BCH 462- Biotechnology & Genetic engineering [Practical]

Lab (4) Western Blot

Remember !!
Antigen X Antibody

Immunoassay

- **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**.

Pause and Think how to prevent the body from rejecting an organ transplant?

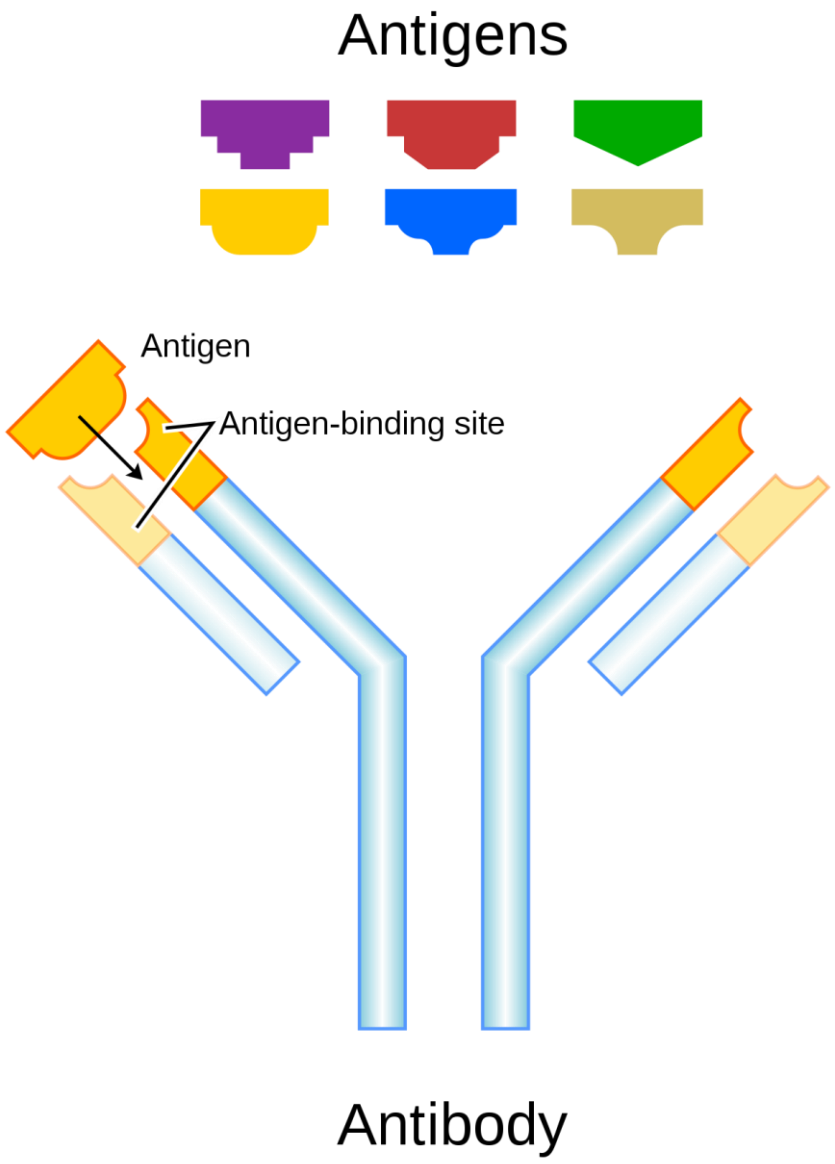
- **Antibody [Ab]:**

- Antibodies are large Y-shaped glycoproteins.

- They are produced by the immune system to identify and neutralize foreign objects (**antigens**).

- **Immunoassay:**

A test that uses highly specific and selective antigen-antibody reactions forming antibody and antigen complexes [**immuno-complexes**] as a means of generating measurable results.



Antibody and antigen complexes [immuno-complexes]

Immunoassay

Remember !!
Antigen = Protein of interest

Primary Antibody "antibody specified to specific antigen"

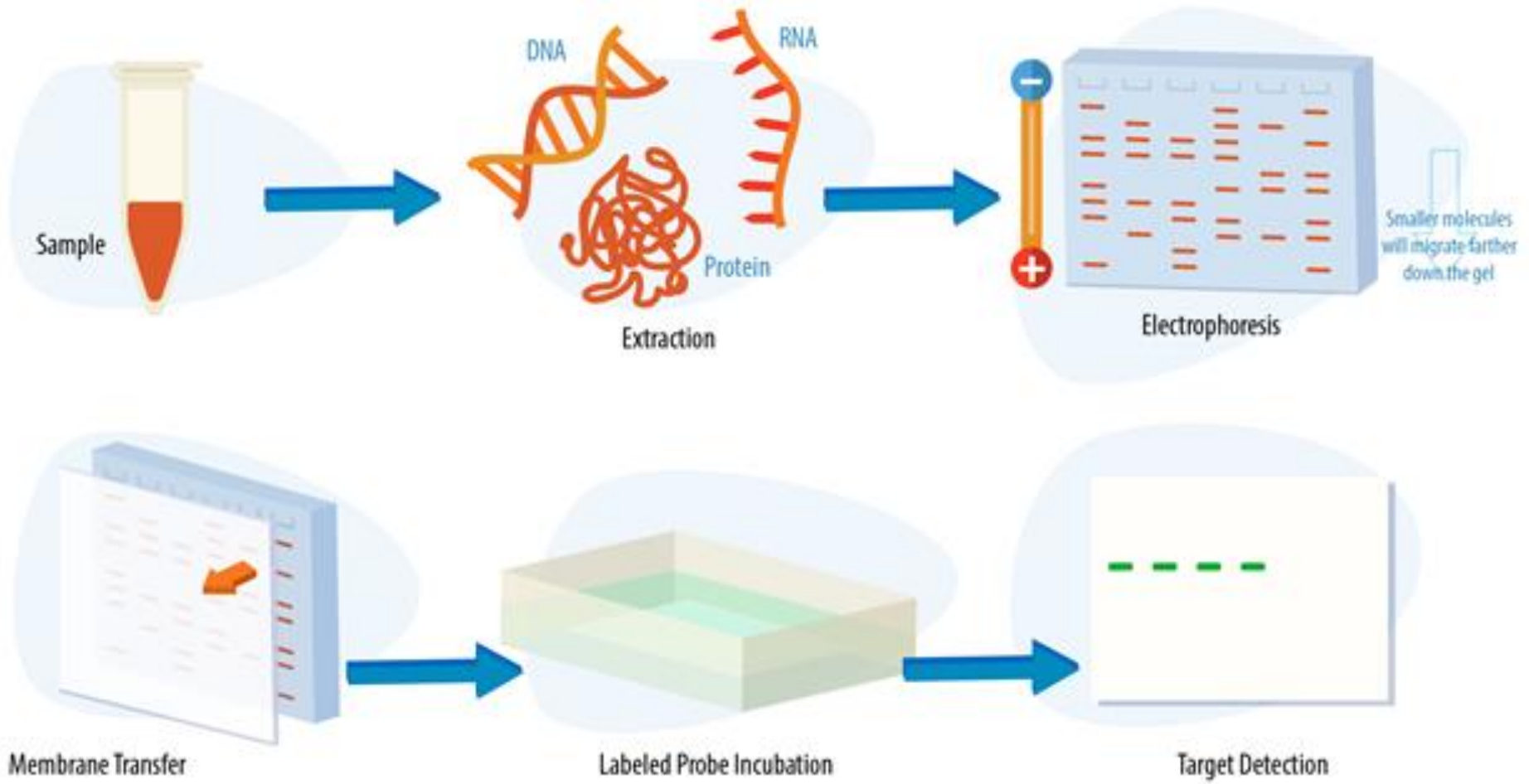
Antigen

Secondary Antibody "antibody specified to Primary antibody"

Blotting

- **Blotting** is the transfer of macromolecules such as nucleic acids and proteins to solid-phase membranous support (for instance, a nitrocellulose, PVDF or nylon membrane).

- **Types**:
  1. Southern blotting used to detect DNA.
  2. Northern blotting used to detect RNA.
  3. Eastern blotting; used to analyze protein post translational modifications (PTM).
  4. **Western blotting used to detect Proteins.**



Overview of blotting technique

Western blot

Remember !!
Antigen = Protein of interest

- Also called **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 to bind to [**antibodies**] resulting in color/florescence indicate the presence of this specific protein.

- **Applications:**

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

Practical Part

Practical part

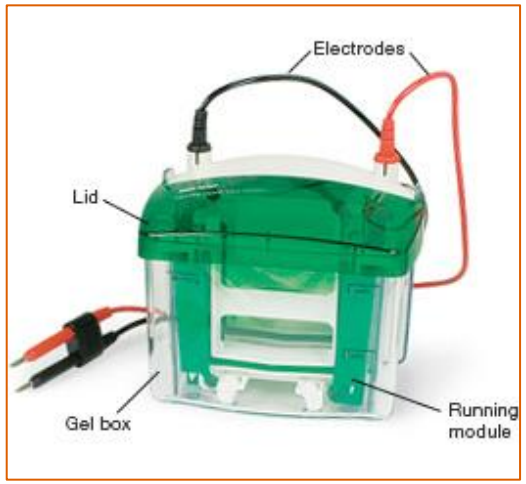
- **Aims:**
  - To understand how proteins (antigens) can be analyzed using antibodies raised against these proteins by immunoblotting technique.
  - To perform the steps of western-blot technique to detect tubulin protein.

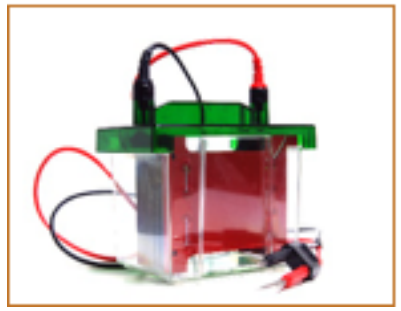
Practical part

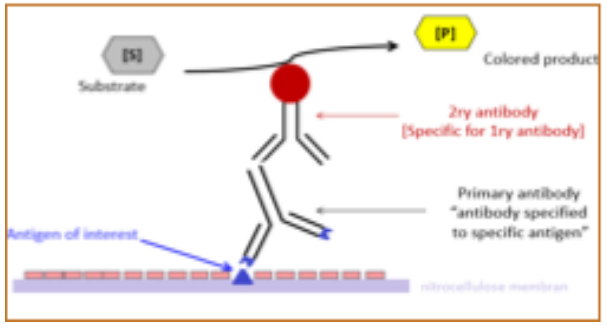
- The mixture of proteins is **separated** based on **molecular weight**.
- These results are then electro-transferred to solid support producing a band for each protein.
- The transferred protein is detected by incubating the membrane **with specific primary antibody** to the protein of interest, **secondary antibody labelled with an enzyme or fluorophore which target the primary antibody**, and **substrate** which in the end you will get colored product.
- The **color/florescence** indicates the presence of the protein of interest.
- The **thickness of the band corresponds to the amount of protein present**.
- Thus, the molecular weight and amount of the desired protein can be characterized from a complex mixture of proteins by western blotting.

Western blot performing steps

The technique uses three elements to accomplish this task







1. **1st phase (SDS-PAGE):**

Separation sample mixture **by size using SDS-PAGE.**

2. **2nd phase (Electro-blotting):**

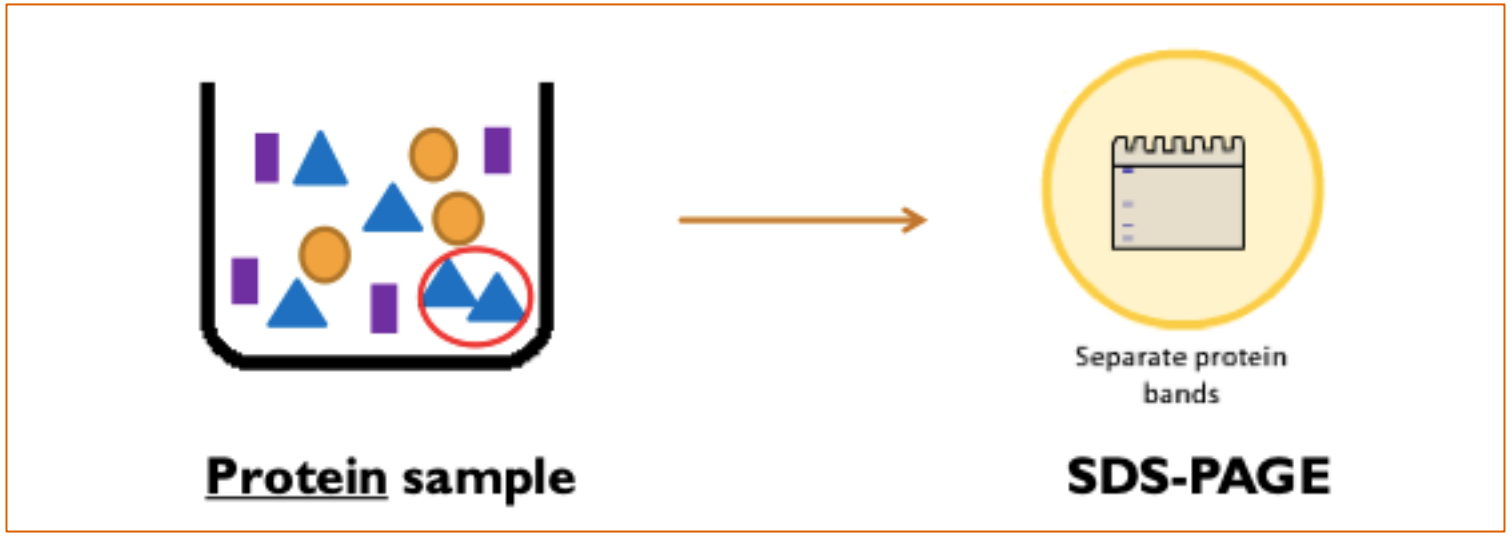
Transfer to a solid support (**electro-blotting**) by transferring the proteins bands from the gel to the membrane.

3. **3rd phase (Marking target protein to visualize):**

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

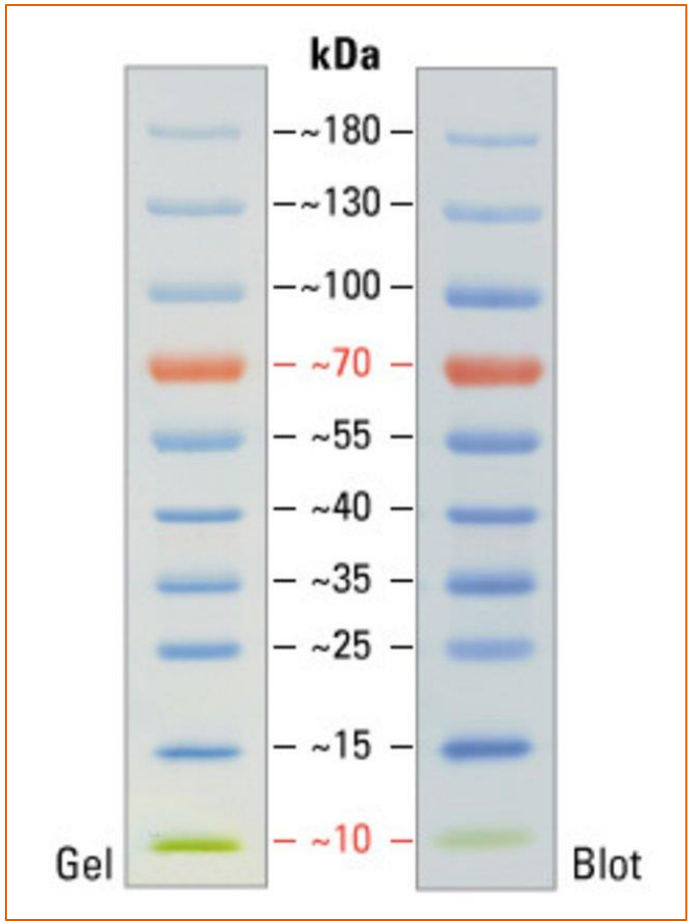
1st phase (SDS-PAGE)

- A protein sample is subjected to polyacrylamide gel electrophoresis.



- **To confirm the separation of samples (since separated proteins are colorless) use:**
  1. Replica of the gel and stain it as usual [with Coomassie brilliant blue R-250].
  2. Using a pre-stained marker.
  3. Reversible staining by Ponceau S.

Pre-stained marker



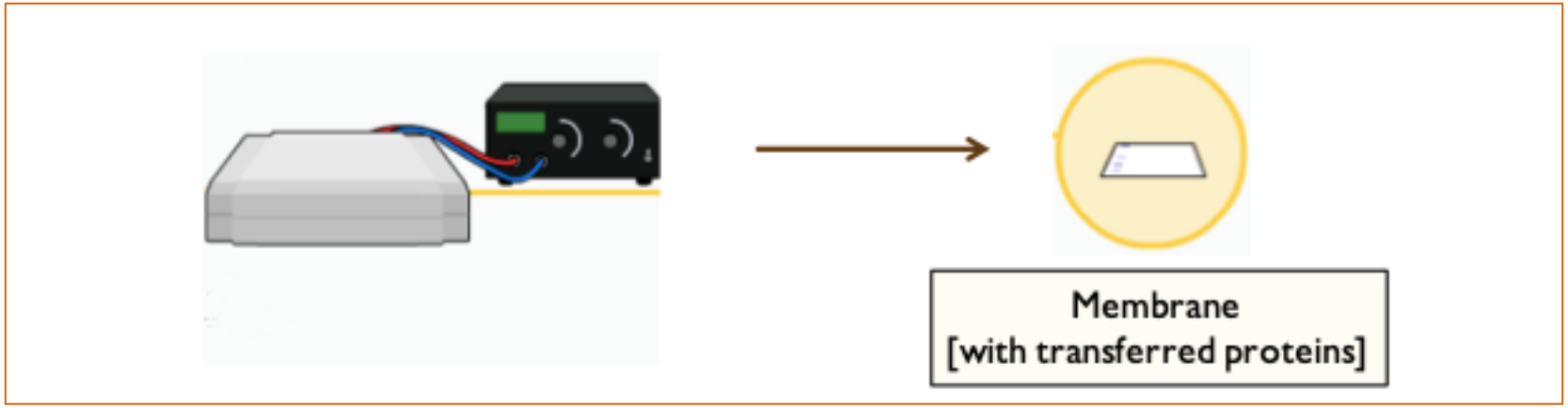
Pre-stained Protein Ladder is a mixture of 10 blue-, orange-, and green-stained proteins (10 to 180 kDa) for use as size standards in protein electrophoresis (SDS-PAGE) and western blotting.

Ponceau staining

Ponceau staining 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).

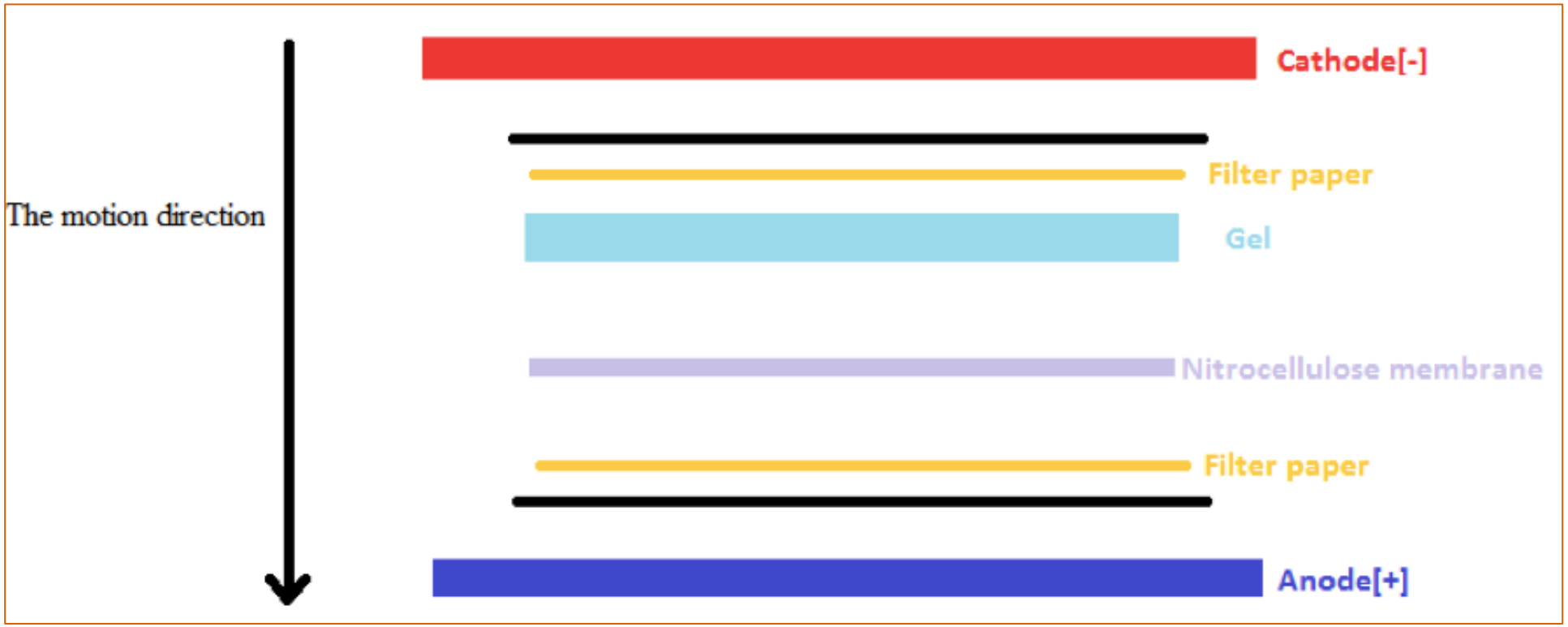
2nd phase (Electro-blotting)

- After that the gel is placed over a sheet of PVDF, the protein in the gel is electrophoretically transferred to the Polyvinylidene fluoride (PVDF) membrane. "**transfer step [Electroblotting]**".



- **Methods of transfer:**
  1. **Wet method** → Most common transfer method, best for proteins >100kDa.
  2. **Semi-wet** → Quick but less efficient.
  3. **Dry** → Quicker, no transfer buffer required.

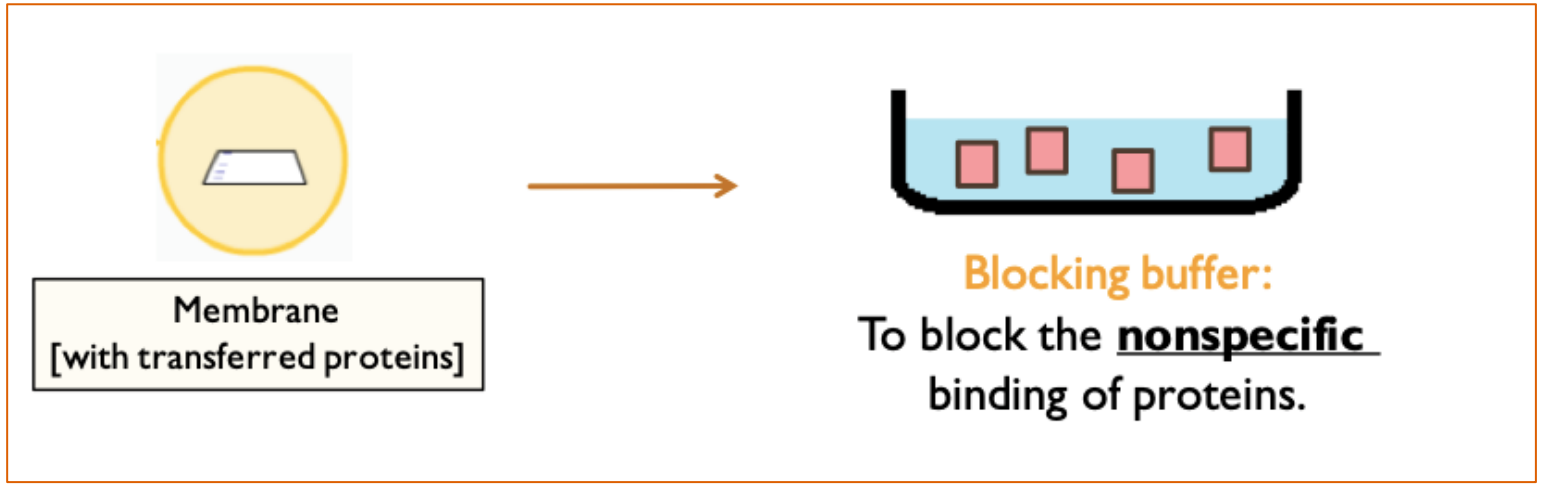
Transfer sandwich



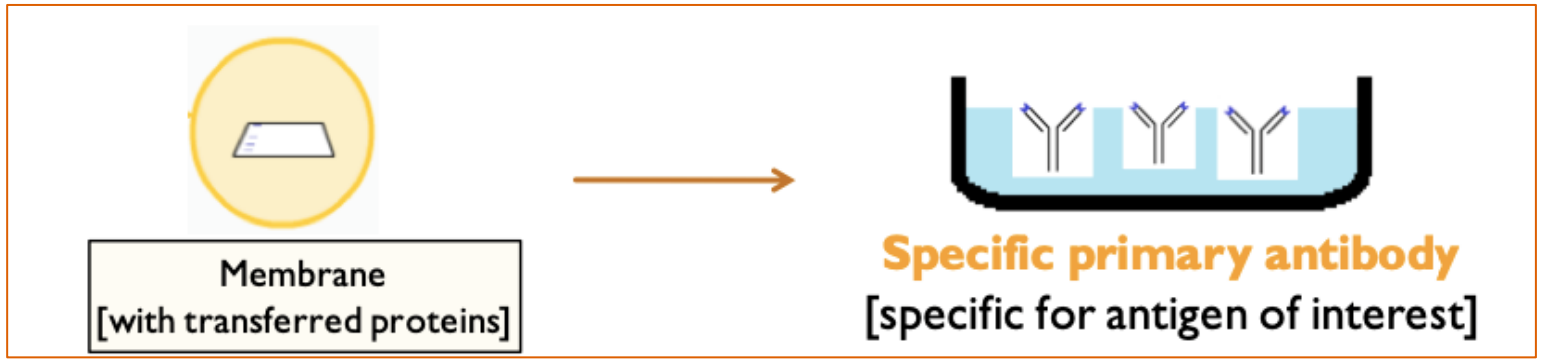
- Because the samples in the gel are **[–ve]** charged , the applied electric current will facilitate their transferring to nitrocellulose membrane, the samples will move toward the **Anode[+].**
- Also the **capillary action** has its effect in the movement of the samples from the gel to the nitrocellulose membrane.
- Note that: [the filter papers, gel and PVDF membrane will be soaked in transfer buffer].

3rd phase (Marking target protein to visualize)

- The PVDF is then soaked in blocking buffer.



- The PVDF is then incubated with the specific primary antibody for the protein of interest.



3rd phase (Marking target protein to visualize)

- The PVDF is then washed and incubated with a secondary antibody, which is specific for the first antibody [primary–antibody].
- **2ry AB** is conjugated with **enzymes** or **fluorescent** that give a subsequent reaction with an applied reagent, leading to a coloring or emission of light, enabling detection.

- The color produced indicate the **presence** of the **antibody-antigen [Ab-Ag]** complex.

3rd phase (Marking target protein to visualize)

Types of western blot detection methods “detection step”:

1- Colorimetric

Secondary antibody labeled with **enzyme** conjugates, such as alkaline phosphatase (AP) or horseradish peroxidase (HRP), when provided with a chromogenic substrate, will cause a color reaction.

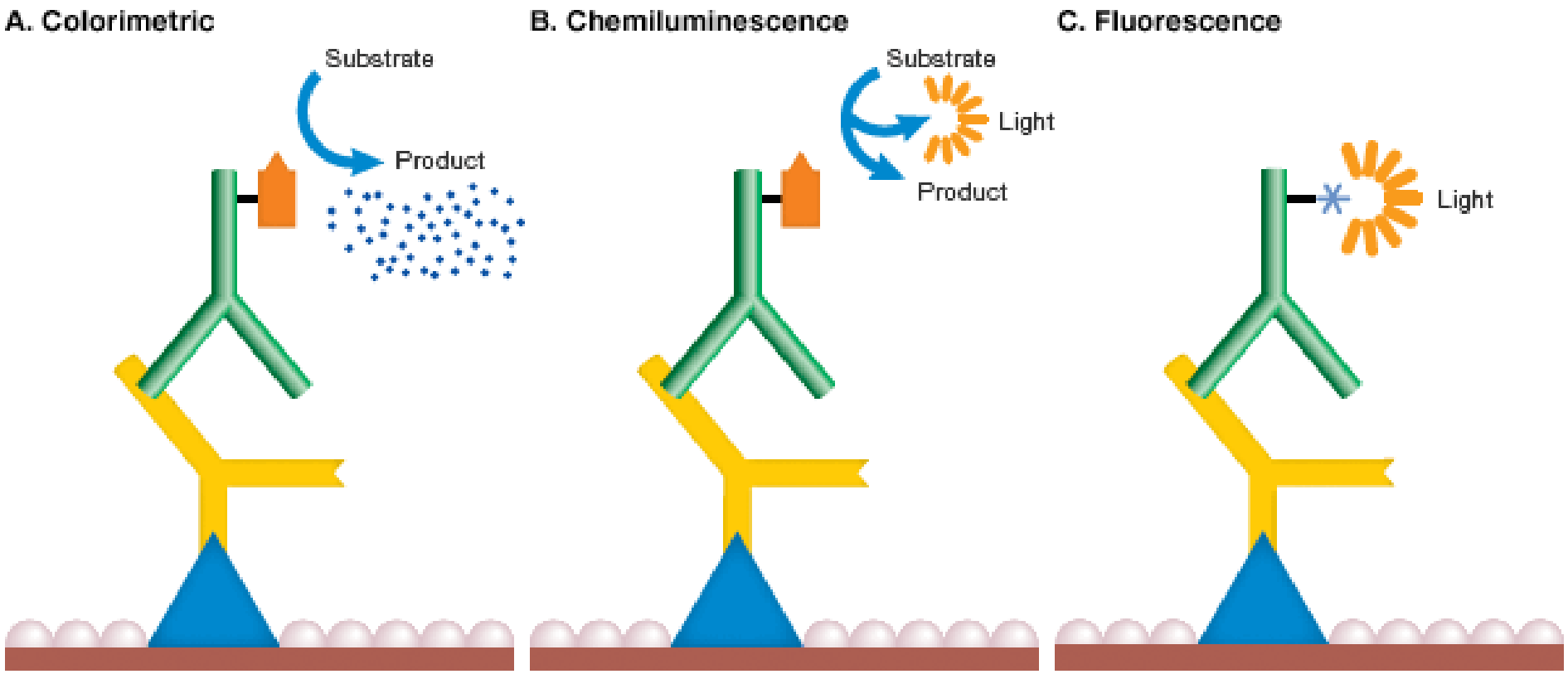
2- Chemiluminescence

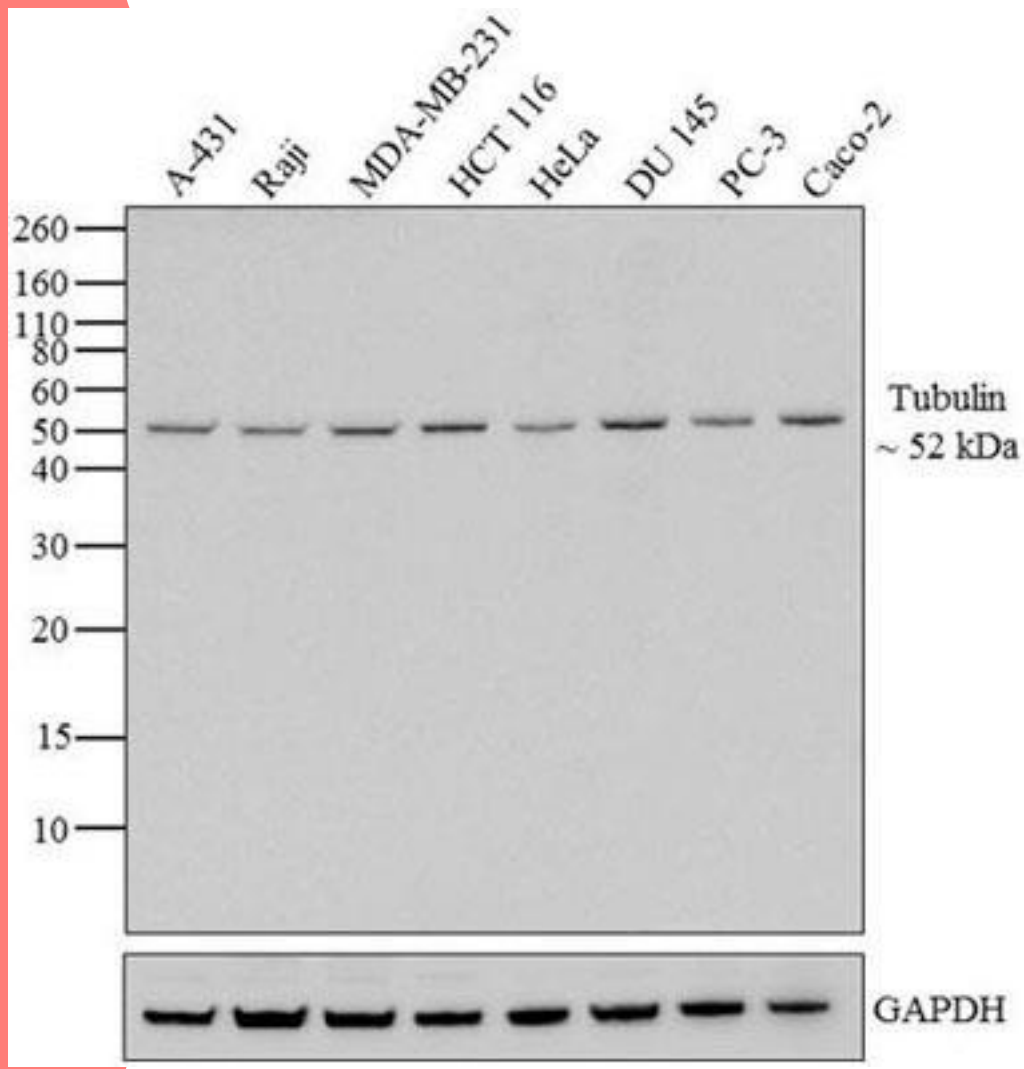
The enzyme attached to the secondary antibody triggers a reaction with a luminescent substrate that produces light as a by-product.

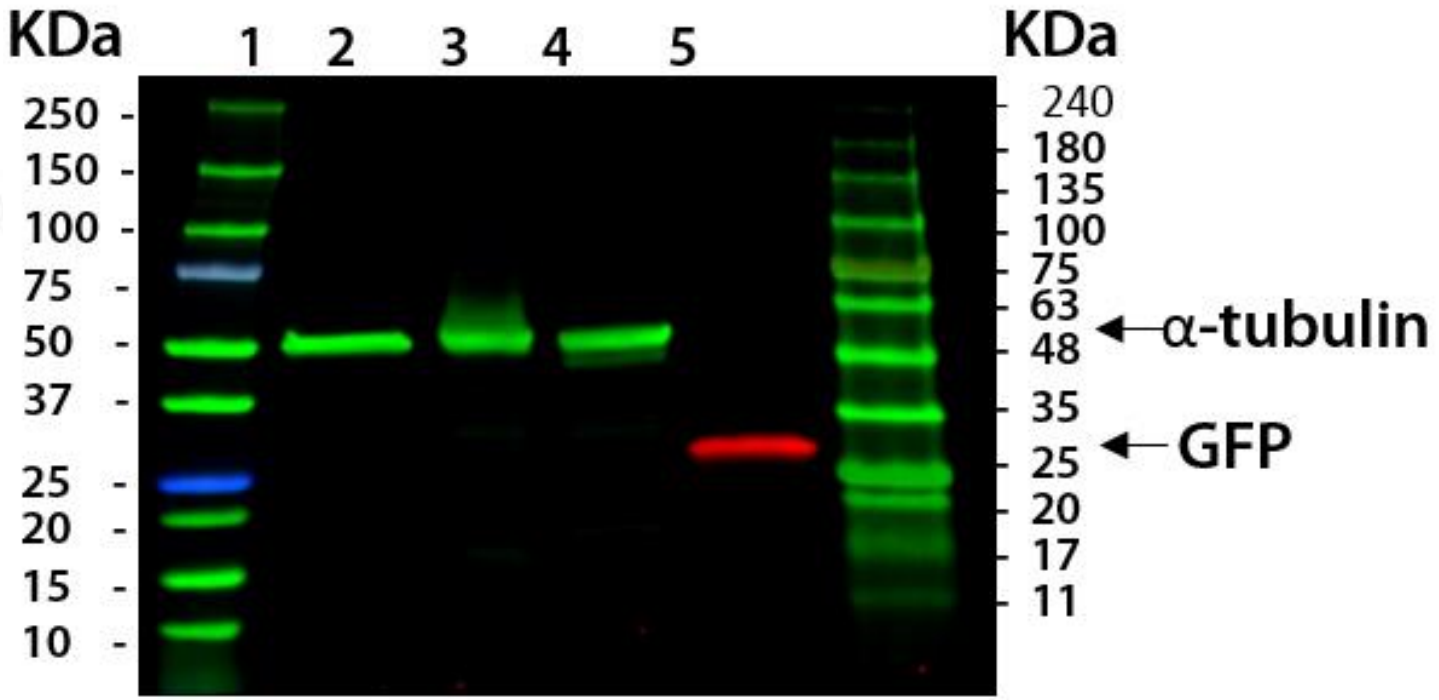
3- Fluorescence

Uses secondary antibodies that are conjugated to specific fluorophores that can absorb and emit light within a range of wavelengths, so no additional substrate is necessary.

Western blot detection methods:



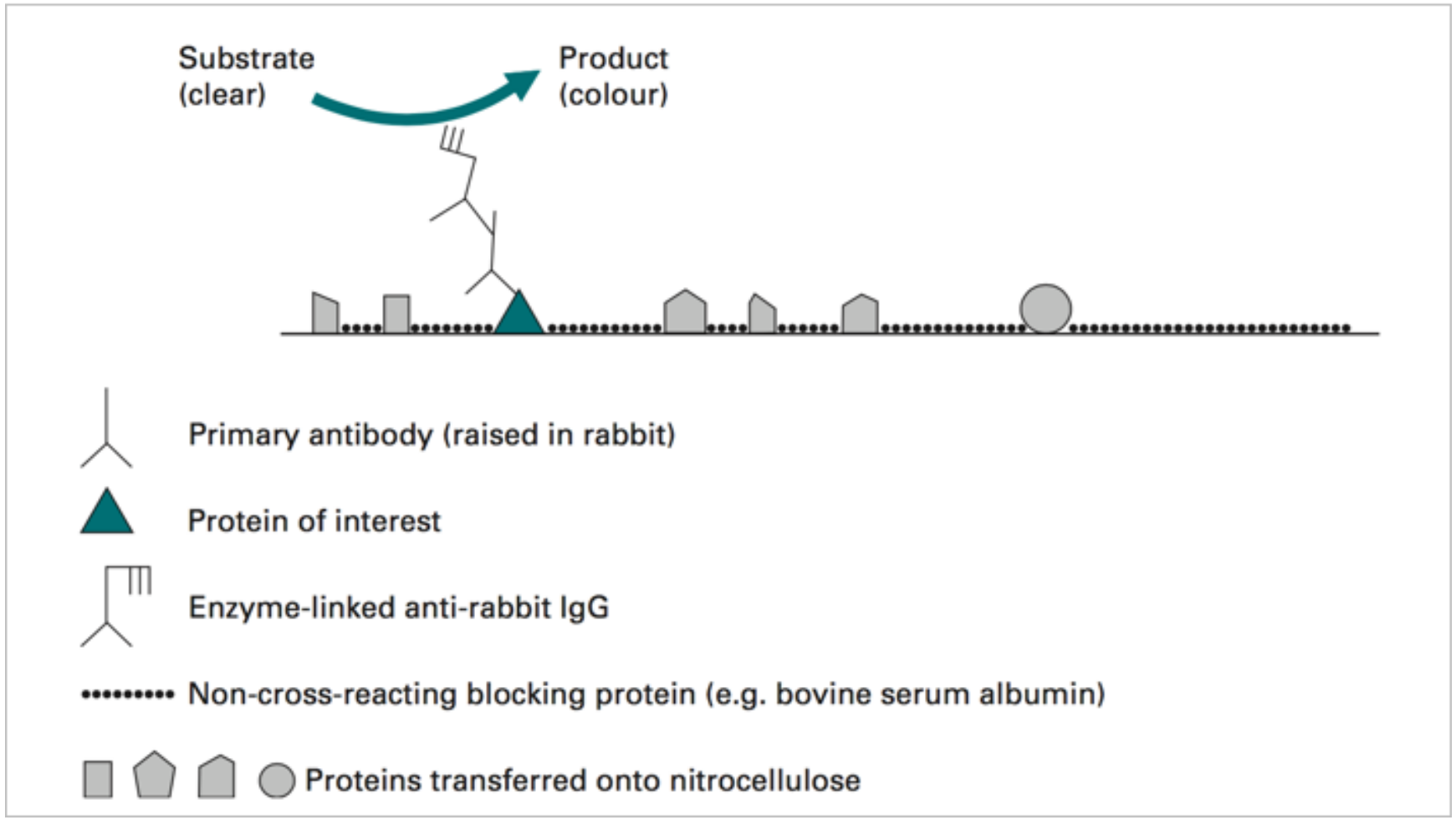




- Thus the **molecular weight** and **amount** of the desired protein can be characterized from a complex mixture of proteins by western blotting.

Pause and Think What is the Mwt of tubulin? Which cell-line has the highest tubulin concentration? What types of test is Western blot? quantitative OR qualitative?

Detection of specific protein using Western blot



Supporting materials:

Performing western blot:

http://www.youtube.com/watch?v=VgAuZ6dBOfs