Protein immunoblotting
(Western blotting)

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Western blotting

- It is an analytical technique used to detect specific proteins in a cell, tissue, organ, or body fluid. The technique depends on the reaction of an antibody with a protein that is immobilized on a thin membrane.

- This technique can be used to identify a target protein in a complex mixture, and to measure its expression level.
-The method originated in the laboratory of George Stark at Stanford

-The name *Western blot* was given to the technique by W. Neal Burnette

-Southern blot?

-Northern blot?

-The transfer of DNA from agarose gel onto NC is called *Southern blot*

- The transfer of RNA from agarose gel onto NC is called *Northern blot*
Western blotting

- Identification of protein based on two distinguishing properties:
  1- Molecular weight
  2- Antibody binding specificity

Western blotting carried out through the following steps:
  1- Sample preparation
  2- SDS-PAGE to separate native proteins
  3- Transfer of protein to a membrane (nitrocellulose or PVDF).
  4- Detection of target proteins by specific antibodies
Sodium Dodecyl Sulfate-Polacrylamide Gel Electrophoresis (SDS-PAGE)
- Proteins usually have a net positive or negative charge (reflects the mixture of charged amino acids they contain)

- Protein will migrate at a rate that depends on its net charge and on its size and shape

- In the mid-1960s SDS polyacrylamide-gel electrophoresis (SDS-PAGE) was developed

- It uses a highly cross-linked gel of polyacrylamide as the inert matrix through which the proteins migrate

- The gel is usually prepared immediately before use by polymerization from monomers
- The pore size of the gel can be adjusted so that it is small enough to retard the migration of the protein molecules of interest.

- The proteins in solution include a powerful negatively charged detergent, sodium dodecyl sulfate (SDS).

- SDS binds to hydrophobic regions of the protein molecules, causing them to unfold into extended polypeptide chains.

- Mercaptoethanol (reducing agent) is usually added to break any S-S linkages in the proteins so that all of the constituent polypeptides in multi-subunit molecules can be analyzed separately.
Sample loading onto the SDS-PAGE gel
Sample preparation

SDS-PAGE

- Lysis
- Centrifugation

Cell supernatant contains soluble proteins
- Cell supernatant
- Cell debris

Cell supernatant is transferred to another tube; cell debris is discarded.

Loading buffer is added and ready to load into wells.

Protein samples along with a protein standard are loaded into wells; gel electrophoresis is run by electric current.
Large proteins are retarded much more severely than small ones.
1-Loading samples onto SDS-PAGE gel

2- Electrophoresis

3-Staining
SDS-PAGE is a more powerful method of protein analysis

- Separate all types of proteins, including those that are insoluble in water

- Membrane proteins, protein components of the cytoskeleton, and that are part of large macromolecular can all be resolved

- SDS-PAGE separates polypeptides according to size (molecular weight and the subunit composition).
The transfer of the proteins onto a membrane

Why not add SDS in the transfer buffer?
The protein bands could not be seen without further staining; this figure is only an illustration showing the location of bands.

Transfer (Semi-dry transfer as an example)

Cathode
- Filter Papers
- Protein gel
- Membrane
- Filter Papers

Anode

Membrane

Protein standard
- Proteins of interest

Protein standard
- Proteins of interest

The protein bands could not be seen without further staining; this figure is only an illustration showing the location of bands.
Protein detection

1- Primary antibody incubation step.
The primary antibodies which specifically recognize the proteins of interest are used.

2- Secondary antibody incubation step.
Use of secondary antibody which recognizes the primary antibody

3- Visualization step
Making the antigen-antibody complex visible (staining).
Protein detection in western blot

1. Coat surface with sample (antigens).
2. Block unoccupied sites with nonspecific protein.
3. Incubate with primary antibody against specific antigen.
4. Incubate with antibody-enzyme complex that binds primary antibody.
5. Add substrate.
6. Formation of colored product indicates presence of specific antigen.

Horseradish peroxidase

Colorless substrate

Secondary antibody (labeled)

Primary antibody (antigen specific)

Colored product (for easy detection)

Protein mixtures are absorbed to an inert surface
Protein detection

The protein bands could not be seen without further staining; this figure is only an illustration showing the location of bands.

Washing is required in each step.
Western blotting to detect a specific antigen protein in a protein mixture using a specific antibody.

Gel-separated antigen proteins are transferred onto a nitrocellulose membrane before being probed with antibodies.