

WESTERN BLOT

Principle

It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electrotransferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labeled antibody and substrate.

A protein sample is subjected to polyacrylamide gel electrophoresis. After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose. The nitrocellulose is then soaked in blocking buffer (3% skimmed milk solution) to "block" the non-specific binding of proteins. The nitrocellulose is then incubated with the specific antibody for the protein of interest. The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody. For example, if the first antibody was raised in mouse, the second antibody might be termed "goat anti-mouse immunoglobulin". What this means is that mouse immunoglobulins were used to elicit an antibody response in goats. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction. Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (e.g. crude cell extract) of other proteins by western blotting.

Electroblotting

Wear gloves while performing electroblotting.

1. A SDS-PAGE gel has been run with the protein samples by the tutor.
2. Remove the glass plates from the SDS-PAGE gel. Carefully separate the plates using a spatula as a lever. Cut at one end of the gel to assist in later orientation of the gel.
3. Cut off the entire stacking gel including the wells.
4. Place the remaining gel in a container containing transfer buffer (Tris, Glycine, 20% Methanol, pH8.6). . Make sure the gel is submerged in the buffer.
5. Soak the nitrocellulose membrane (NC) membrane, 2 Scotch Brite pads, 4 filter papers in a container containing transfer buffer. The filter papers and the NC must be thoroughly wet.
6. Open the transfer cassette with the black end (negative electrode) on the table. Assemble the cassette from black end to the white end (Positive electrode) in the following manner:-

Black end (negative electrode)

- 1 Scotch Brite pad
2 sheets of filter paper
1 gel
1 NC membrane
2 sheets of filter paper
1 Scotch Brite pad

White end (Positive electrode)

7. Smoothen the assembly at each step to prevent the trapping of air bubbles between either the NC membrane or the gel.
8. Close and secure the transfer cassette.

9. Fill the transfer chamber with transfer buffer almost to the top.
10. Place the transfer cassette slowly into the transfer chamber containing the transfer buffer. Make sure that the black end of the transfer cassette is facing the black button on the transfer chamber. Place the lids and turn on current at 250 milliamps for 1 hour.

Protein detection

Wear gloves.

1. At the end of the transfer, take out the transfer cassette and place the white end on the table.
2. Open the cassette. Remove the Scotch brite pad and the filter papers. **Do not remove the gel yet.** Using a ball-point pen, mark on the NC the corners of the gel so that you can detect the size of the gel.
3. Remove the gel and stain it with Coomassie for 30 mins and destain to detect efficiency of transfer.
4. With the transferred NC, draw the 'alignment' line with the blue ink.
5. Submerge the entire membrane into Ponceau S stain provided, for 2-5 mins. Make sure the NC is completely red.
6. Wash the excess stain in distilled water long enough to detect bands. Do not destain bands for too long or they will disappear.
7. Place the membrane onto the filter paper. Cut out the portion containing m.wt marker.
8. Destain the remaining membrane in distilled water until the bands disappear.

Blotting analysis

1. Place the membrane into a container containing 3% skimmed milk solution to block the non-specific sites on the membrane
2. Place the container on the shaker for 1 hour at 37°C or overnight at 4°C and gently rock the membrane.
3. Remove the skimmed milk solution and wash the membrane with 1X PBS containing 0.05% Tween 20 (Non-ionic detergent)
4. Repeat this step 3 for 5 times.
5. Place the membrane in primary antibody (anti-His antibody) solution for 1 hour at 37°C
6. Remove the primary antibody solution. Wash the membrane with 1X PBS containing 0.05% Tween 20 (Non-ionic detergent) for five times
7. Add secondary antibody (anti-mouse -IgG HRP 1: 1600). Rock gently for 1 hr at room temperature.
8. Remove the secondary antibody solution. Wash the membrane with 1X PBS containing 0.05% Tween 20 (Non-ionic detergent) for five times
9. Add 1 ml of colour development solution (4-Chloro-1- Naphthol and hydrogen peroxide). Cover with aluminium foil and rock gently for 15 mins.
10. Once the desired bands appears. Discard the color development solution and stop the reaction by adding water

Determination of mol. weights

Aligned the developed membrane with the molecular weight markers and determine the molecular weight of the protein using the following calculation

$$R_f = \frac{\text{distance traveled by the protein}}{\text{distance traveled by tracker dye}}$$

Distance traveled is measured from the beginning of the resolving gel in cm.

Sizes of the molecular weight markers provided are:

Mol weight markers (kD)	Dist. travelled (cm)	Rf
250		
148		
60		
42		
30		
22		
17		
Distance traveled by Tracking dye		

Plot a graph of Rf (on X-axis) and m.wt (on Y-axis) , on the log graph paper provided. Draw the best-fit linear line. Prepare a table of distance travelled by your antigenic bands, calculate its Rf and read off the m.wt from the graph.

What is the mol. weight of the antigenic bands?
