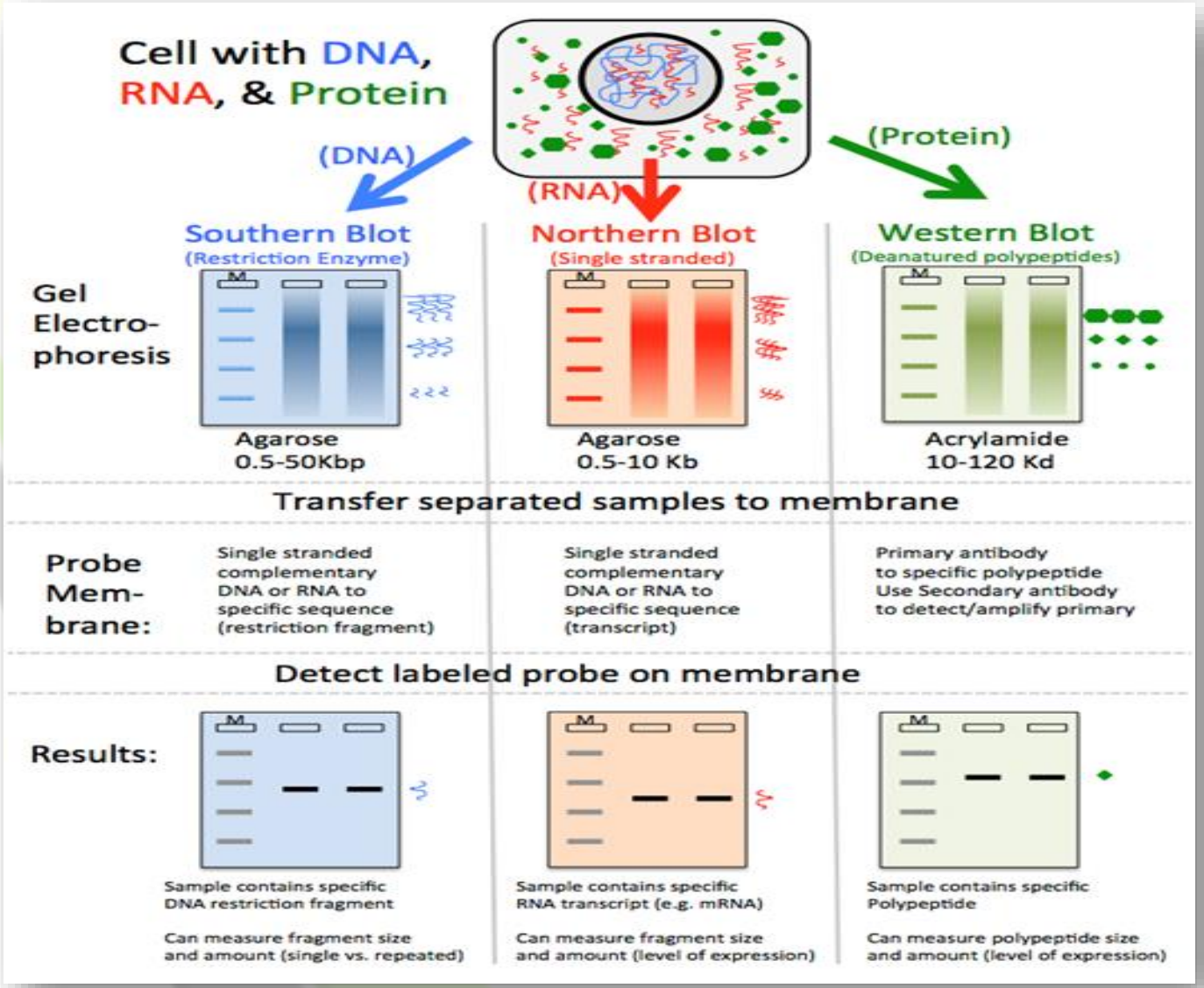
BCH 462

Western Blot

Blotting


Cell with DNA, RNA, & Protein
(DNA)
(RNA)
(Protein)
Southern Blot
(Restriction Enzyme)
Northern Blot
(Single stranded)
Western Blot
(Deanatured polypeptides)
Gel Electro-phoresis
M
Agarose
0.5-50Kbp
Agarose
0.5-10 Kb
Acrylamide
10-120 Kd
Transfer separated samples to membrane
Probe Mem-brane:
Single stranded complementary DNA or RNA to specific sequence (restriction fragment)
Single stranded complementary DNA or RNA to specific sequence (transcript)
Primary antibody to specific polypeptide
Use Secondary antibody to detect/amplify primary
Detect labeled probe on membrane
Results:
Sample contains specific DNA restriction fragment
Can measure fragment size and amount (single vs. repeated)
Sample contains specific RNA transcript (e.g. mRNA)
Can measure fragment size and amount (level of expression)
Sample contains specific Polypeptide
Can measure polypeptide size and amount (level of expression)

Immunoassay:

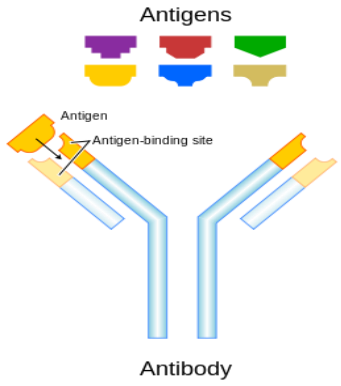
A test that uses antibody and antigen complexes [immuno-complexes] as a means of generating measurable results.

Antigens [Ag]:

A substance that when introduced into the body stimulates the production of an antibody. Antigens include toxins, bacteria, foreign blood cells, and the cells of transplanted organs.

Antibody [Ab]:

Antibodies are large Y-shaped glycoproteins. They are produced by the immune system to identify and neutralize foreign objects (antigens).



Western blot or protein immunoblot :

It is a widely used immunoassay technique, used to identify specific proteins [antigens] in a sample of tissue homogenate or extract, based on their ability [the antigens] to bind to antibodies resulting in color indicate the presence of this specific protein.

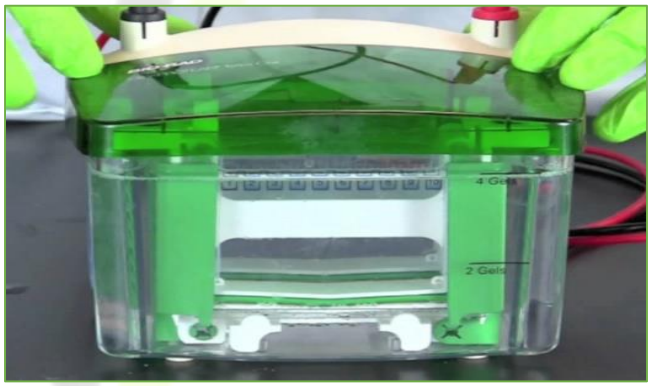
Principle:

It is an analytical method where in a protein sample is electrophoresed on an SDS-PAGE then electro-transferred onto a membrane. The transferred protein is detected using specific primary antibody, secondary antibody labeled with an enzyme, and substrate which in the end you will produce a colored product. The color indicate the presence of the protein of interest.

Western blot Applications

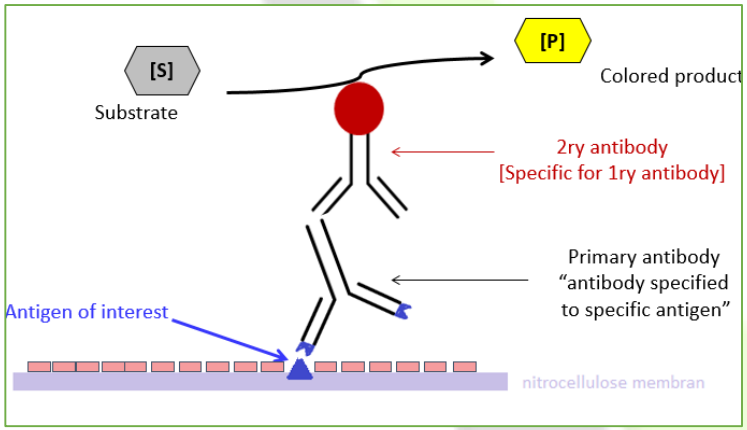
- Analyzing, identifying target proteins and estimating their molecular weight.
- To compare the amounts of a protein of interest among different samples.
- Used in clinical laboratories for assisting identification of certain antigen proteins (pathogen or biomarker).
- Used to detect changes in protein expression under different biological conditions (e.g. in disease, stress, etc.).

The technique uses three elements to accomplish this task



1. Separating the sample mixture using SDS-PAGE.

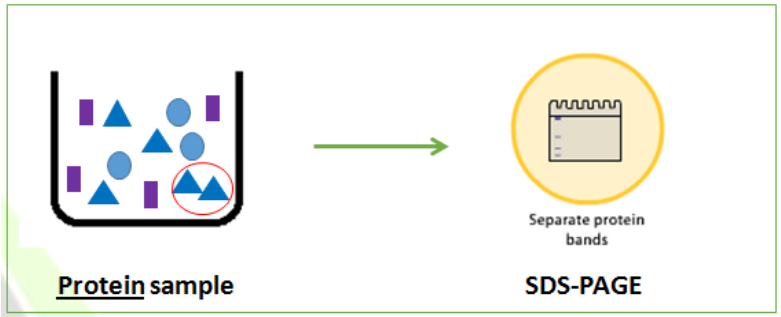
2. Transfer step [Electroblotting], by transfering the proteins bands from the gel to the membrane.



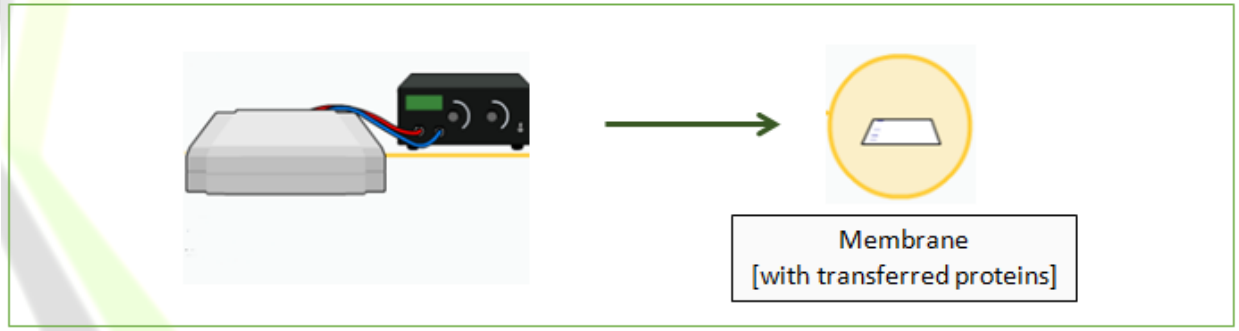
3. Marking target protein using a proper primary and secondary antibody to visualize.

Steps of detection of specific protein using Western bolt

1. A protein sample is subjected to polyacrylamide gel electrophoresis.



2. After that the gel is placed over a sheet of nitrocellulose , the protein in the gel is electrophoretically transferred to the nitrocellulose. “transfer step **[Electroblotting]**”



Transfer

• Membrane can be Nitrocellulose or PVDF

•**<u>Differences between them :</u>**

Nitrocellulose → cheaper, easier to use.

PVDF → needs more work, but binds most proteins more effectively.

<u>Types of transfer</u>:

Wet

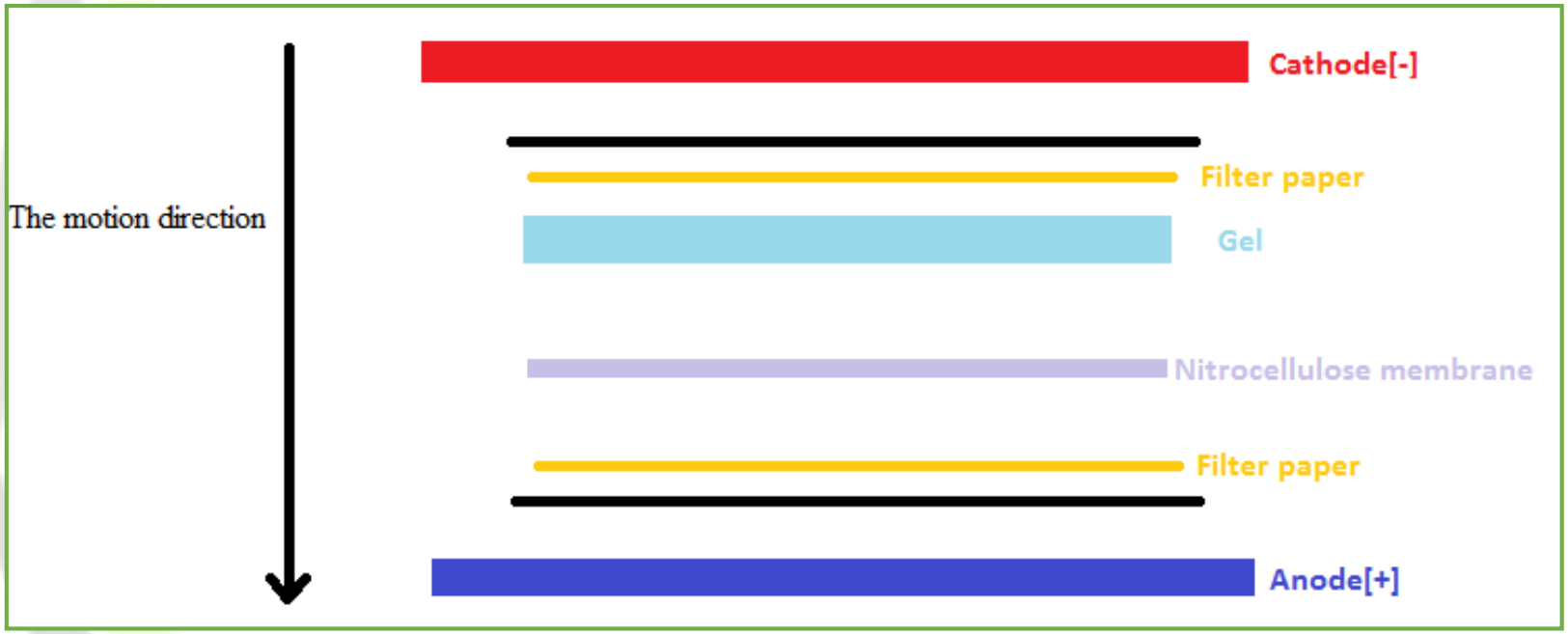
Best for proteins >100kDa

Semi-dry

Quick

Dry

Even quicker



Because the samples in the gel are [–ev] 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.

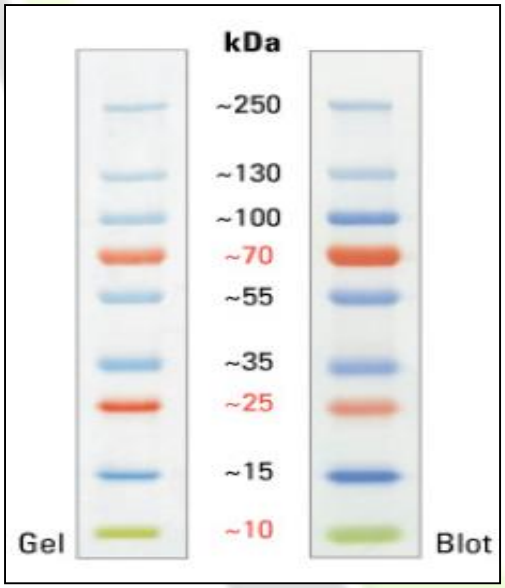
Note that: [the filter papers, gel and nitrocellulose membrane will soaked in transfer buffer].

To confirm if the samples are transferred from the gel to the membrane (Since separated proteins are colorless) either by:

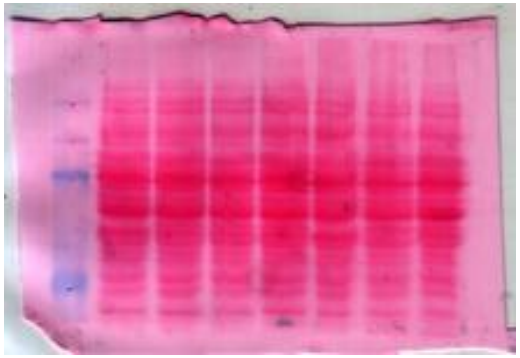
1- making a replica of the gel and stain it as usual [with Coomassie brilliant blue R-250] .

2- using a prestained marker.

3- reversible staining by Ponceau stain.



prestained marker

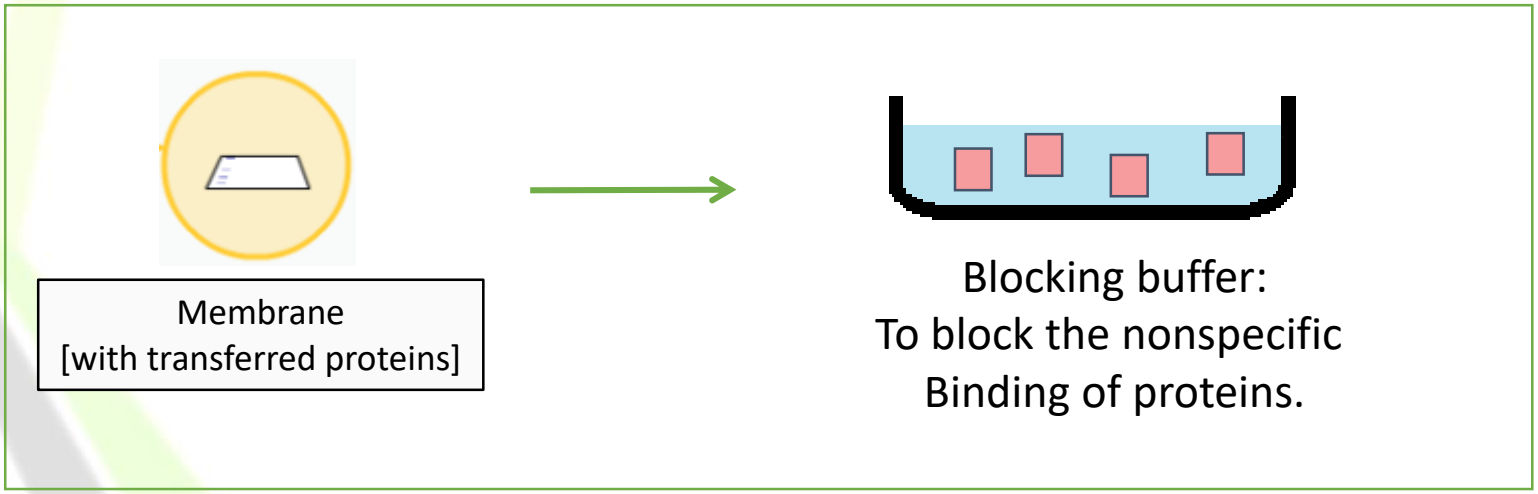


Ponceau staining

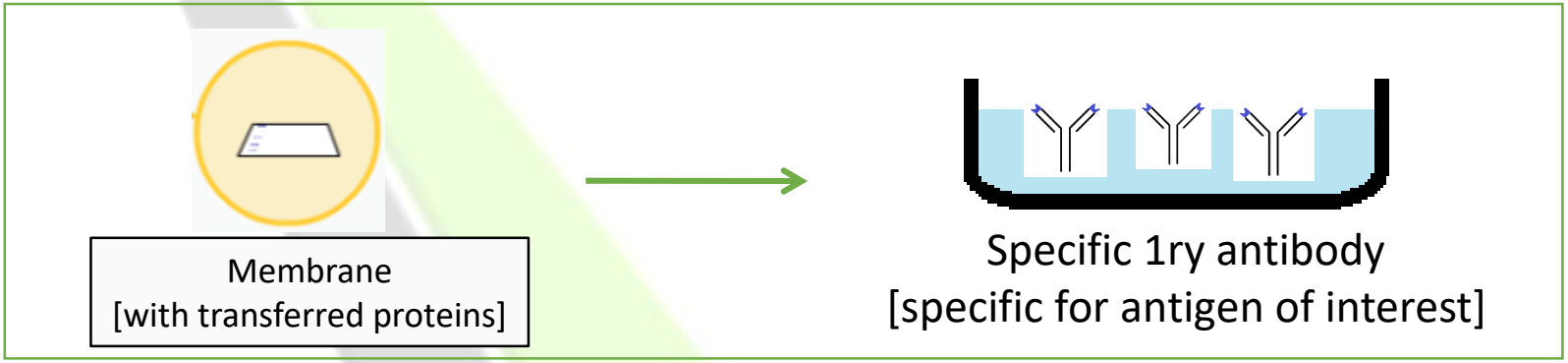
It is a **washable** light red colored dye , that may be used to prepare a stain for rapid detection of protein bands on nitrocellulose or polyvinylidene fluoride (PVDF) membranes (Western blotting).

3.The nitrocellulose is then soaked in blocking buffer to Fill up the space on the membrane to prevent non-specific antibody binding.

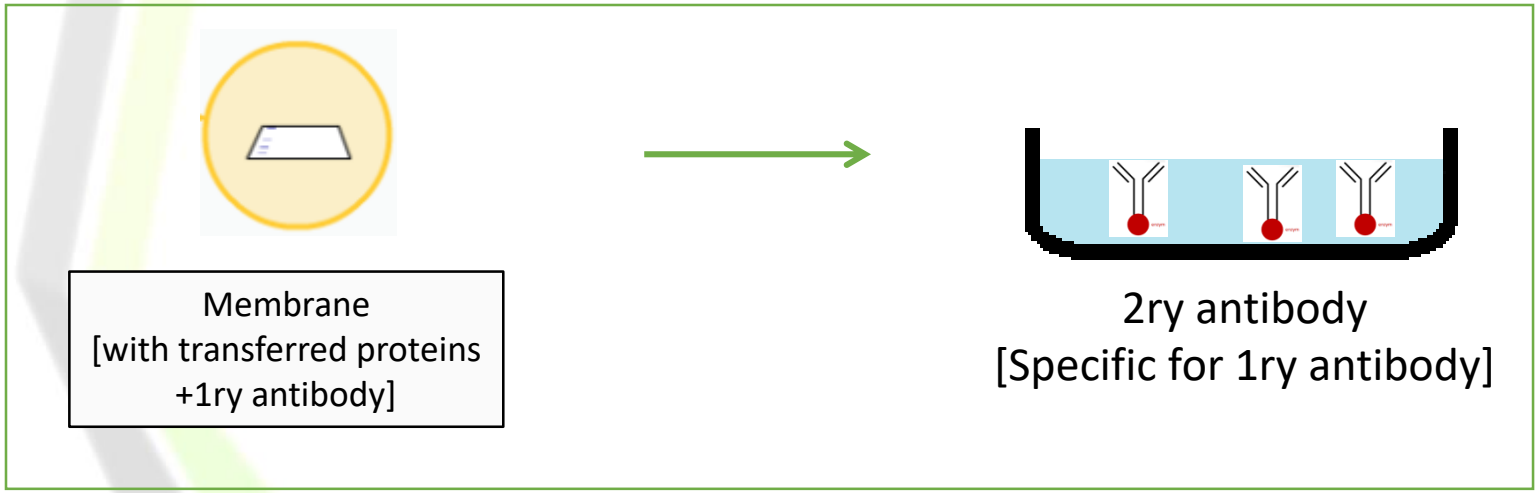
e.g. milk , BSA



4.The nitrocellulose is then incubated with the specific primary antibody for the protein of interest.



5.The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody[1ry –antibody].



- Note that The enzyme linked will convert colorless substrate to colored product.
- The color produced indicate the presence of the antibody - antigen [Ab-Ag] binding complex.

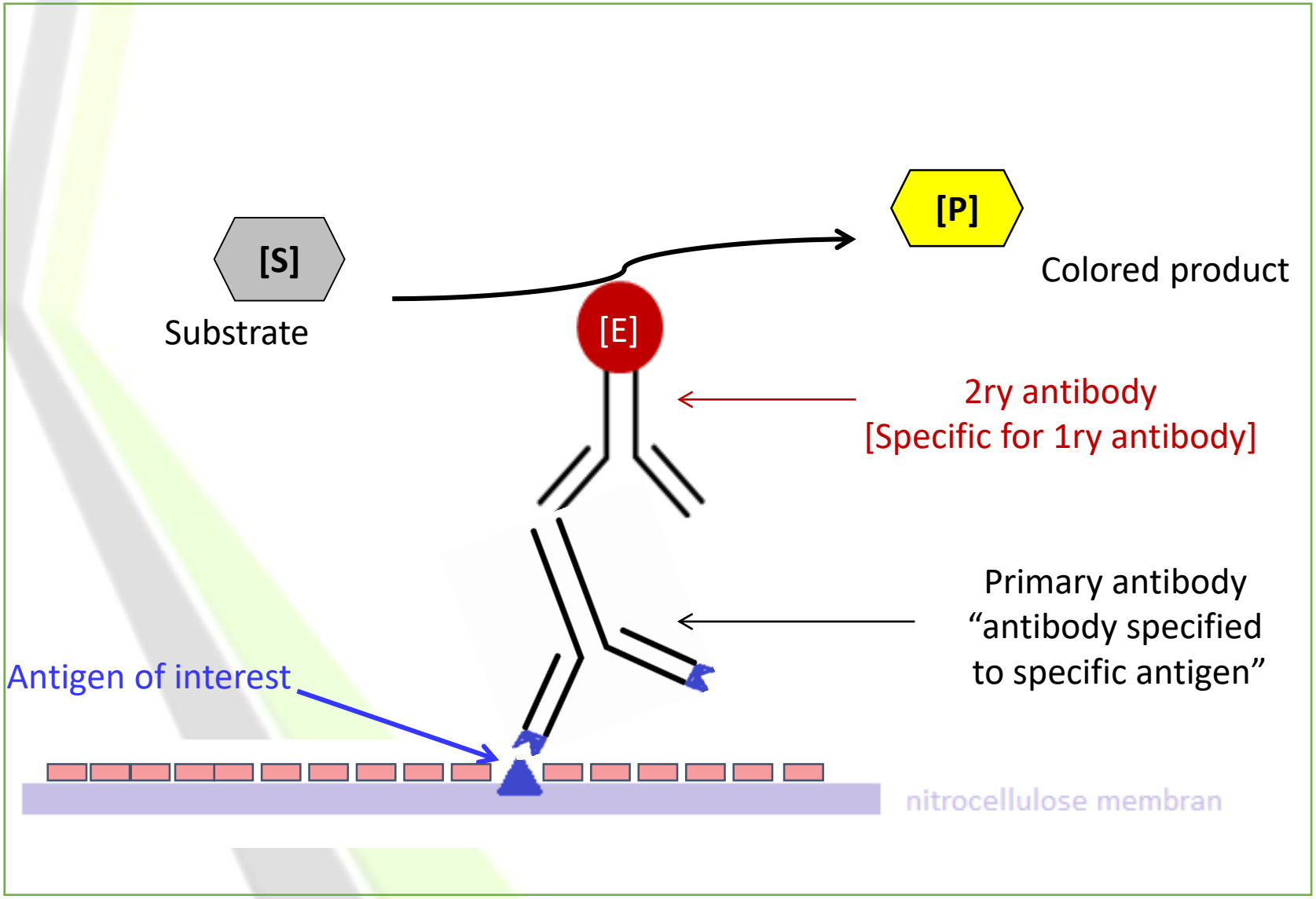
6.The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction. “detection step”.

-Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection.

-**Detection can be :**

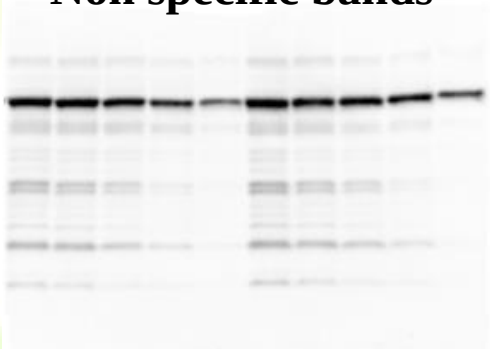
- Colorimetric
- Radioactive label
- Fluorescently labelled secondary antibody
- Chemiluminescent – HRP or AP labelled secondary antibody - very sensitive (emits light can be detected by X-ray film)

Detection of specific protein using Western bolt



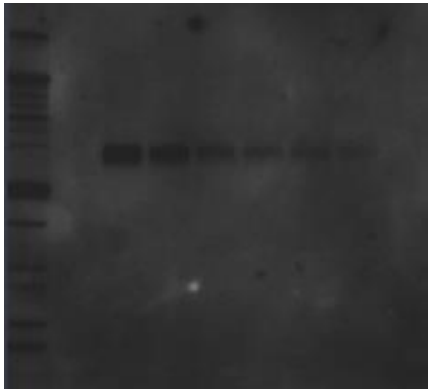
Common problems

Non specific bands



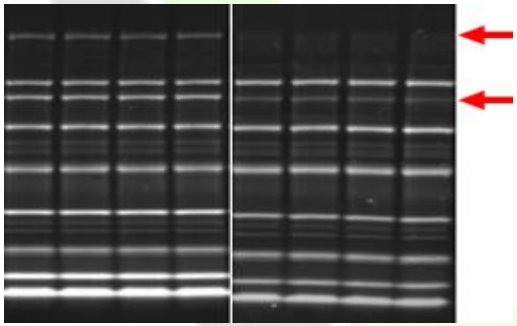
Probably too much antibody
Or insufficient blocking

High background



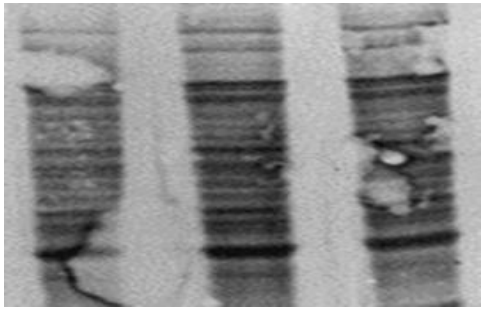
Probably too much antibody
Or insufficient blocking
Or insufficient washing

Incomplete transfer



Transfer time too short
Transfer current too low

Blotchy transfer



Air bubbles between gel and membrane

Video of wet transfer- western blot:

https://youtu.be/4BE0CWdfxw0