WESTERN BLOTTING

PRINCIPLE: Western Blotting was performed by the rapid method of Towbin et al., (1979) to detect the expression pattern of a protein. To detect the antigens blotted on a nitrocellulose membrane with the use of an antibody.

Western blot is the analytical technique used in molecular biology, immunogenetics and other molecular biology to detect specific proteins in a sample of tissue homogenate or extract. Western blotting is called so as the procedure is similar to Southern blotting. While Southern blotting is done to detect DNA, Western blotting is done for the detection of proteins. Western blotting is also called protein immunoblotting because an antibody is used to specifically detect its antigen.

REAGENTS AND MATERIALS:

1. Nitrocellulose membrane,

2. Plastic staining box,

3. Electroblotting apparatus.

4. Whatmann No.1 filter paper.

5. Transfer buffer (500 ml, pH 8.3) Tris–HCl -25 mM Glycine -192 mM Methanol-20%.

6. 10X Tris buffered saline (TBS) (100 ml, pH 7.6) Tris -2.4 g NaCl -8 g They were dissolved in low amount of double distilled water,and the total was made upto 100 ml with double distilled water.

7. Blocking solution (50mL) 5% Non-dry fat milk powder - 0.25g 1X TBS (pH 7.6) - 50mL 0.1% Tween-20 - 0.05mL.

8. Washing buffer (100mL) (TBS) 1X TBS (pH 7.6) -100 ml 0.1% Tween - 20 - 0.1 ml.

10. Prepared primary antibodies.

11. Prepared secondary antibodies.

12. Colour indicator solution 0.05% 0f 3’3- diaminobenzidine tetra hydrochloride (DAB) substrate and 0.01% of H_2O_2 were dissolved in 1X PBS (pH 7.6). This chromogen substrate was prepared just prior to the treatment.

13. Ponceau S red solution (100mL) Ponceau S red - 0.5 g Glacial acetic acid - 5%.

PROCEDURE :

The technique consists of three major processes:

1. Separation of proteins by size (Electrophoresis).
2. Transfer to a solid support (Blotting)
3. Marking target protein using a proper primary and secondary antibody to visualize (Detection).

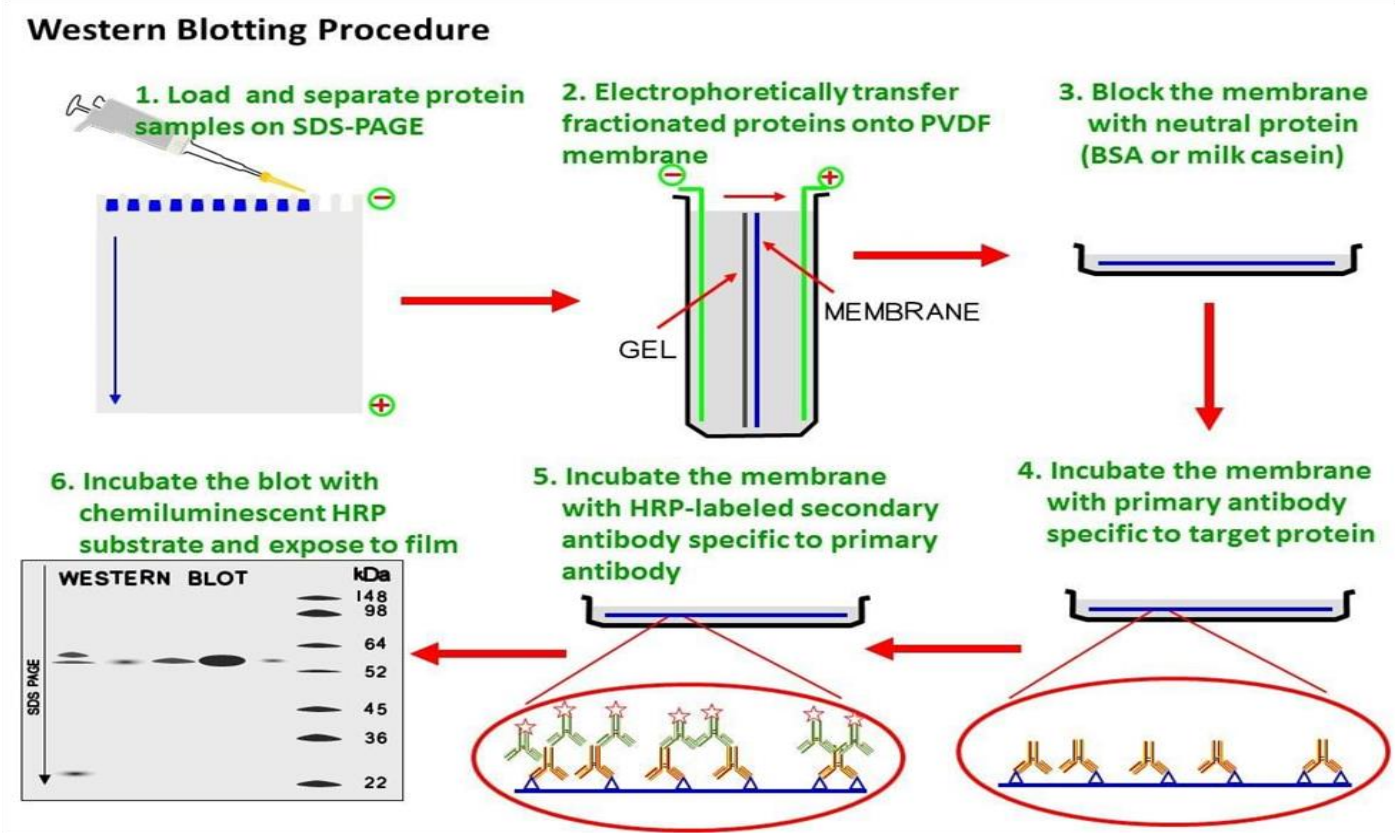
1. After SDS-PAGE, the gel was equilibrated in blotting buffer for 20 min at room temperature. While the gel was equilibrating, a piece of nitrocellulose membrane was cut into the same dimension as the gel it was wet slowly by sliding it at 45o angle into transfer buffer and was soaked for 20 min.

2. The pieces of Whatmann No.1 filter paper, four pads were also soaked in transfer buffer for 20 min.

3. Then, the pads, filter paper, nitrocellulose membrane and gel were assembled in the semi-dry blot apparatus in the following order: The two pre soaked pads were placed at the bottom and a glass pipette was rolled over the surface of the pad to remove air bubbles.

Then, the Whatmann No.1 filter paper was placed followed the nitrocellulose membrane. Carefully, the equilibrated gel was placed on top of the nitrocellulose membrane. The second Whatmann No.1 filter paper and followed it, the second set of pad were placed on top of the gel. (After each step care was taken to remove the bubbles). The transfer cell and plug was assembled and the gel transferred for 2h at 25V/130.

After the transfer, protein were visualized by staining in ponceau S solution for 5 min, destained in the distilled water and the molecular marker was marked with in delible ink and destained for 10 min. The membrane was blocked in blocking buffer for 1h at room temperature. Then, the membrane was washed again with washing buffer and incubated with primary antibody overnight at 4oC. The next day, the membrane was washed again with washing buffer and incubated with HRP-conjugated secondary antibody for 2 h at room temperature. The membrane was washed and DAB solution was added and incubated at room temperature and watched for colour development, which is usually completed with in 5–10 min. The membrane was rinsed with distilled water to stop the reaction of DAB. It was then placed on filter paper to air dry. Dilutions of the primary and secondary antibody were standardized after several trials. The specific protein was detected as a band in the nitrocellulose membrane.



RESULT: The presence of specific protein or the presence of antigen or specific antibody was visualised as a bluish grey coloured band.

APPLICATION:

1. Identification of a specific protein in a complex mixture of proteins. In this method, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotted onto nitrocellulose. The separated bands of known antigens are then probed with the sample suspected of containing antibody specific for one or more of these antigens. Reaction of an antibody with a band is detected by using either radiolabeled or enzyme-linked secondary antibody that is specific for the species of the antibodies in the test sample.
2. Estimation of the size of the protein as well as the amount of protein present in the mixture.
3. It is most widely used as a confirmatory test for diagnosis of HIV, where this procedure is used to determine whether the patient has antibodies that react with one or more viral proteins or not.
4. Demonstration of specific antibodies in the serum for diagnosis of neurocysticercosis and tubercular meningitis.