Experiment (4): Western Blot

Aims:

- To understand how proteins (antigens) can be analysed using antibodies raised against these proteins by Immunoblotting technique.
- To perform the steps of western-blot technique to detect the specific protein.

Introduction:

Immunoassay is a test that uses the highly specific and selective antigen-antibody reactions forming antibody and antigen complexes [immuno-complexes] as a means of generating measurable results. Western blot (also called protein immunoblot) is a widely used immunoassay technique, used to identify proteins specific proteins [antigens] in a sample of tissue homogenate or extract, based on their ability [the antigens] to bind to antibodies resulting in colour indicate the presence of this specific protein. [1,2]

Western blot has various applications for research use such as protein expression level, epitope mapping and to detect the phosphorylation signal and structure domain analysis.[3]

- PAUSE AND THINK ➔ Is there other reactions with high specificity than antigen-antibody reactions?

Principle:

The mixture of proteins is separated based on molecular weight, and thus by type, through SDS-PAGE. These results are then electro-transferred to a nitrocellulose polyvinylidenedifluoride (PVDF), or nylon membrane producing a band for each protein. The transferred protein is detected by incubating the gel with specific primary antibody to the protein of interest, secondary antibody labelled with an enzyme which target the primary antibody, and substrate which in the end you will get coloured product. Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. The colour indicates the presence of the protein of interest. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present. [4]

Thus, the molecular weight and amount of the desired protein can be characterized from a complex mixture of proteins by western blotting.

Western blot performing steps:

The technique uses three elements to accomplish this task: (1) separation by size using SDS-PAGE, (2) transfer to a solid support (electro-blotting), and (3) marking target protein using a proper primary and secondary antibody to visualize.

- **1st phase (SDS-PAGE):** A protein sample is subjected to polyacrylamide gel electrophoresis. The separation of the sample can be confirmed by: 1.Replica of the gel and stain it as usual [with Coomassie brilliant blue R-250] , 2.prestained marker and 3.Ponceau S.

- **2nd phase (Electro-blotting):** After that the gel is placed over a sheet of nitrocellulose, the protein in the gel is electrophoretically transferred to the nitrocellulose membrane. The transfer can be done by wet method or semi-wet method. This done by Creating a transfer sandwich: filter papers-gel-nitrocellulose membrane-filter papers. The filter papers, gel and nitrocellulose membrane will be soaked in transfer buffer. Because the samples in the gel are negatively charged, the applied electric current will facilitate their transferring to nitrocellulose membrane, the samples will move toward the anode. Also, the capillary action has its effect in the movement of the samples from the gel to the nitrocellulose membrane.

- **3rd phase (Marking target protein to visualize):** The nitrocellulose is then soaked in blocking buffer to block the nonspecific binding of the proteins. The nitrocellulose is then incubated with the specific primary antibody for the protein of interest. After that the nitrocellulose will washed and then incubated with a second antibody, which is specific for the primary antibody. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction (detection step) by converting a colorless substrate to a colored product. Several substrates can be converted to colored precipitate "product" by (AP) and (HRP) enzymes. As the precipitate accumulate on the membrane, a visible band develops.

Material

Transfer buffer: (25mM Tris, 190 mM glycine, 20% methanol, 0.1% SDS)
Adjust the pH to 8.3

Block buffer: (10% milk with 0.5% Tween 20) **Or** 5% BSA (with fluorescent system)

Washing buffer (TBST): (25mM Tris, 0.15M NaCl, 0.05% Tween-20)
Adjust the pH to 7.5

10X PBS: (NaCl, KCl, Na_2HPO_4, KH_2PO_4)
Adjust the pH to 7.4

Protocol

I. Crude extraction of protein from animal tissue

1. Weight the sample, wash it with normal saline, and cut it into small pieces.
2. For each 1g of the sample add 5ml of the extraction buffer (0.1 M Tris-HCl, pH 7.4) and homogenized it in the blender.
3. Transfer the homogenized tissue/buffer mixture into centrifuge tubes.
4. Centrifuge your homogenate for 15 minutes at 3000 xg.
5. Measure the volume of the supernatant.

II. Separation of the protein by SDS-PAGE

1. Prepare 12% of separating gel and 7% of stacking gel.
2. Mix the sample with 10X loading buffer (1:1), then heat them at boiling for 5-10 min.
3. Load 20ul of prepared samples into wells and in different well load 10ul of pre-stained protein marker
4. Run at 120 volts for 60 – 90 minutes

According to: http://www.assay-protocol.com/molecular-biology/electrophoresis/denaturing-page.html

III. Electro-blotting of separated protein (Semidry blotter)

1. Wet the filter papers with transfer buffer.
2. The prewetted nitrocellulose / PVDF membrane is put on top the filter. (i.e PVDF membrane need to be activated with 100% methanol)
3. The gel is put on top of the membrane.
4. Ensure that no air bubbles are anywhere in this stack of membranes.
5. Then wetted filter papers should be placed on top of the gel.
6. Again, remove any bubbles.
7. Put it onto the apparatus.
8. apply a continuous voltage of 25V for 15-20 minutes

IV. Visualization of target protein

1. Block the membrane for 2 hrs at room temperature or overnight at 4°C using blocking buffer.
2. Wash the membrane in three times using TBST, 5 min each. (Optional)
3. Incubate the membrane with 1:1000 dilutions of primary antibody, prepared in 1XPBS for 2 hrs at room temperature or overnight incubation at 4°C.
4. Wash the membrane in three times using TBST, 5 min each.
5. Incubate the membrane with the 1:10000 dilution of secondary antibody, prepared in 1XPBS at room temperature for 1 hr.
6. Wash the membrane in three times using TBST, 5 min each.
7. Put the membrane in 1XPBS and visualize it in ODYSSET CLx device.

Supporting materials:

- Performing western blot: http://www.youtube.com/watch?v=VgAuZ6dBOfs
- Ponceau S staining: http://www.youtube.com/watch?v=Jj_37cDsO7o

References:

1. Sosnik A, Biomedical Applications of Functionalized Nanomaterials, 2018.
2. https://www.thermofisher.com/sa/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-western-blotting.html
3. http://www.sinobiological.com/western-blot-applications-for-research.html
4. Mahmood, T., & Yang, P.-C. (2012). Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences*, *4*(9), 429–34.