Western blotting

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The Western blot (or protein immunoblot) is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein.

Steps

1. **Tissue preparation**

Samples can be taken from whole tissue or from cell culture. Solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication. (Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturing and degradation.)

2. **Gel electrophoresis**

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel.

The most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure.

Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size. (The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration, the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration, the better the resolution of higher molecular weight proteins.)

3. **Transfer**

In order to make the proteins accessible to antibody detection, they are moved from the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The primary method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from the gel onto the membrane while maintaining the organization they had within the gel.

4. **Blocking**

Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the

antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This leads to clearer results, and eliminates false positives.

5. **Detection**

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate, this enzyme drives a colourimetric reaction and produces a color.

- **Primary antibody-**

After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) is incubated with the membrane.

- **Secondary antibody-**

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

The enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane.

Applications

- The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample.
- A western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- Some forms of Lyme disease testing employ western blotting.
- Western blot can also be used as a confirmatory test for Hepatitis B infection.
- In veterinary medicine, western blot is sometimes used to confirm FIV+ status in cats.

Advantages of Western Blotting

- Western blotting is effective and useful method to detect and characterize proteins in small amounts.
- Immunogenic responses from infectious agents (ex. viruses, bacteria) are hard to find since they are difficult to isolate from patient sample. But Western blotting can detect this.
- Western Blotting utilizes not only antigens, but also antisera as a diagnostic tool. Antisera is widely used in the test for HIV presence.
- Compared to ELISA, Western blotting has higher specificity.

Disadvantages of Western Blotting

- A non-intended protein has a slight chance of reacting with the secondary anti-body, resulting in the labeling of an incorrect protein.
- Incidental phosphorylation or oxidation of proteins may result in multiple bands appearing.
- The appearance of bubbles may occur when transferring the sample from the gel/membrane sandwich and may also occur when incubating the sample with antibodies.

- If the transfer time is not sufficient when transferring proteins to the membrane, the larger proteins of higher molecular weight will not transfer properly, resulting in an incorrect or no band reading at all.

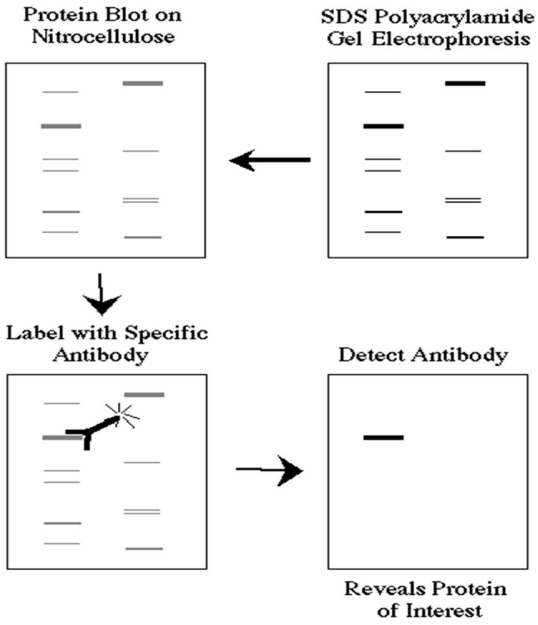

Fig: Western Blotting