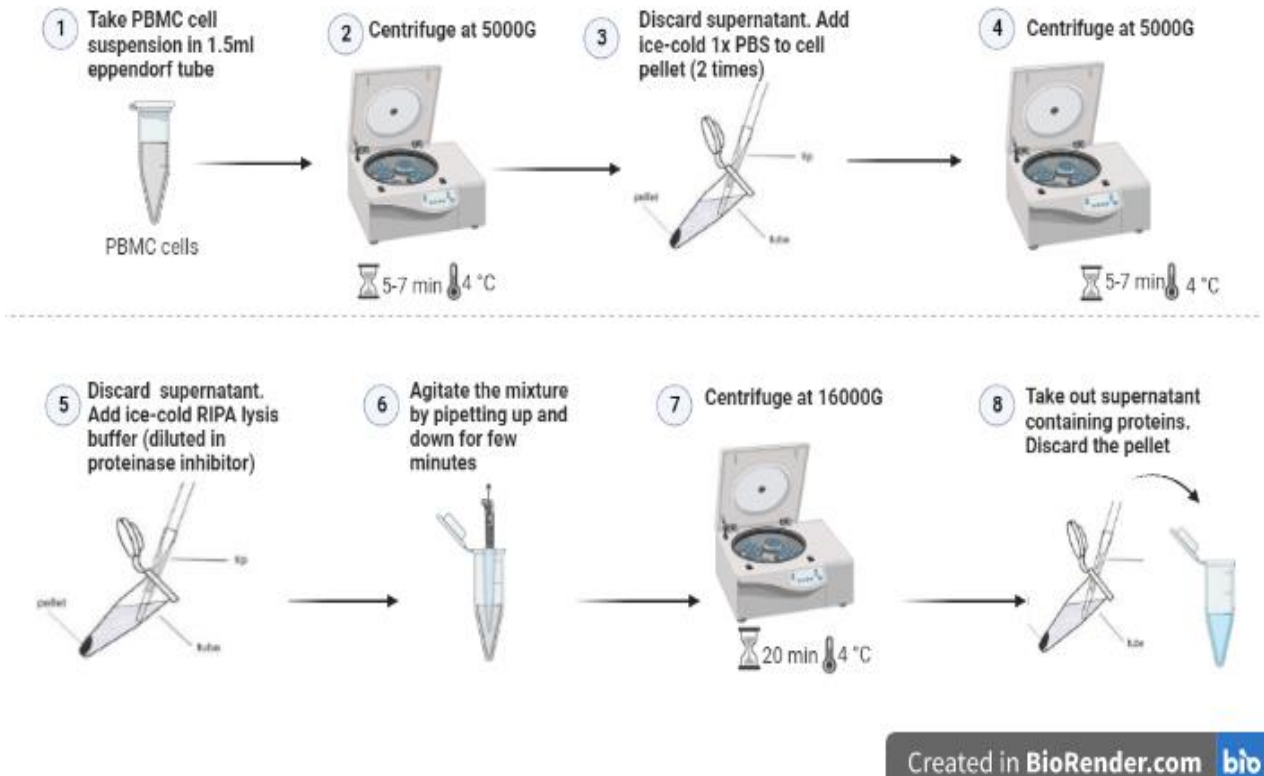


Figure 5: Steps involved in whole protein extraction from PBMCs using RIPA lysis buffer.

2.4 Protein quantification and estimation assay

Protein quantification is required in a wide range of scientific fields. A variety of methodologies have been developed to quantify complex protein mixtures as well as a single type of protein. Protein concentration assays include absorbance at 280 nm, Lowry Assay, Bradford Assay, and Bicinchoninic Assay [12]. Bradford assay, a commonly used assay in laboratories is covered here.

2.4.1 Bradford or Coomassie brilliant blue protein assay (range: 20-2000 µg/ml)

To determine protein concentration, the Bradford assay relies on the formation of a compound between Coomassie brilliant blue G-250 dye and proteins in solution. The protein concentration is determined by the amount of dye in the blue anionic form as measured by the absorbance of the solution at 595 nm with a spectrophotometer. The color binds to the amino acids arginine, tryptophan, tyrosine, histidine, and phenylalanine most of the time [13].

The assay is based on the fact that when Coomassie Brilliant Blue G-250 binds to protein, the maximum absorbance in an acidic solution shifts from 465 nm to 595 nm. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, resulting in a noticeable color change [14], [15], and [16].

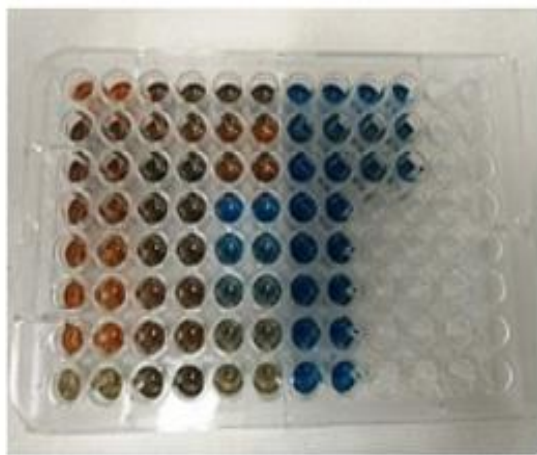
Reagents:

Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman paper just before use. Commercially available Bradford reagent can also be used. Both 96 well plate as well as cuvettes can be used for this assay as per the availability.

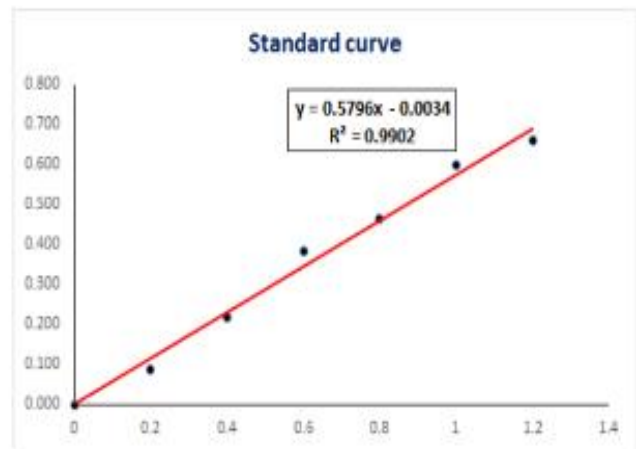
Procedure: (96 Well Plate Assay Protocol)

96-well plate (Figure 6a) allows rapid analysis of multiple protein samples while using a small sample volume (5 μ l). Bradford reagent is light sensitive hence assay to be performed in a dark room.

- i. Prepare BSA stock solution (10 mg/ml).
- ii. Prepare protein standards 1-7 (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mg/ml) using BSA stock solution.
- iii. Dilute unknown protein samples to obtain between 30-35 μ g concentrations. Dilution should be 1:10.
- iv. Shake the Bradford Reagent and bring to room temperature before use.
- v. To each well add 200 μ l of the Bradford Reagent.
- vi. Add 5 μ l of the standards, blank and unknown protein samples in duplicates to the wells having Bradford reagent. Use 5 μ l distilled H₂O or Milli-Q water as blank.
- vii. Incubate the samples at room temperature for 5-10 minutes. Then measure the absorbance at 595 nm using Spectrophotometer.
- viii. The absorbance of the samples must be recorded before the 60 minute time limit as the protein-dye complex is stable for 60 minutes.
- ix. Note the OD values of each standard and sample.
- x. Plot a standard curve between the net absorbance (OD) and the protein concentration of each standard in Excel sheet (Figure 6b).
- xi. Determine the protein concentration of the unknown sample(s) by using the formula “ $y=mx+c$ ”, where, ‘x’ is concentration of samples, ‘y’ is OD value of samples, ‘c’ is intercept and ‘m’ is slope.



(a)



(b)

Fig. 6: (a) Bradford assay using 96 well plate. (b) Protein standard curve.

2.5 SDS-PAGE (Sodium Dodecyl sulphate- polyacrylamide gel electrophoresis)

SDS-PAGE is an electrophoresis method that allows protein separation by mass, ranging from 5 to 250KDa. The use of sodium dodecyl sulphate (SDS) and polyacrylamide gel together eliminates the influence of structure and charge, allowing proteins to be separated solely on the basis of molecular weight differences. The relative migration distance (Rf) of a protein in PAGE is inversely related to its MW log (Molecular weight). Proteins with known MW (Protein marker/ladder) must be run on the gel simultaneously to determine the MW of proteins on SDS-PAGE [17], [18].

Procedure:**2.5.1 Casting the Gel**

Before starting the experiment, clean all the things like glass plates, spacers, combs etc. using 75% ethanol with tissue paper. Prepare the reagents such as 30% acrylamide-Bisacrylamide (**Table 1a**), 4X Tris (pH: 8.8) for separating gel (**Table 1b**), 4X Tris (pH: 6.8) for stacking gel (**Table 1c**), 10% SDS (**Table 1d**), 10% APS (**Table 1e**) for Separating and Stacking gels.

- i. Assemble glass plates and spacers in gel casting apparatus.
- ii. Mix the components for the **Separating gel (resolving gel) (Table 2a)**.
- iii. Pour the separating gel mixture into the gel plates to a level 2 cm below the top of the shorter plate.
- iv. Place a layer of ddH₂O over the top of the separating gel to prevent meniscus formation in the separating gel.
- v. Allow separating gel to stand 30 min at room temperature.
- vi. Drain the ddH₂O from top of the separating gel. Rinse with ddH₂O, drain, and wick any remaining ddH₂O away with a wipe.
- vii. Mix components for **Stacking gel (Table 2b)**.
- viii. Pour stacking gel solution into gel plates (on top of separating gel), so that gel plates are filled. Insert comb to the top of the spacers.
- ix. Allow gel to stand for at least 1 hour at room temperature, or overnight at 4 °C.

Table 1: Reagents required for the preparation of recipes for separating and stacking gels

30% Acrylamide: Bisacrylamide recipe	
Acrylamide	29.2g
Bisacrylamide	0.8g
ddH ₂ O	100ml
(a)	
4X Separating gel buffer	
1.5M Tris base	18.15g
Dissolve in 80ml ddH ₂ O, adjust pH to 8.8, make the final volume up to 100ml.	
(b)	
4X stacking gel buffer	
0.5M Tris base	3.0g
Dissolve in 30ml ddH ₂ O, adjust pH to 6.8, make the final volume up to 50ml.	
(c)	
10% SDS recipe	
SDS	10g in 100ml dH ₂ O
(d)	
10% APS recipe	
Ammonium per sulphate (APS)	0.1g in 1ml dH ₂ O
(e)	

Table 2: Separating and Stacking gel recipes

10% Separating gel (Resolving gel) recipe	
ddH ₂ O	4ml
30% acrylamide: Bisacrylamide	3.33ml
4X Tris (pH: 8.8)	2.50ml
10% SDS	100µl
TEMED	5µl
10% APS	50µl
(a)	
4% Stacking gel	
ddH ₂ O	3ml
30% acrylamide: Bisacrylamide	0.61ml
4X Tris (pH: 6.8)	1.25ml
10% SDS	25µl
TEMED	2.5µl
10% APS	50µl
(b)	

Note: Store acrylamide: Bisacrylamide in brown bottle at room temperature. APS (Ammonium per sulphate) should be prepared fresh in a 1.5 ml eppendroph tube and wrapped with aluminum foil. TEMED (Catalyst) should be stored in brown bottle at 4 °C.

2.5.2 Preparing Samples

- i. Take 20-25 μl (Approx. 30-35 μg conc.) of each protein sample (conc. determined by Bradford assay using 1:10 dilution) and add 5 μl of **2X Sample buffer** to make the final volume 30 μl (**Table 3a**).
- ii. Incubate at 95 $^{\circ}\text{C}$ for 10 minutes in thermostat.
- iii. Centrifuge at 3000 G for 1 minute.

Note: Well combs will hold up to 30 μl of prepared sample.

2.5.3 Running the Gel

- i. Fix the gel plates into the running apparatus.
- ii. Add 1x running buffer (**Tank Buffer**), approximately 300 ml to both chambers of the apparatus (**Table 3b**).
- iii. Remove combs and load the molecular weight marker (Protein ladder) in first well and prepared samples into the remaining wells of the gel.
- iv. Run the gel at 80-90 V until the dye front migrates into the separating gel (~15 min) and increase to 200 V until the dye front reaches the bottom of the gel (~45 min).

Table 3: Reagents required for making sample buffer and tank buffer.

2X sample buffer	
4X (0.5M)Tris, pH: 6.8	2.5ml
10% SDS	4g
100% glycerol	2ml
B-mercaptoethanol	0.8ml
0.1% Bromophenol blue	300 μl
ddH ₂ O up to 10ml	

(a)

Tank Buffer	
Tris base	6.05g
Glycine	28.80g
10% SDS	10ml
ddH ₂ O up to 1000ml	

(b)

2.5.4 Gel fixation solution

- i. Remove the gel from the apparatus and remove the spacers and glass plates.
Note: Never use a metal spatula to separate the glass plates.
- ii. Place the gel into a small tray.
- iii. Pour the **gel fixation solution** into that tray. Keep the gel in the gel fixation solution for about 1 hour (**Table 4a**).

2.5.5 Staining & Destaining the Gel

- i. Discard the gel fixation solution from the tray, then add gel staining solution.
- ii. Add ~20 ml **staining solution (Table 4b)** and stain for 30 min with gentle shaking on a shaker.
- iii. Pour off and save the stain for another gel.
- iv. Add ~5 ml **destain solution (Table 4c)** to the tray in which the gel is present and leave it for overnight on a shaker with gentle shaking at 4 $^{\circ}\text{C}$.
- v. Pour off and discard the destain solution.
- vi. Rinse with ddH₂O. Add ~30 ml ddH₂O and rinse for 5 min with gentle shaking.
- vii. Visualize the gel bands in Gel doc. Gel can be stored for about 3 days in **gel storing solution (Table 4d)** at 4 $^{\circ}\text{C}$.

Note: Gel wash can also be done by using a solution of Glycerol and Ethanol.

Table 4: Reagents required for making the Recipes for gel stain, destain and storage.

Gel fixation solution recipe			
Ethanol	500ml	50ml	25ml
ddH ₂ O	400ml	40ml	20ml
Glacial Acetic acid	100ml	10ml	5ml
Total volume	1000ml	100ml	50ml

(a)

Gel Staining solution recipe		
Coomassie Brilliant blue (G-250/R-250)	(G-	0.3g
Methanol (Carbinol)		80ml
Glacial acetic acid		20ml
ddH ₂ O up to 100ml		
Dissolve Coomassie dye in methanol and Shake it on a shaker for about 60 min. Then add acetic acid and ddH ₂ O and filter through Whatman filter paper. Store in brown bottle at room temperature.		

(b)

Gel Destaining solution recipe	
Acetic acid	100ml
Methanol (Carbinol)	300ml
ddH ₂ O up to 1000ml.	

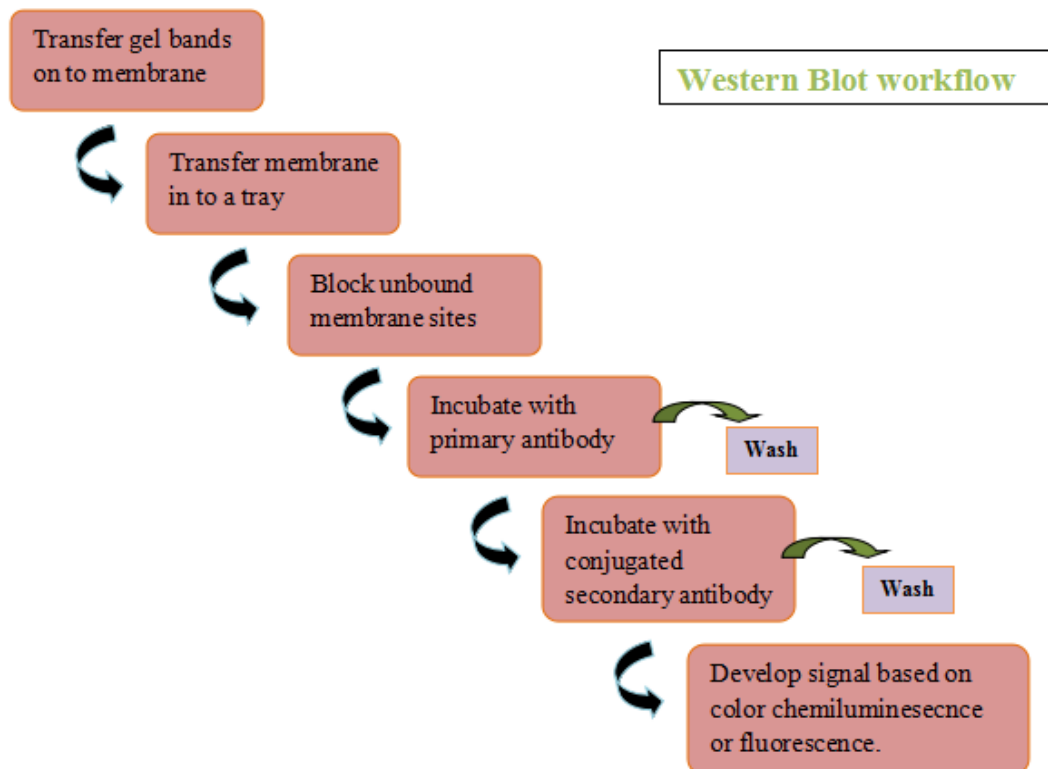
(c)

Gel storing solution recipe	
Acetic acid	5ml
ddH ₂ O	95ml
Total Volume	100ml

(d)

2.6 Western Blotting

Western blotting is a technique that uses specific antibodies to detect proteins that have been separated by gel electrophoresis based on size. This immunoassay employs a nitrocellulose or PVDF (polyvinylidene fluoride) membrane. Western blot workflow is given in **Figure 7**.

**Figure 7:** Steps involved in Western blotting.

2.6.1 Transfer of bands from gel to membrane

The proteins are transported from within the gel onto a membrane, a solid support, to make them accessible to antibody detection [19]. Electro blotting is the most important method used for transferring proteins as it pulls negatively charged proteins from the gel towards the positively charged anode and into the PVDF or NC membrane using an electric current.

- i. In a tray filled with **transfer buffer (Table 5a)**, make a sandwich of gel and membrane (sponge, blotting papers, gel, Membrane, blotting papers and sponge from anode to cathode).
- ii. Assemble the transfer sandwich, making sure there are no air bubbles.
- iii. In the transfer tank, put the sandwich cassette, fill it with transfer buffer and place one or two ice blocks in the tank. Transfer buffer should be ice-cold.
- iv. Allow proteins to transfer from gel to membrane by keeping the sandwich for roughly about 2 hours at 4 °C (cold room or fridge) with a voltage of 90 V.
- v. With the **Trans-Blot® Turbo™ transfer system** (Bio-Rad 690BR023060), proteins can be transferred from gel on to the blot membrane within 7 minutes. So, it saves time.

2.6.2 Blocking the membrane:

- i. After the run, take out the sandwich cassette from the running apparatus, carefully remove the membrane using a sterile forceps and place it in a small tray.
- ii. Add blocking buffer (4% BSA) (**Table 5b**) to it and place it on a shaker for about 30 minutes.
- iii. Before using the blocking buffer, filter it twice with a syringe filter.

Table 5: Reagents required for transfer buffer, blocking buffer and wash buffer.

1X Transfer buffer recipe	
Tris	6.05g
Glycine	28.80g
Methanol	200ml
dH ₂ O	800ml
SDS	0.1%
Total volume	1000ml
Adjust dH ₂ O accordingly	

(a)

Blocking buffer recipe	
BSA	4g
1X TBS	10ml
dH ₂ O	90ml
Prepare 1X TBS (Tris buffered saline). Take 10ml of 1XTBS and mix it with 90ml dH ₂ O. Lastly, add 4g BSA to 100ml total volume.	

(b)

Wash buffer (1X TBST) recipe	
10X TBS (Table 5d)	20ml
(0.1%) Tween 20	0.2ml
dH ₂ O	180ml

(c)

10X TBS (Tris buffered saline) recipe	
Tris base	2.4g
NaCl	8.8g
Dissolve in 60ml dH ₂ O. Check pH and then make the final volume up to 100ml.	

(d)

2.6.3 Antibody Incubation

- i. After blocking step, incubate the membrane for 2 hours at room temperature or overnight at 4 °C in the primary antibody solution against the target protein. Primary antibody should be diluted in the blocking buffer in the ratio of 1:2000 before use or as per manufacturer's recommendation ratio.
- ii. Rinse the membrane 3 to 5 times for 15 minutes with wash buffer (TBST) after primary antibody incubation.

- iii. After three wash steps, incubate the membrane in HRP conjugated-secondary antibody solution for 2 hours. Secondary antibody dilution in blocking buffer should be 1:5000 or as per manufacturer's recommendation ratio.
- iv. Rinse the membrane 2-3 times with wash buffer (1x TBST) (**Table 5c**).

The secondary antibody recognizes and binds to the primary antibody's species-specific region. The secondary antibody is usually coupled to biotin or a reporter enzyme such as alkaline phosphatase or horseradish peroxidase (HRP) to detect the target protein. HRP is frequently used in conjunction with secondary antibodies to allow chemiluminescence detection of the target protein by cleaving the chemiluminescent substrate and producing luminescence. As a result, the amount of secondary antibody conjugated with HRP used determines the amount of luminescence produced, which indirectly evaluates the presence of the target protein [20].

2.7 Detection and visualization

To see whether the transfer process was successful or not, Ponceau S staining (**Table 6**) can be used directly after the transfer of proteins from gel to membrane to visualize the protein bands by naked eye. For Ponceau staining there is no need for antibodies. It is a reversible method for staining and detecting proteins on nitrocellulose and PVDF membranes. The stain has few nonspecific interactions with the membrane surface and thus provides a reliable method for observing protein transfer to a membrane. The staining procedure is straightforward and quick, yielding reddish-pink bands that are easily photographed. The stain can be easily removed in less than five minutes with incubation in 0.1% NaOH.

Table 6: Reagents required for making Ponceau S stain.

Ponceau S stain recipe	
Ponceau S tetrasodium salt	0.5g
Glacial acetic acid	25ml
dH ₂ O	400ml
Total volume	500ml
Add 25ml of glacial acetic acid to 400 ml of dH ₂ O. Then add 0.5g of Ponceau S tetrasodium salt to the prepared acetic acid. Stir the mixture to dissolve. Make up the solution to 500 ml using distilled water.	

Procedure

- i. After transferring proteins from the gel to the membrane, place the membrane in a tray and add a 5 ml Ponceau stain solution, incubate for 10 minutes at room temperature on a shaker.
- ii. The red/pinkish bands on the membrane can then be seen with the naked eye.
- iii. The presence of bands indicates that your protein has been transferred, and if you want to proceed with a western blot, you can remove the Ponceau S stain by incubating the membrane in 0.1% NaOH or dH₂O (2-3 times) for 5 minutes.

Following the secondary antibody incubation and wash steps, the membrane can be visualized in a variety of ways. Colorimetric, chemiluminescence, and fluorescence are the three main methods for observing a western blot membrane. Chemiluminescent detection is becoming more popular in laboratories. It has two detection methods: film detection and CCD detection. It is extremely sensitive and can detect proteins in the femtogram range. Chemiluminescent detection is an indirect enzymatic method that employs secondary antibodies labelled with horseradish peroxidase (HRP) as an enzymatic reporter [21].

In our lab, we use The ECL (Enhanced Chemiluminescence) western blotting substrate, a highly sensitive, non-radioactive chemiluminescent substrate based on enhanced luminol for the detection of

horseradish peroxidase (HRP) on immunoblots/membranes. ECL Western Blotting Substrate [WesternBright™ ECL-200 ml (**advansta**) K-12045-D20] Includes:

- 100 ml WesternBright™ Peroxide.
- 100 ml WesternBright™ ECL.

Procedure

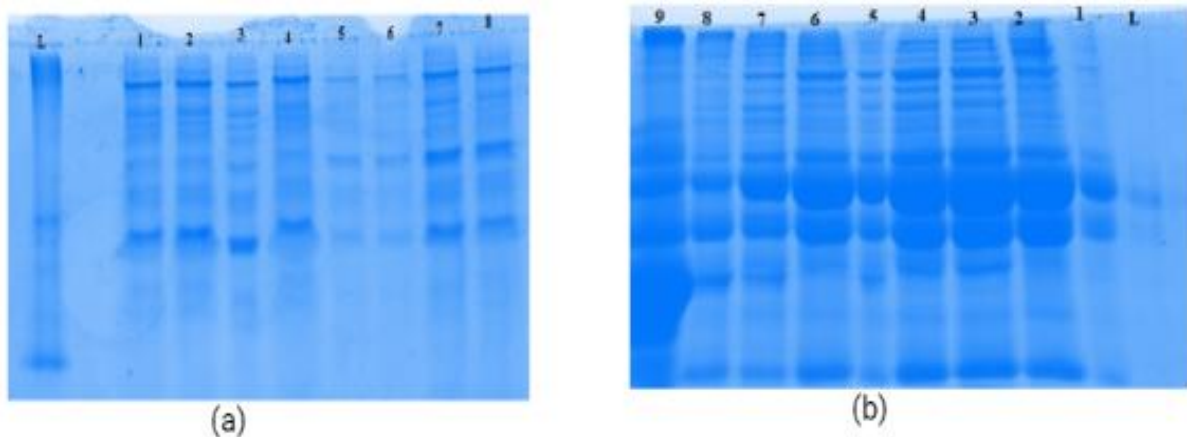
- Remove the blotting membrane from the tray and place it inside the chemidoc in a dark room.
- Pipette 500 μ l WesternBright™ Peroxide and 500 μ l WesternBright™ ECL into a 1.5 ml eppendroph tube and mix.
- Inside the chemidoc chamber, add 1 ml developer (ECL+Peroxide) to the membrane and incubate for 1 minute before visualizing.

Note: The membrane should not be dry, it should be dipped in wash buffer (1x TBST) before adding developer. Use clean forceps and gloves to handle the membrane.

- Chemidoc can be used to observe the band intensity of the target proteins during chemiluminescence.
- Visualize the membrane immediately after adding developer.

3. Results

We observed protein bands with protein concentration of 30 μ g/ μ l and 50 μ g/ μ l in both the gels (SDS-PAGE) as shown in Figure 8 (a, b). Further, after transferring the protein bands from gel on to nitrocellulose membrane and incubation with GAPDH antibody, we observed a 38KDa band on the NC-membrane under chemiluminescence in Chemidoc and by naked eye using Ponceau S stain as shown in Figure 9 (a, b, c, d).



(a): 30ug protein loaded in SDS-PAGE.

(b): 50ug protein loaded in SDS-PAGE.

Figure 8: (a), (b) are SDS-PAGE gel images with the protein concentration of 30 μ g/ μ l and 50 μ g/ μ l respectively. L is protein marker, 1-8 in (a) and 1-9 in (b) are protein samples.

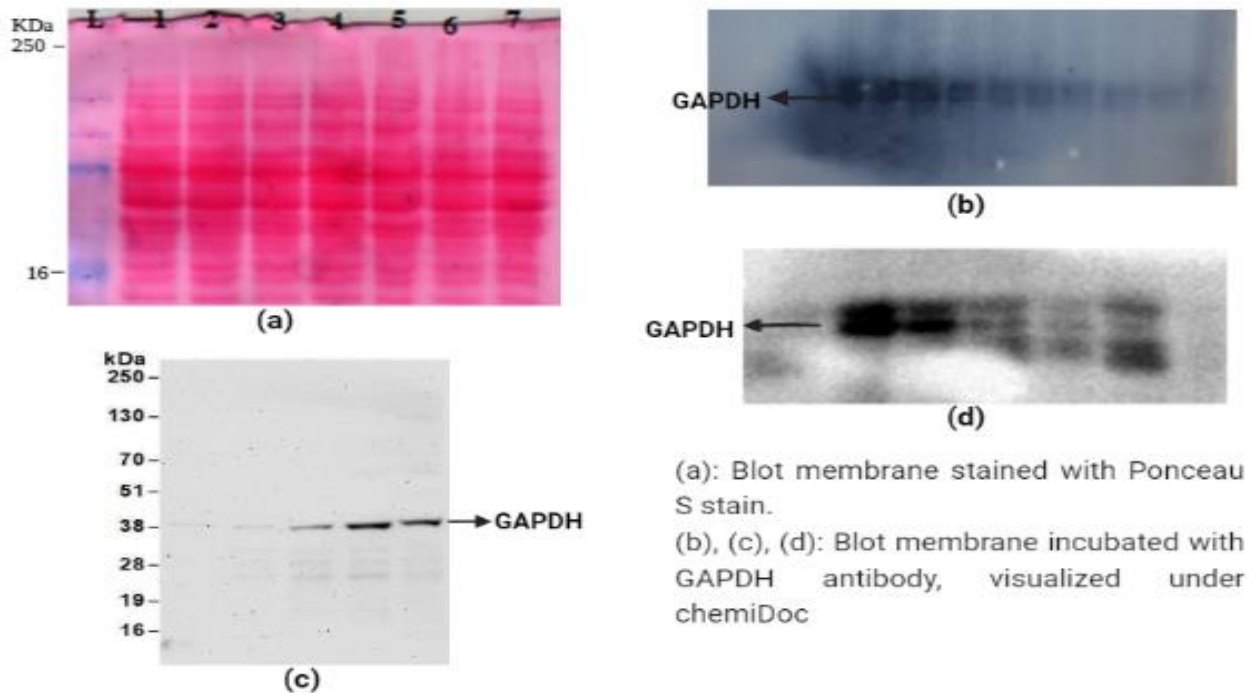


Figure 9: (a) is the NC-membrane stained with ponceau S dye, L is protein marker and 1-7 are protein samples. (b), (c), and (d) are NC-membranes incubated with GAPDH antibody with 38KDa band size, visualized under chemidoc.

General considerations:

- i. When handling the membrane, always wear gloves and use clean forceps.
- ii. Throughout the procedure, make sure the membrane does not dry out.
- iii. Sodium azide inhibits HRP, it should not be used as a preservative for antibodies or buffers.
- iv. Add 0.05-0.1% Tween-20 to blocking buffer and diluted antibodies to reduce background.
- v. The working solution for the substrate is light sensitive. Avoid prolonged exposure to bright light.

4. Discussion

In the present study, we have made an attempt to cover the methodologies standardized in our lab based on earlier accessible protocols, beginning with the separation of peripheral blood mononuclear cells (PBMCs) from human blood samples, cell viability testing, cell counting, total protein extraction methods, and protein quantification and estimation techniques, which enable a researcher to find the protocol from PBMCs to Western blot in a single manuscript.

PBMCs are used in a variety of studies including infectious diseases, vaccine development, immunology, primary cancers of the blood and blood-related organs, personalized medicine, toxicology, transplant therapy, and biomarker discovery [22], [23]. Clinicians can modify therapies by creating a genetic immunological profile from PBMCs, allowing for more specific and successful treatment. Cell viability is a measure of the proportion of live, healthy cells within a population and these assays are used to evaluate the overall condition of the cells and measure the rate of cell survival after exposure to substances, such as those found in a drug screen [24]. By conducting Bradford assay, a colorimetric protein assay that exploits a dye's interaction with protein, protein concentration is quantifiable [25].

The Western blot technique is based on the tenets of equal protein loading, protein separation by molecular weight, electrophoretic transfer to an appropriate membrane, and antibody probing. In western blots, GAPDH is a frequently used loading control, with a molecular weight (MW) of about 38KDa. It is a multifunctional protein that regulates many cellular processes, including membrane fusion, transport, apoptosis, DNA replication and repair, transcription and translation, and its expression is constant and

widespread because these processes are necessary for fundamental cell function, which makes it the perfect housekeeping gene [26].

Band visualization has many analytical applications. In other words, the presence of bands can verify the expression of a protein, and the density of bands can show differences in relative protein expression. Our SDS-PAGE and western blot results of extracts (30 ug and 50 ug) from different protein samples, isolated from human PBMCs are showing 38KDa band size of GAPDH, which indicates that the expression of GAPDH is constant.

5. Conclusion

The most common methods used in research labs for protein based experiments are discussed in this manuscript. A sample will have a variety of non-protein components, depending on the source material and methodologies applied before the protein assay, it is essential to be aware of these factors in order to select the best assay method. Understanding the function of proteins and the consequences of their presence, absence, and alterations depends heavily on protein analysis. The development of the fundamental methods for protein isolation, identification, and quantitation has made it possible to identify biomarkers for a better understanding of health and disease.

6. Declarations

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6.2 Funding Source

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6.3 Ethics Declaration

The ethical approval was obtained in accordance with the Helsinki Declaration from a government hospital in Hyderabad, as well as institutional ethical clearance (Ethical approval reference no.: Reg No ECR/300/Inst/AP/2013/RR-16).

6.4 Informed Consent Statement

Informed consent was taken from the patient at the time of sampling;

6.5 Competing Interests

The authors declare no competing interests.

6.6 Publisher's Note

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References

- [1] M. J. Lopez and S. S. Mohiuddin, "Biochemistry, Essential Amino Acids," *StatPearls*, Mar. 2022.
- [2] T. Sanvictores and F. Farci, "Biochemistry, Primary Protein Structure," *StatPearls*, pp. 1–9, 2020.
- [3] N. Boodhun, "Protein analysis: key to the future," *BioTechniques*, vol. 64, no. 5, pp. 197–201, May 2018, doi: 10.2144/BTN-2018-0055.

- [4] C. H. Lee, "A Simple Outline of Methods for Protein Isolation and Purification," *Endocrinology and Metabolism*, vol. 32, no. 1, p. 18, Mar. 2017, doi: 10.3803/ENM.2017.32.1.18.
- [5] K. Kallberg, H. O. Johansson, and L. Bulow, "Multimodal chromatography: An efficient tool in downstream processing of proteins," *Biotechnol J*, vol. 7, no. 12, pp. 1485–1495, Dec. 2012, doi: 10.1002/BIOT.201200074.
- [6] S. S. Nielsen, *Food Analysis*, 5th ed., . 5th ed., in Food Science Text Series. Springer Cham, 2017. doi: 10.1007/978-3-319-45776-5.
- [7] A. Puleo, C. Carroll, H. Maecker, and R. Gupta, "Isolation of Peripheral Blood Mononuclear Cells Using Vacutainer® Cellular Preparation Tubes (CPTM)," *Bio Protoc*, vol. 7, no. 2, 2017, doi: 10.21769/BIOPROTOCOL.2103.
- [8] Y. Grigoryev, "Cell Counting With a Hemocytometer: As Easy as 1, 2, 3...," *Bite Size Bio*, Jun. 29, 2021. <https://bitesizebio.com/13687/cell-counting-with-a-hemocytometer-easy-as-1-2-3/> (accessed Mar. 28, 2023).
- [9] L. L. Y. Chan, W. L. Rice, and J. Qiu, "Observation and quantification of the morphological effect of trypan blue rupturing dead or dying cells," *PLoS One*, vol. 15, no. 1, p. e0227950, Jan. 2020, doi: 10.1371/JOURNAL.PONE.0227950.
- [10] M. Betti, C. Ciacci, S. Abramovich, and F. Frontalini, "Protein Extractions from *Amphistegina lobifera*: Protocol Development and Optimization," *Life (Basel)*, vol. 11, no. 5, May 2021, doi: 10.3390/LIFE11050418.
- [11] L. C. M. Ngoka, "Sample prep for proteomics of breast cancer: proteomics and gene ontology reveal dramatic differences in protein solubilization preferences of radioimmunoprecipitation assay and urea lysis buffers," *Proteome Sci*, vol. 6, Oct. 2008, doi: 10.1186/1477-5956-6-30.
- [12] M. Johnson, "Protein Quantitation," *Materials and Methods*, vol. 2, Feb. 2012, doi: 10.13070/MM.EN.2.115.
- [13] F. He, "Laemmli SDS PAGE," *Bio Protoc*, vol. 1, no. 11, pp. 1–4, Jun. 2011, Accessed: Mar. 28, 2023. [Online]. Available: <https://bio-protocol.org/pdf/Bio-protocol80.pdf>
- [14] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal Biochem*, vol. 72, no. 1–2, pp. 248–254, May 1976, doi: 10.1016/0003-2697(76)90527-3.
- [15] C. M. Stoscheck, "[6] Quantitation of protein," *Methods Enzymol*, vol. 182, no. C, pp. 50–68, Jan. 1990, doi: 10.1016/0076-6879(90)82008-P.
- [16] S. C. Silvério, S. Moreira, A. M. F. Milagres, E. A. Macedo, J. A. Teixeira, and S. I. Mussatto, "Interference of some aqueous two-phase system phase-forming components in protein determination by the Bradford method," *Anal Biochem*, vol. 421, no. 2, pp. 719–724, Feb. 2012, doi: 10.1016/J.AB.2011.12.020.
- [17] A. B. Nowakowski, W. J. Wobig, and D. H. Petering, "Native SDS-PAGE: High Resolution Electrophoretic Separation of Proteins With Retention of Native Properties Including Bound Metal Ions," *Metallomics*, vol. 6, no. 5, p. 1068, 2014, doi: 10.1039/C4MT00033A.
- [18] "SDS-PAGE Protocol | Rockland." <https://www.rockland.com/resources/sds-page-protocol/> (accessed Mar. 29, 2023).
- [19] T. Mahmood and P.-C. Yang, "Western blot: technique, theory, and trouble shooting," *N Am J Med Sci*, vol. 4, no. 9, pp. 429–434, Sep. 2012, doi: 10.4103/1947-2714.100998.
- [20] A. K., "Improving quantification accuracy for western blots," *Image Analysis*, Sep. 2006.
- [21] A. Lal, S. R. Haynes, and M. Gorospe, "Clean western blot signals from immunoprecipitated samples," *Mol Cell Probes*, vol. 19, no. 6, p. 385, Dec. 2005, doi: 10.1016/J.MCP.2005.06.007.
- [22] J. Pourahmad and A. Salimi, "Isolated Human Peripheral Blood Mononuclear Cell (PBMC), a Cost Effective Tool for Predicting Immunosuppressive Effects of Drugs and Xenobiotics," *Iranian Journal of Pharmaceutical Research*, vol. 14, no. 4, p. 979, 2015, Accessed: Mar. 29, 2023. [Online]. Available: <https://pubmed.ncbi.nlm.nih.gov/26664364/>
- [23] S. P., "PBMCs: Applications in Research," *News Medical Life Sciences*. <https://www.news-medical.net/life-sciences/PBMCs-Applications-in-Research.aspx> (accessed Mar. 29, 2023).
- [24] T. L. Riss *et al.*, "Cell Viability Assays," *Assay Guidance Manual*, Jul. 2016. <https://www.ncbi.nlm.nih.gov/books/NBK144065/>
- [25] K. Gavini and K. Parameshwaran, "Western Blot," *StatPearls*, Apr. 2022. <https://www.ncbi.nlm.nih.gov/books/NBK542290/>
- [26] M. R. White and E. D. Garcin, "The sweet side of RNA regulation: glyceraldehyde-3-phosphate dehydrogenase as a noncanonical RNA-binding protein," *Wiley Interdiscip Rev RNA*, vol. 7, no. 1, p. 53, Jan. 2016, doi: 10.1002/WRNA.1315.

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