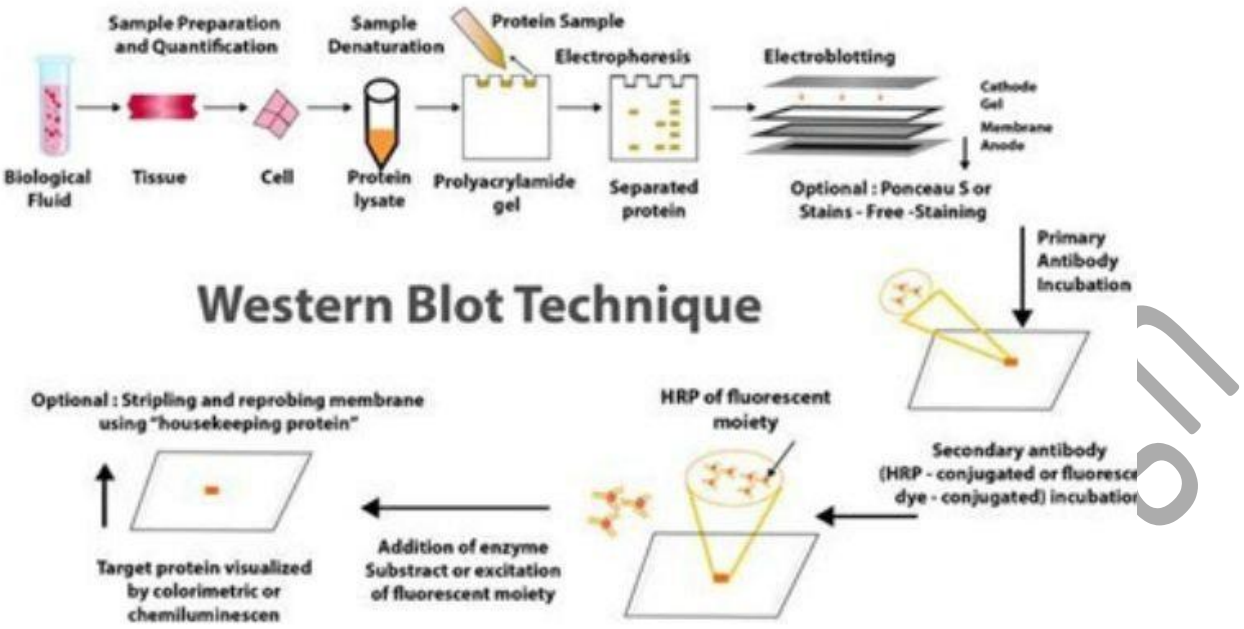
Western Blotting:

Principle: It is used to identify a particular protein from a mixture of proteins. Labelled antibodies against a particular protein is used to identify that protein. It is a very specific identification technique also known as immunoblotting.

Procedure:

- Cell lysate is obtained and protein is extracted.
- The concentration of protein is determined by UV-Vis spectroscopy and when sufficient amount is available, it is mixed with loading buffer containing glycerol which helps in sinking of sample in the well. The loading buffer also contains a tracking dye called as Bromophenol Blue (BPB) which is used to track the gel progress.
- Sample is loaded in wells and run through SDS-PAGE.
- Proteins are separated on the basis of molecular weight.
- Proteins move towards anode as they are negatively charged.
- When proteins are separated on gel, a nitrocellulose membrane or PVDF membrane is placed below the gel.
- Proteins get transferred to nitrocellulose paper by capillary action or with the help of current depending on the procedure used for transfer.
- The membrane is non-specifically saturated/masked using casein or Bovine serum albumin (BSA).
- This step is called blocking. It is done before adding antibodies so that antibodies do not bind to free nitrocellulose membrane and give false results. This is possible because nitrocellulose membrane binds proteins and antibodies are also proteins.
- Primary antibodies specific to desired protein are added and form Ag-Ab complex.
- Secondary antibodies which are enzyme labelled (alkaline phosphatase or Horseradish peroxidase) are added.
- A substrate is added to the reaction mixture and allowed to incubate.
- Visible coloured product is formed as a precipitate and the protein of interest can be identified and isolated.

The workflow for the Western blot is given in the figure below.



Note:

- Sometimes there are two proteins which have same molecular mass so they will be found at the same position in the gel and on the membrane after transfer. In such cases, if we need to perform the western blot for both the proteins then we use a method called as stripping and reprobing.
- Stripping refers to removal of the primary and secondary antibody used to probe earlier. This is done using mild detergent treatment.
- After this, we can use the primary and secondary antibodies for the detection of the next protein at the same position.
- When Western blotting is being used for the quantitative analysis then we use internal controls to remove the variability in the samples with respect to the experimental proteins.
- The internal controls are the proteins whose expression is ubiquitous in all the cells in the body and their expression is unaffected by the experimental conditions.
- The most common internal controls used are β-actin, γ-tubulin, GAPDH etc. We use antibodies against these proteins as well whenever we are using internal controls.

Dot blot: It is a method for detection, analysis and identifying proteins. It is similar to the Western blot but in this method protein samples are not separated by electrophoresis but are spotted in circular manner on a membrane directly as crude extract.

This method is qualitative and semiquantitative in nature. The outline of the method is given below:

- Grid is marked on the nitrocellulose or PVDF membrane using a pencil and the spots are made in a circular manner.

- Using the pipette tip, crude extract is placed in the grid in a predefined manner. The volume is kept small usually 2µl is placed on the grid and after that membrane is allowed to dry.
- After this, blocking is performed to prevent non-specific binding of the antibodies.
- Now, primary antibody is added and incubated overnight. This is monoclonal and unlabelled with enzyme.
- After the incubation is over, membrane is washed and incubated with the secondary antibody which is labelled with enzymes.
- After this incubation is over unbound secondary antibody is washed and substrate is added to develop the signal.