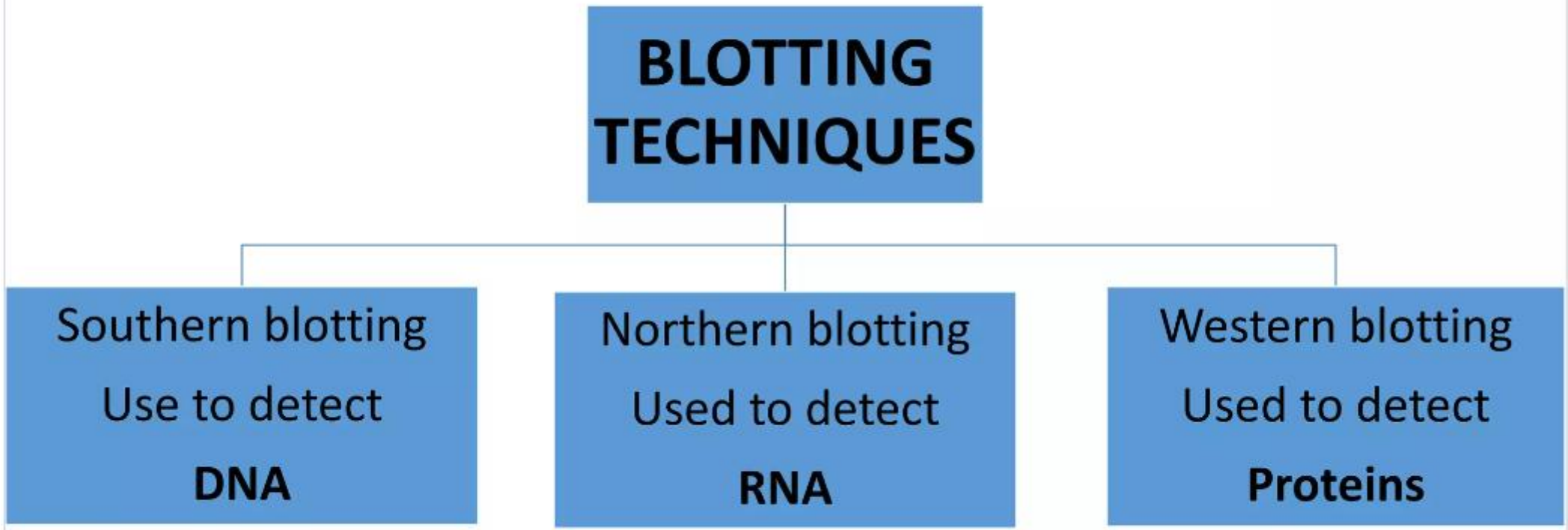
Western Blotting

INTRODUCTION

Blotting

- ✓ **Blotting** is a method of transferring proteins, DNA or RNA, onto a **carrier** (for example, a nitrocellulose or PVDF or nylon membrane)

Types of blotting techniques

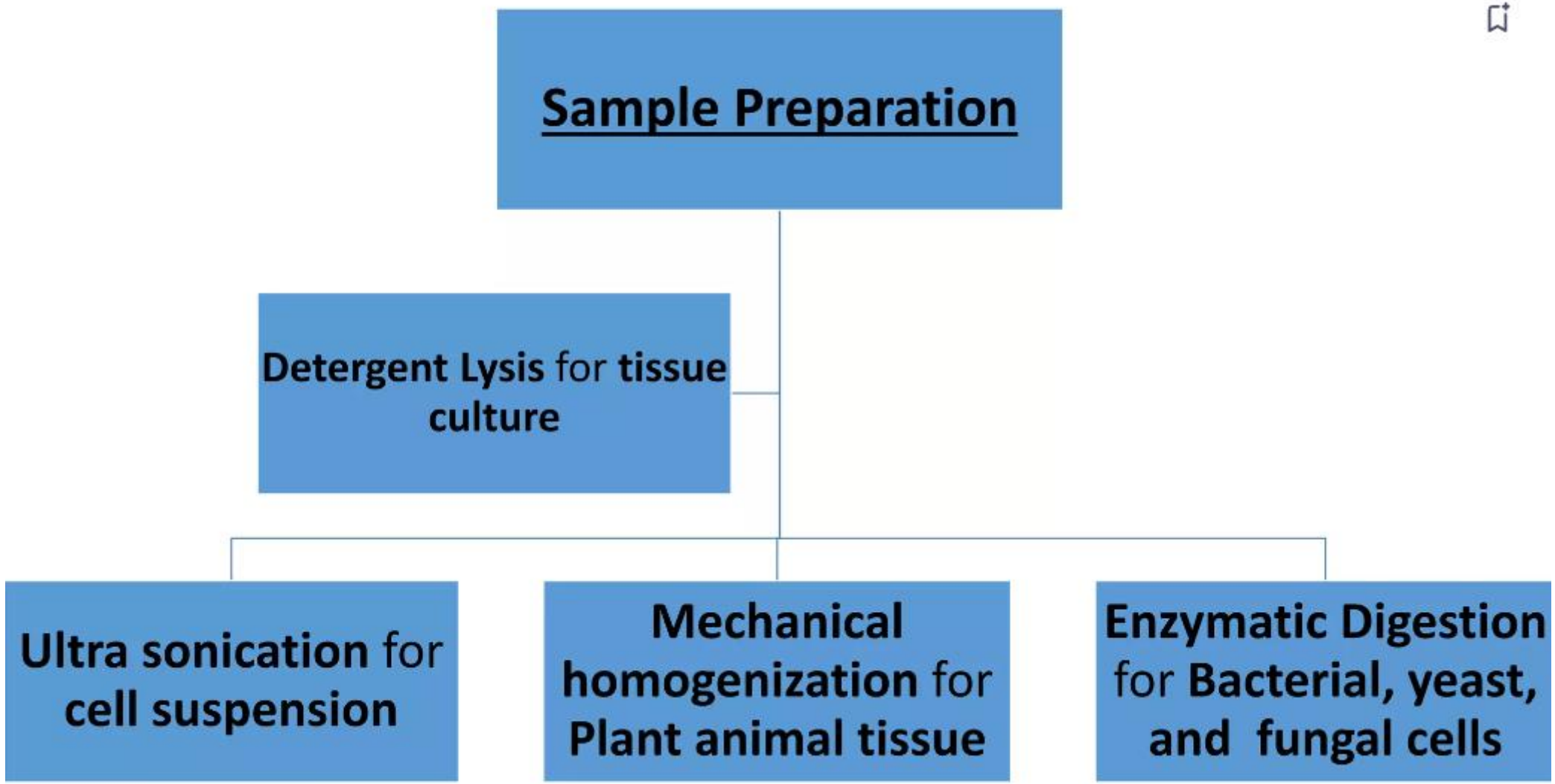


WESTERN BLOTTING

- ✓Western blotting is a widely used analytical technique in molecular biology to detect specific protein in a sample of tissue homogenate or extract.
- ✓It works on the principle of gel electrophoresis.
- ✓Proteins are separated based on their size on polyacrylamide gel.

Steps involved in western blotting

1. Sample preparation
2. Gel Electrophoresis
3. Blotting (or transfer)
4. Blocking
5. Antibody Probing
6. Detection

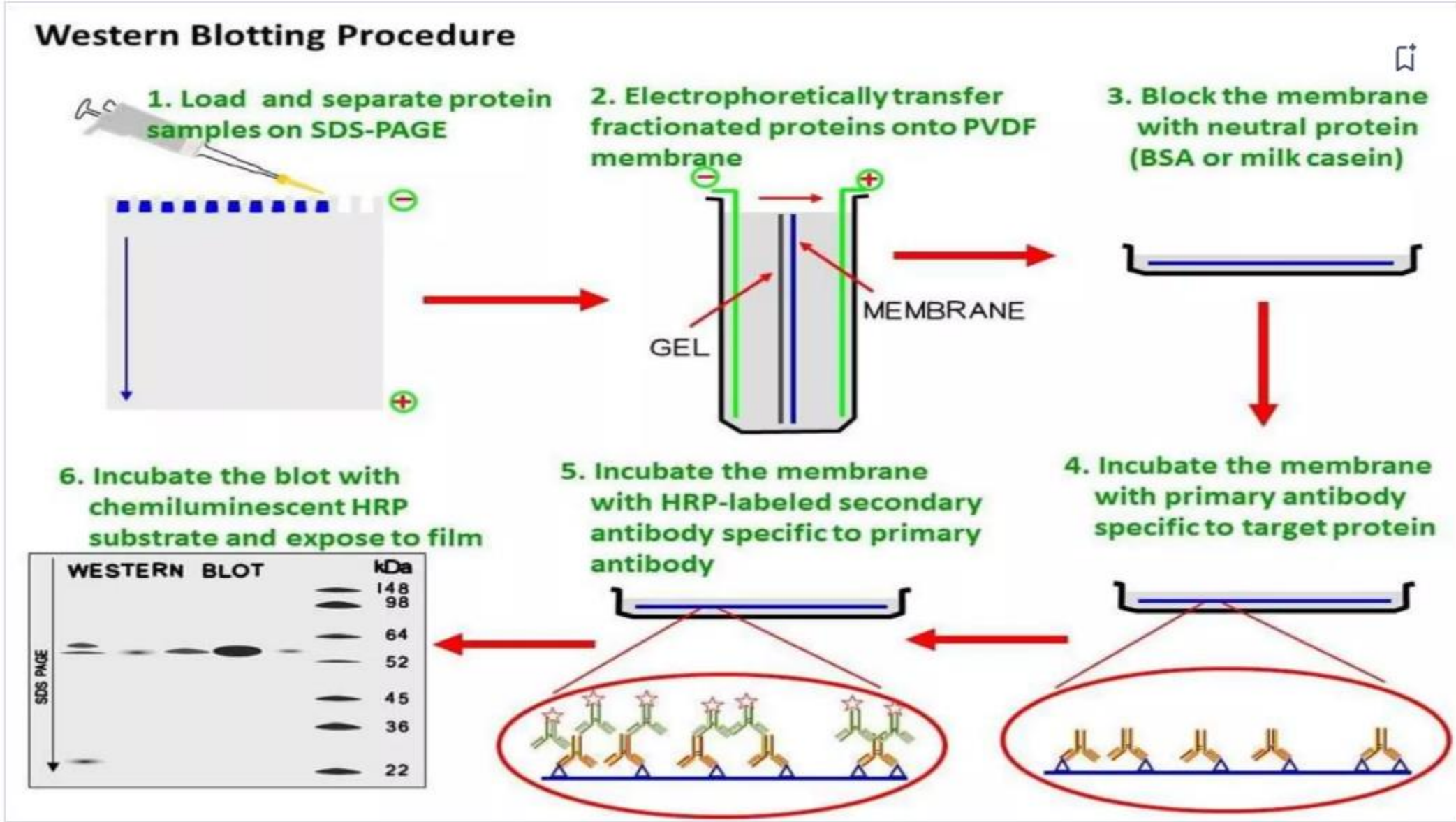

Sample Preparation
Detergent Lysis for tissue culture
Ultra sonication for cell suspension
Mechanical homogenization for Plant animal tissue
Enzymatic Digestion for Bacterial, yeast, and fungal cells

Gel Electrophoresis

- ✓Electrophoresis is commonly used method for separating proteins on the basis of size, shape or charge.

- ✓In SDS Gel Electrophoresis, protein sample are separated according to there molecular weight.

Western Blotting Procedure



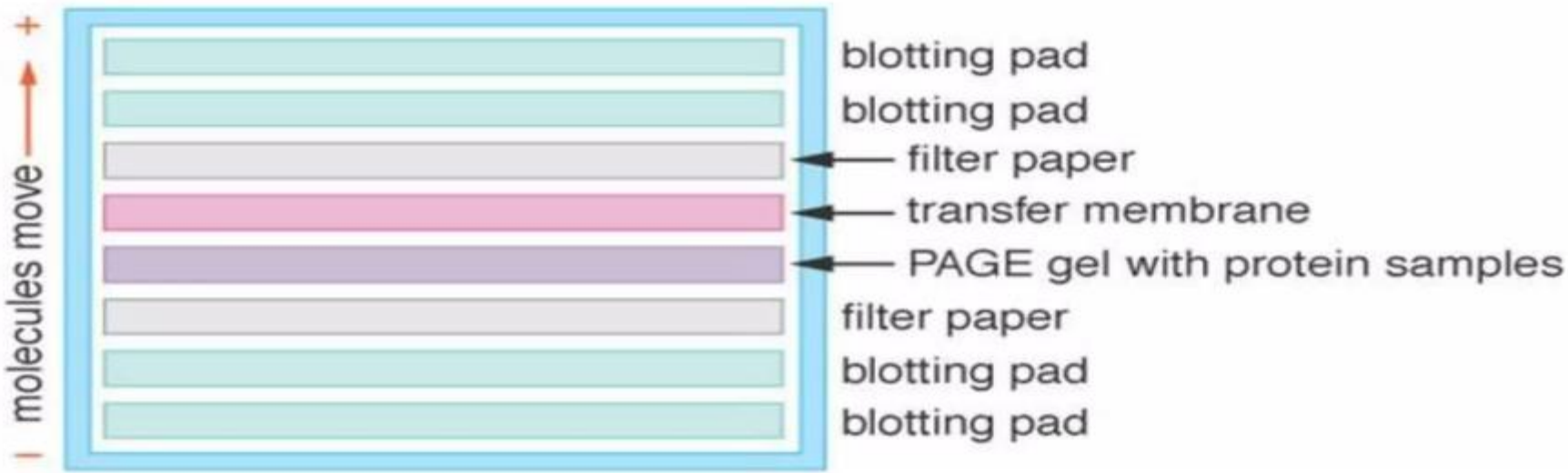
PROTEIN TRANSFER

- ✓On completion of the separation of proteins by Polyacrylamide gel electrophoresis, the next step is to transfer the proteins from the gels to solid support membrane.
- ✓Usually made up of a chemically inert substances, such as NITROCELLULOSE or PVDF.
- ✓The process of transferring proteins from a gel to a membrane while maintaining their relative position and resolutions is known as blotting.

3- Blotting- Blotting Membranes

- The solid support onto which the separated proteins are transferred is usually of two types, both of which bind proteins with high affinity:
  - Nitrocellulose membrane
    - has excellent protein binding and retention capabilities
    - is brittle and thus it is usually less effective when blots need to be reused
  - Polyvinylidene fluoride (PVDF) membrane
    - PVDF demonstrates superior mechanical strength making it suitable for stripping/reprobing

Western Blot Gel/Transfer Membrane Sandwich



Western Blot Gel/Transfer Membrane Setup Diagram.
During a Western blot, electrical current carries protein bands from the PAGE gel to the blot transfer membrane.

PROTEIN STAINING

- ✓After gel electrophoresis, it may be necessary to confirm that all the proteins in the gel have been completely eluted.
- ✓As proteins are not directly visible in the gel, the gel must be stained.
- ✓Proteins are usually stained with dyes such as coomassie blue, silver stain, or deep purple.
- ✓After staining, a permanent record may be made by imaging the gel with suitable instrument.

BLOCKING

- ✓For meaningful result, the antibodies must bind only to the protein of interest and not to the membrane.
- ✓Non-specific binding (NSB) of antibodies can be reduced by blocking the unoccupied sites of membrane with an inert protein or non-ionic detergent.
- ✓Blocking agents should possess greater affinity towards membrane than the antibodies.

BLOCKING AGENTS

✓The most common blocking agents are:-

a) Bovine serum albumin (BSA)

b) Non-fat milk

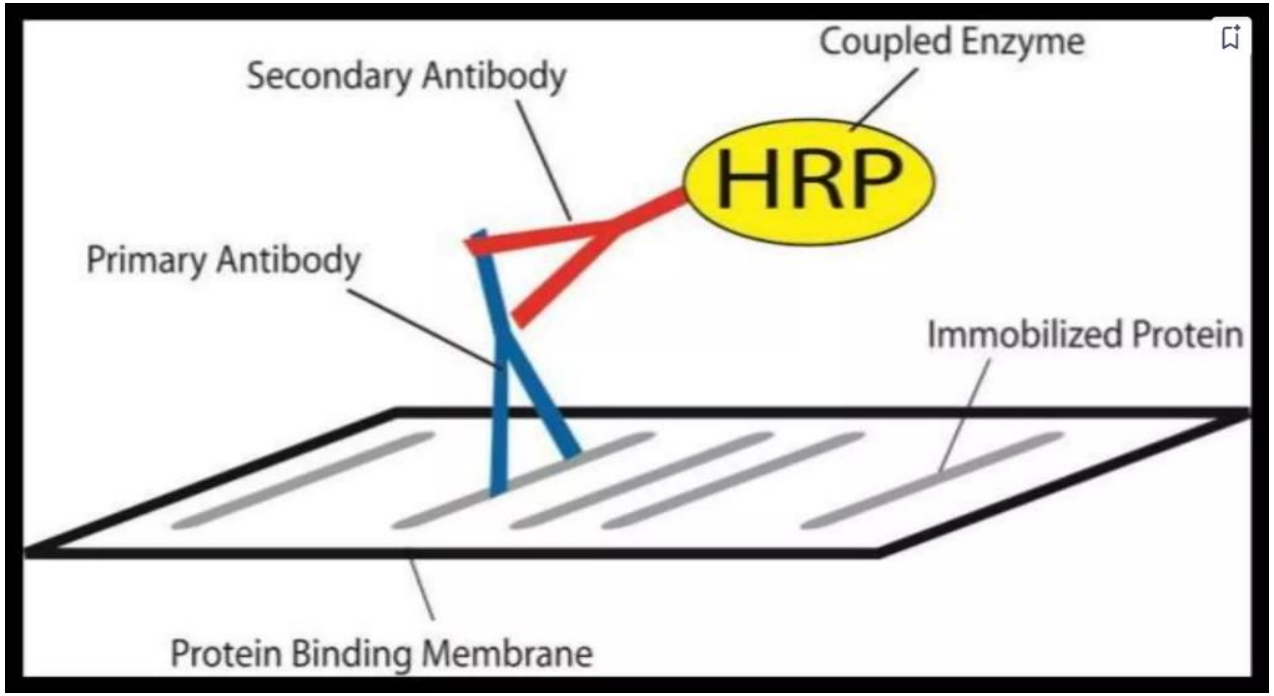
c) Casein

d) Gelatine

e) Dilute solution of Tween 20.

ANTIBODY PROBING

- ✓After blocking, the blot is incubated with one or more antibodies.
- ✓This uses specific antibody to detect a localize the protein blotted to a membrane.
- ✓The specificity of antigen-antibody binding permits the identification of a single protein in a complex sample.
- ✓The non-labelled primary antibody directed against the target protein, and specific labelled secondary antibody binds to the primary antibody.
- ✓The secondary antibody is conjugated to an enzyme that is used to indicate the location of the protein.



- ✓Secondary antibodies can be a monoclonal or polyclonal antibodies.
- ✓The secondary antibodies not only serves as a carrier of the label, but it is also helps to amplify the emitted signals.
- ✓The signal emitted by the labelled secondary antibody is then measured and is proportional to the quantity of protein of interest present on the membrane.

WASHING

- ✓Unbound antibodies can cause high background and poor detection.
- ✓Hence, washing the blot removes unbound antibodies from the membrane.
- ✓A dilute solution of tween-20 in TBS or PBS buffer is commonly used for washing.

PROTEIN DETECTION

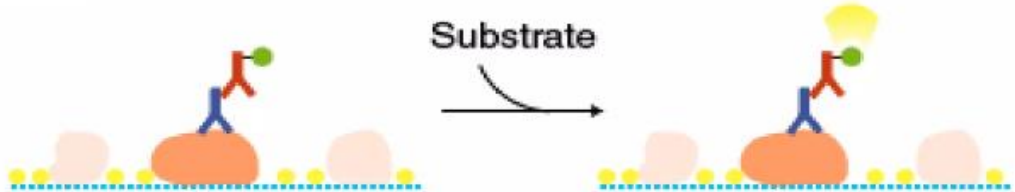
- ✓After the unbound probes are washed away, the western blotting is now ready for detection of the probes that are labelled and bound to the protein of interest.
- ✓Enzyme such as alkaline phosphatase (AP) & Horse Radish Peroxidase (HRP) are widely used in detection of proteins.
- ✓There are four methods of detection can be done & they are as follows:-

TYPES OF PROTEIN DETECTION

- ✓Chromogenic detection
- ✓Chemiluminescence detection
- ✓Fluorescent detection
- ✓Radioactive detection

Chemiluminescence method

✓ A reaction mixture containing a substrate is added to the membrane. The enzyme attached to the antibody catalyzes a reaction that emits light is detected by X-ray film.



- More routinely, HRP is used with ECL (enhanced chemiluminescence) detection
- For ECL detection, the substrate is luminol which is oxidized by HRP in the presence of H_2O_2 to produce light
- The emitted light is detected by exposing the Western blot to X-ray film, or by using a CCD camera for light capture

COLORIMETRIC METHOD

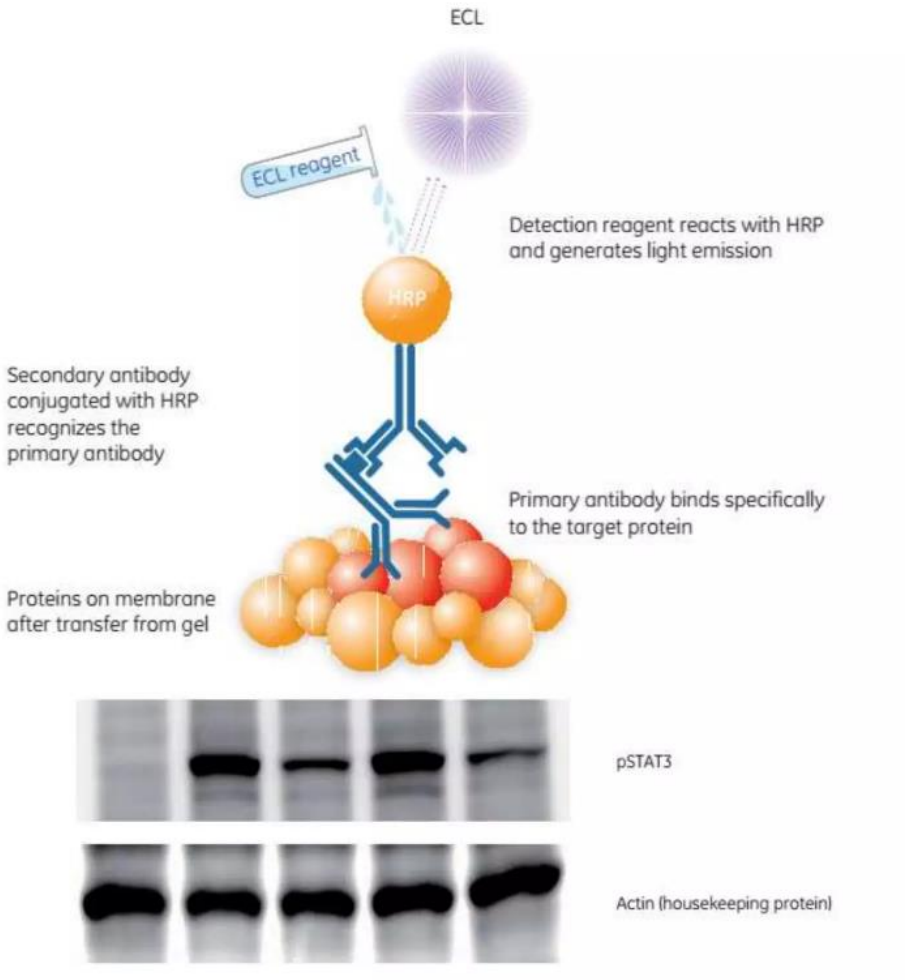
✓A chromogenic substrate is used as a detection reagent.

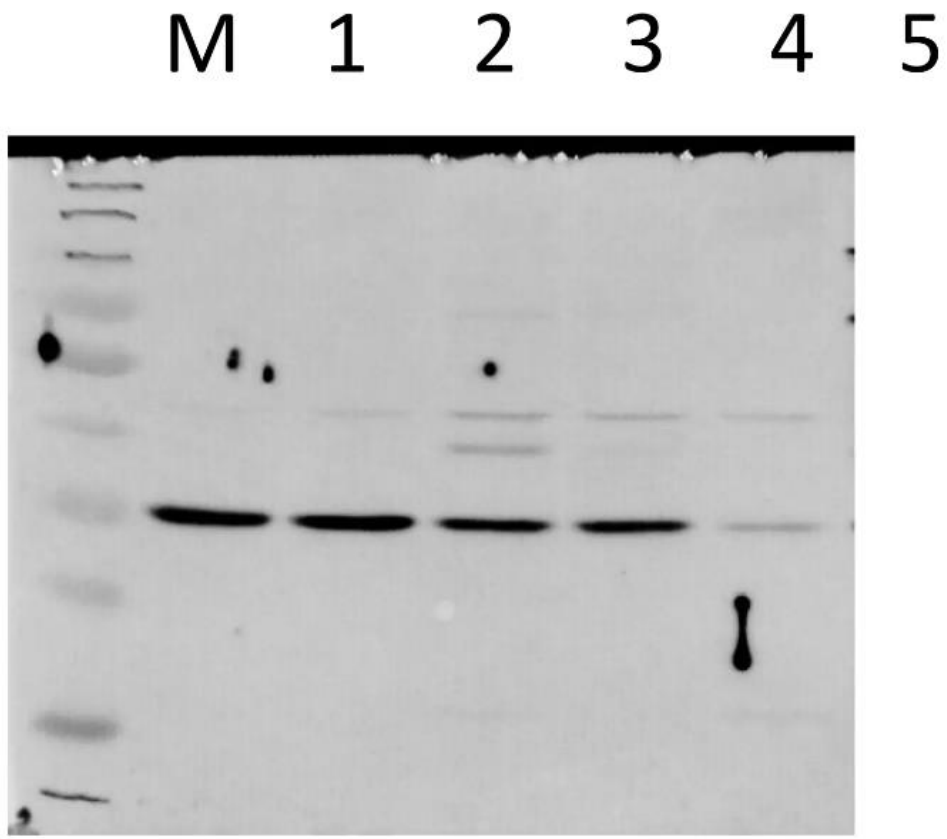
✓Diaminobenzidine (DAB) or TMB are commonly used as substrates of HRP.

- Colorimetric substrates for HRP {eg. Tetramethylbenzidine(TMB)} produce purple/black bands directly on the surface of the blot

ANALYSIS & IMAGING

- ✓This is the last & major step of the western blotting technique.
- ✓Detection of signals using either X-ray film, scanners or a CCD, results in one or more visible proteins bands on the membrane image.
- ✓The molecular weight of the protein can be estimated by comparison with marker proteins and the amount of protein can be determined as this is related to band intensity.
- ✓Qualitative & quantitative analysis can be done in order to verify the absence or presence of specific proteins of interest.


ECL
ECL reagent
Detection reagent reacts with HRP and generates light emission
HRP
Secondary antibody conjugated with HRP recognizes the primary antibody
Primary antibody binds specifically to the target protein
Proteins on membrane after transfer from gel
pSTAT3
Actin (housekeeping protein)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APPLICATION OF BLOTTING

- ✓Analysis of IgG fractions purified from human plasma.
- ✓Diagnosis of HIV by ELISA, involves the western blotting technique.
- ✓Western blotting technique is also used to detect some forms of Lyme disease.
- ✓Western blotting technique is used in defence test for BSE, which is commonly known as Mad cow disease.
- ✓Confirmatory test for Hepatitis-B involves western blotting technique.
- ✓This technique is also employed in the Gene Expression Studies.

LIMITATION OF WESTERN BLOTTING

- Very delicate and time consuming process.
- Incorrect labelling of protein can happen due to the reaction of secondary antibody.
- Well trained technicians are required for this technique.