CLS 468 Immunology Clinical Practice

جامعة الملك سعود
King Saud University

Western Blotting Technique

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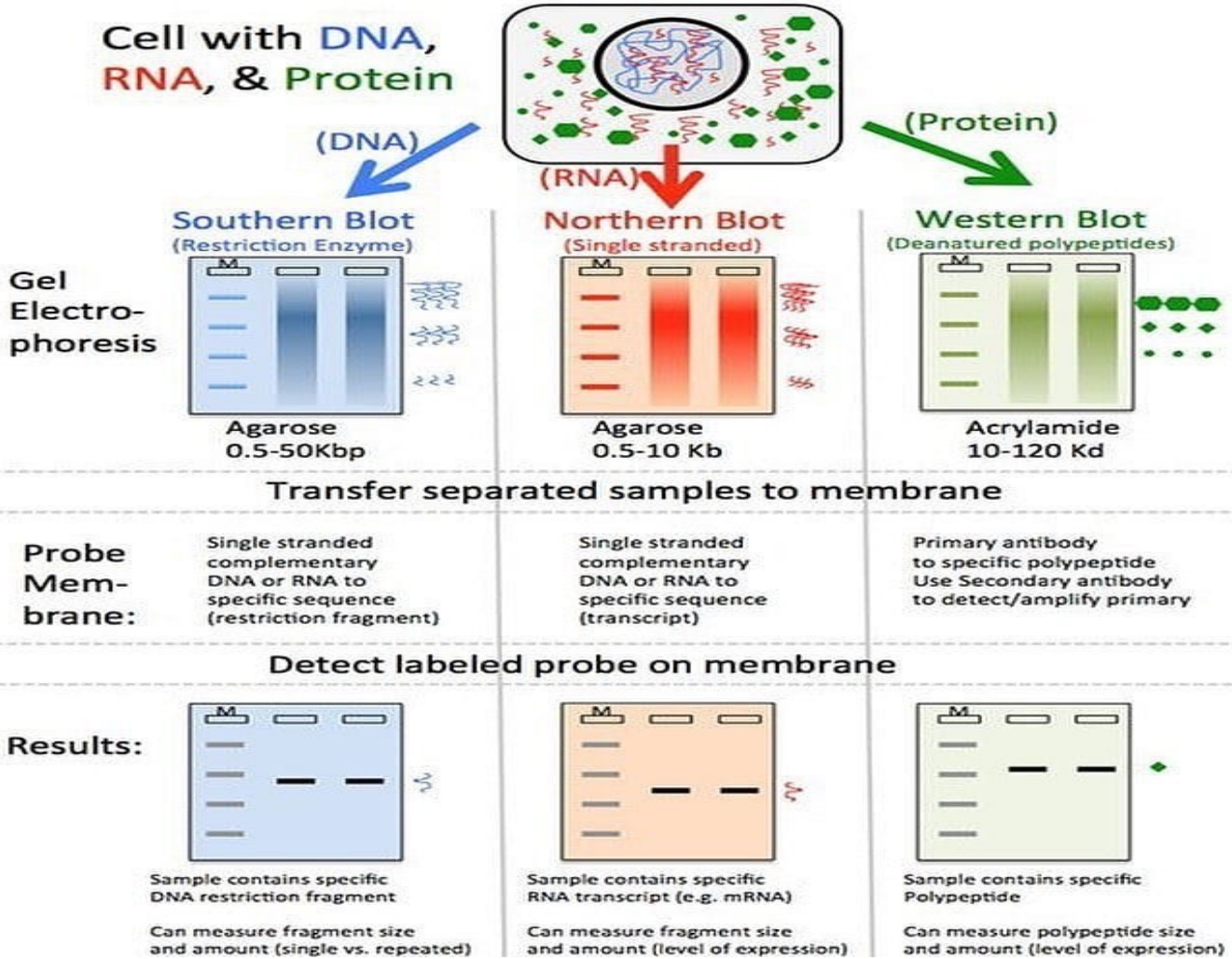
Outlines

I. Background
II. Sample preparation
III. Gel Electrophoresis
IV. Transfer
V. Antibody probing
VI. Detection techniques
VII. Imaging
VIII. Application of Western Blotting in Clinical Immunology
IX. Line blot immunoassay
X. HIV I/II Confirmatory Testing


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)

I. Background

- Western blot is a technique used to identify specific proteins in a sample containing a complex mixture of proteins.

- **The three main steps of Western blot are:**
  1. Separation of protein by size.
  2. Transfer of protein to a solid support.
  3. Labeling target protein using a proper primary and secondary antibody to visualize.

II. Sample preparation

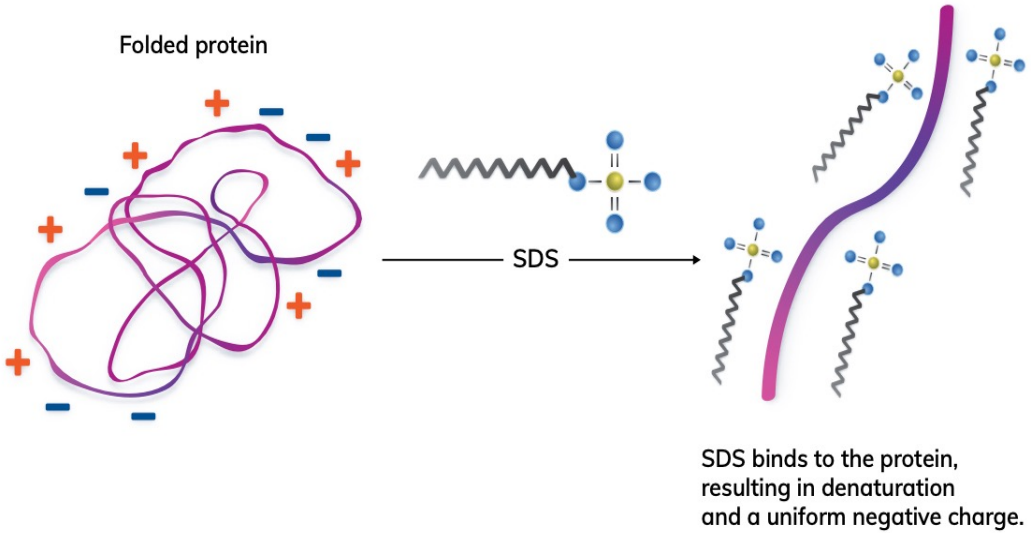
- **Extraction of protein from a biological sample:**
  - The selection of extraction methodology depends on:
    - The **source** and **protein** of interest.
    - The **yield** and **purity** of protein.

Sample	Typical lysis options
Tissue culture	Detergent lysis
Cell suspensions	Ultrasonication
Most plant and animal tissues	Mechanical homogenization (e.g. Waring** blender or Polytron**)
Soft animal tissues and cells	Dounce (manual) and/or Potter-Elvehjem (mechanical) homogenization
Bacterial and mammalian cells	Freeze/thaw lysis
Bacteria, erythrocytes, cultured cells	Osmotic shock lysis
Solid tissues and plant cells	Manual grinding with mortar and pestle
Cell suspensions, yeast cells	Grinding with abrasive component (e.g. sand, glass beads, alumina)
Bacteria, yeast, plant tissues, fungal cells	Enzymatic digestion
Bacteria, yeast, plant cells	Explosive decompression (nitrogen cavitation)
Microorganisms with cell walls	French press
Plant tissues, fungal cells	Glass bead milling

II. Sample preparation

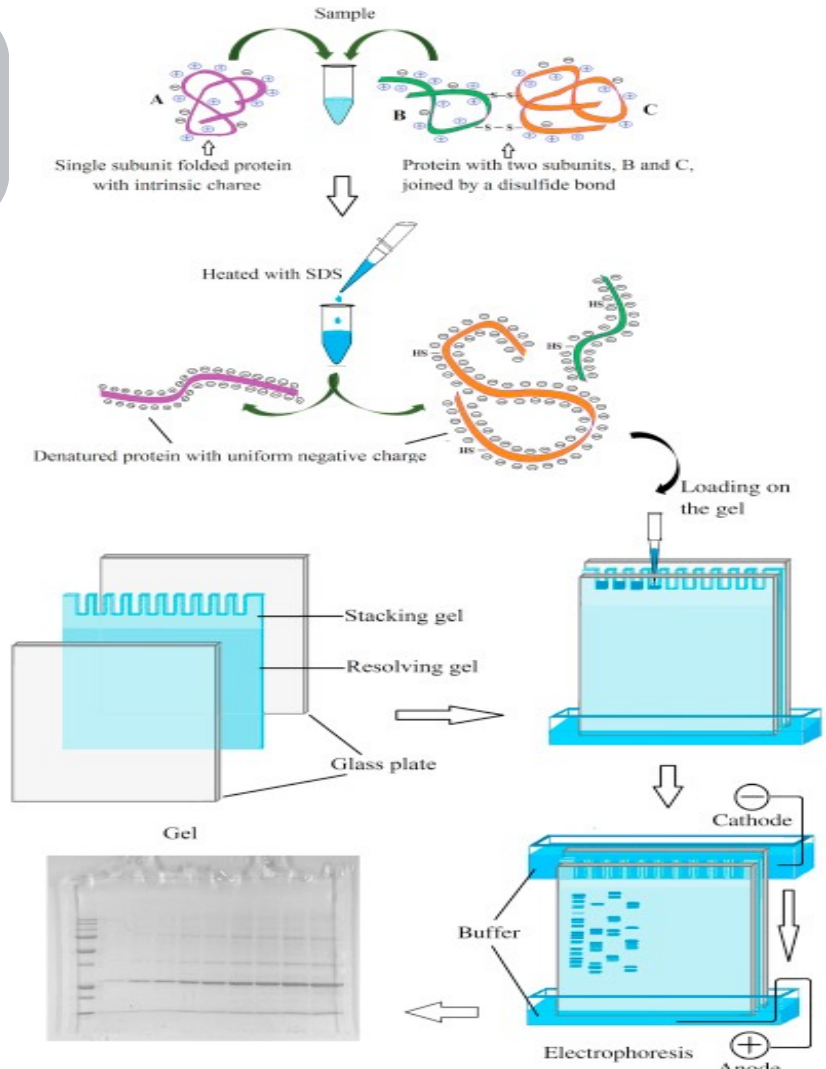
- Proteins are **denatured** prior to loading in polyacrylamide gel electrophoresis **using sodium dodecyl sulfate (SDS).**

- SDS linearizes the protein and gives the protein a **negative charge.** Thus, proteins are separated in electrophoresis <u>primarily by their molecular weight.</u>

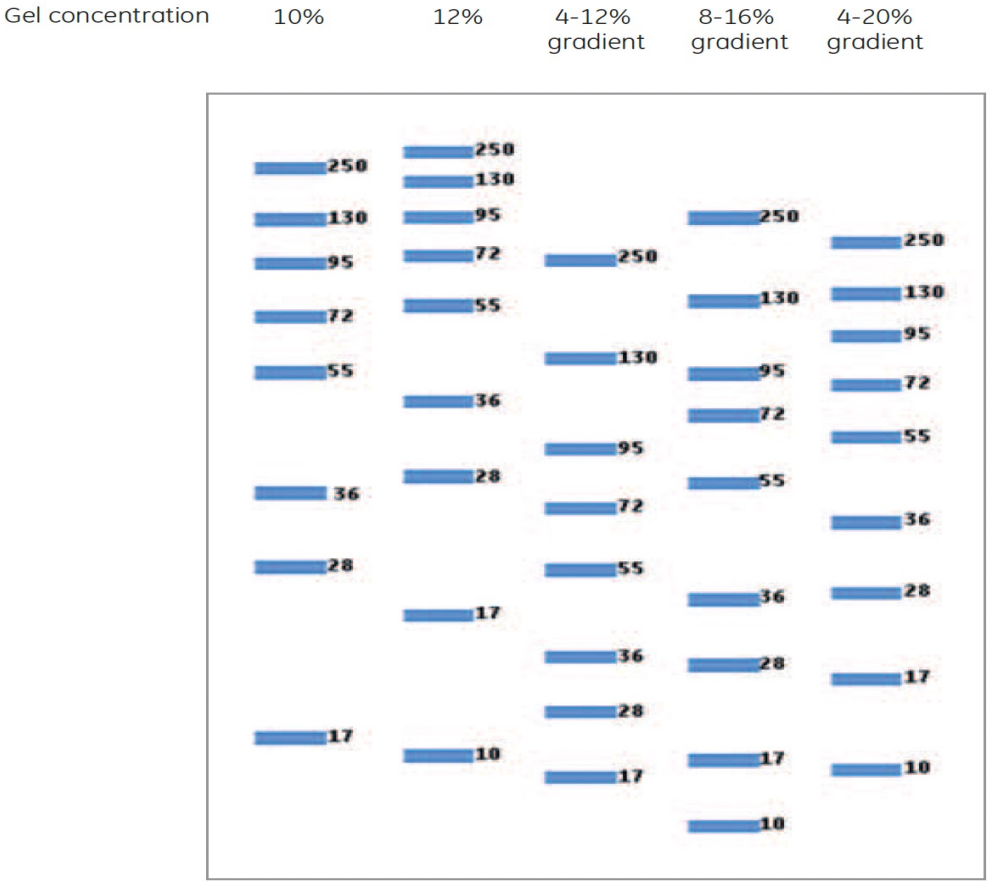


III. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

- The first step of a Western blot is based on <u>separating the proteins</u> across a gel matrix in a process called **gel electrophoresis** based on their mass.
- The used gel type is polyacrylamide gel electrophoresis (**PAGE**).
- A protein sample is mixed with a **loading buffer**, loaded onto the gel, and then subjected to an electrical current.
- The negatively charged proteins will travel through the gel toward the positive electrode.



Gel Concentration



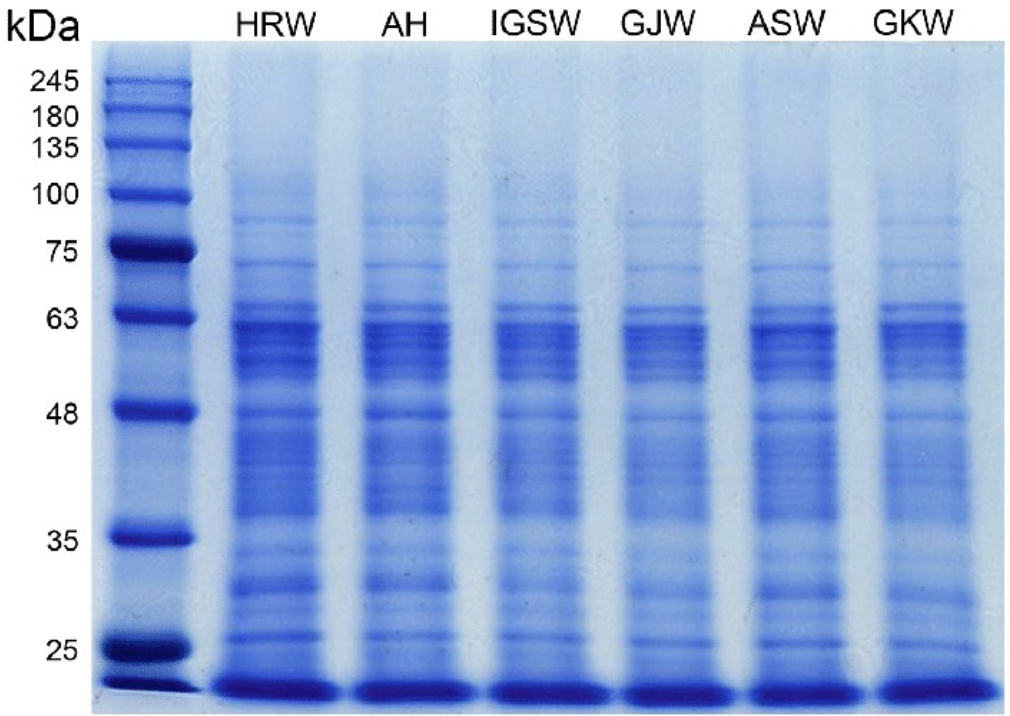
Resolving power of different concentration of acrylamide.

MW Range, kDa	Gel Percentage
10–43	15
12–60	12
20–80	10
30–95	8
50–200	6

Coomassie Brilliant Blue

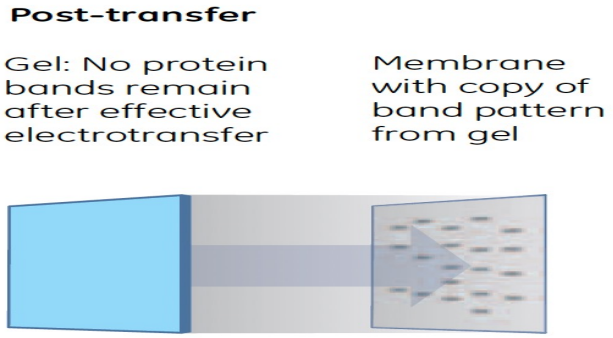
- **Coomassie Brilliant Blue** is used to stain total protein in the polyacrylamide gels.

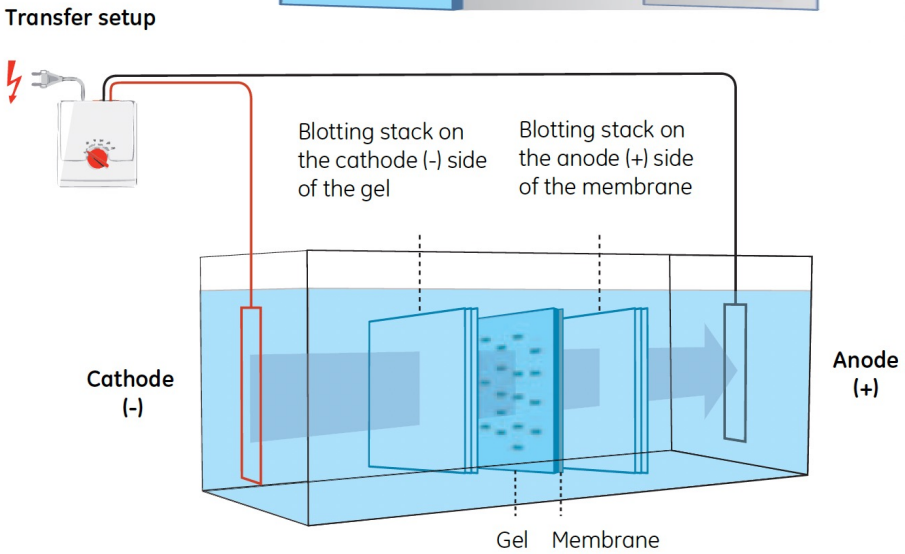
- The stained gel is then **destained** using a mixture of 25% methanol and 10% acetic acid diluted in 65% water.



IV. Protein transfer

- After protein separation a **transfer (blotting)** of the proteins from the gel to a solid support membrane.
- The aim of the blotting is to make it possible to detect the proteins on the membrane using specific antibodies.
- The most commonly used method of transfer is by using an electrical field (**Electrotransfer**), which relies on the electrical field used to migrate protein from gel to the membrane.



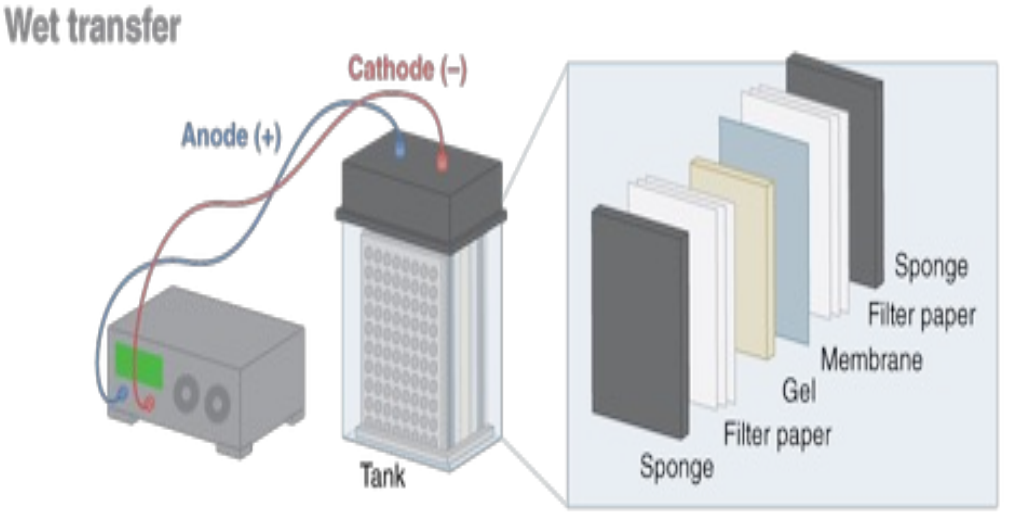


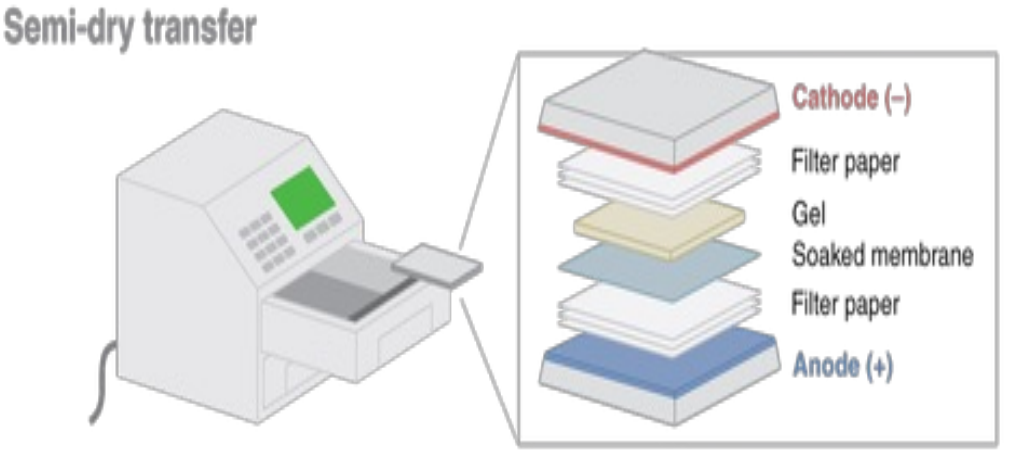
IV. Protein transfer

- The gel, membrane, and electrodes are assembled in a **sandwich.**
- Proteins then migrate toward the positively charged **anode (+)** in an electric field.

Types of Electrotransfer Transfer:

1. Wet transfer
2. Semi-dry transfer

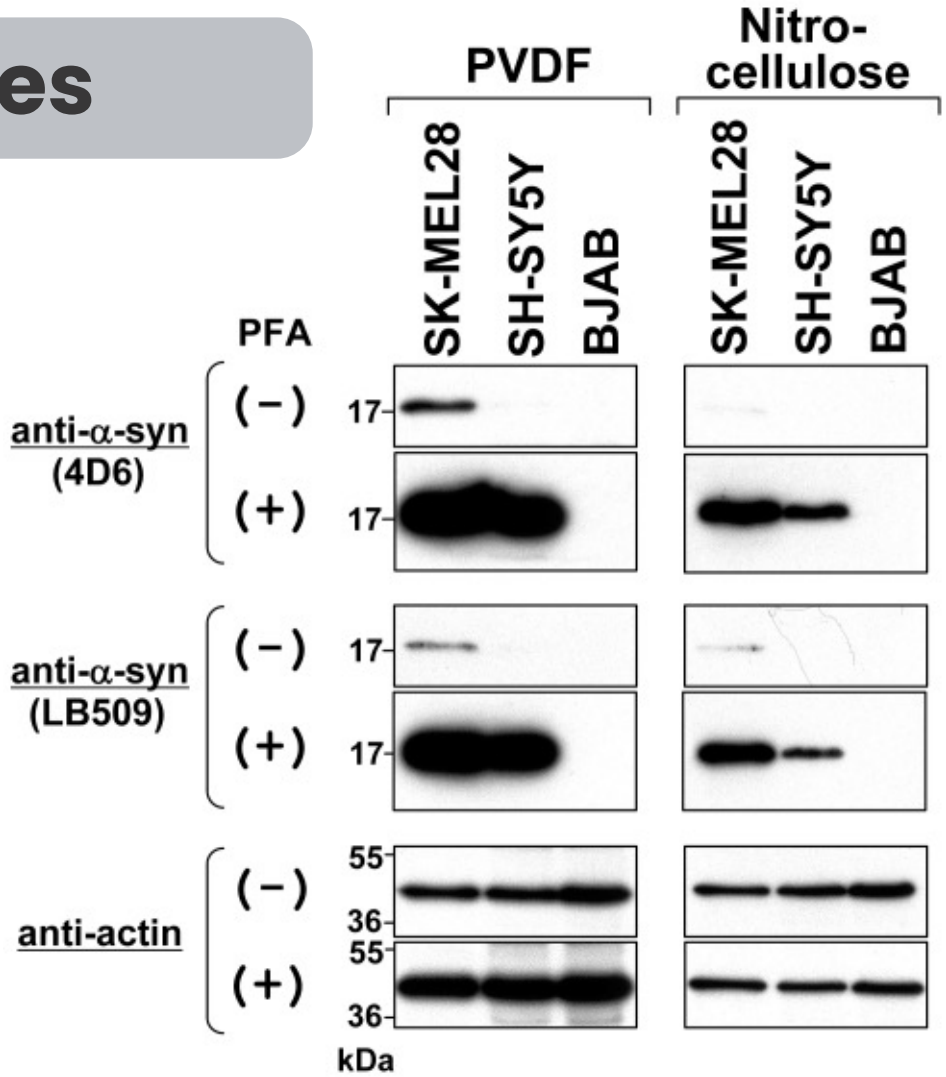




IV. Transfer Membranes

- Membranes are porous materials with pore sizes from 0.05 to 10 µm in diameter.

- Types of membranes used for Western blotting are:
  - Nitrocellulose membranes
    - Most frequently used.
    - Low background.
  - Polyvinylidene difluoride (PVDF) membranes
    - Higher protein binding capacity.
    - Have a higher background.
    - Need to be prewetted in either methanol or ethanol before use.

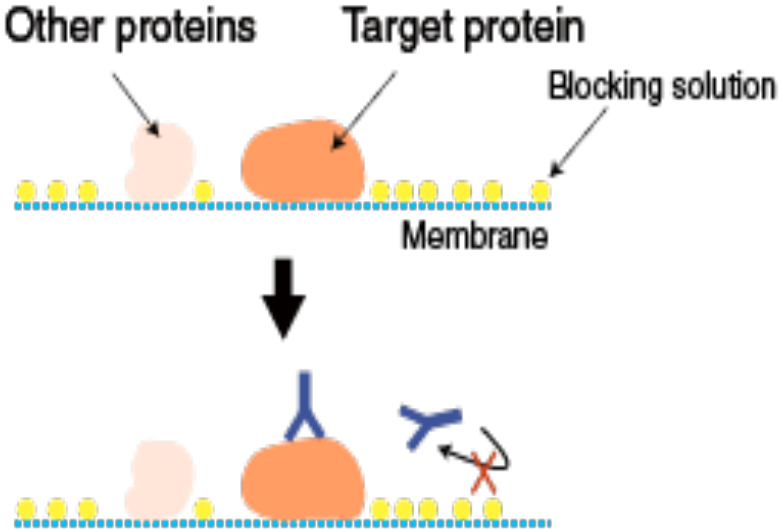


Blocking

- To prevent non-specific binding of antibodies to the membrane **" blocking"** spaces not already occupied by proteins.

- Two main classes of blocking agents:
  1. Proteins
     - Permanent blocking agents.
     - Examples: bovine serum albumin (BSA), non-fat milk, and casein.

  2. Non-ionic detergents
     - Non-permanent blocking agents can be removed in a simple washing step.
     - Examples: Tween-20, Triton X-100, sodium dodecyl sulfate (SDS).



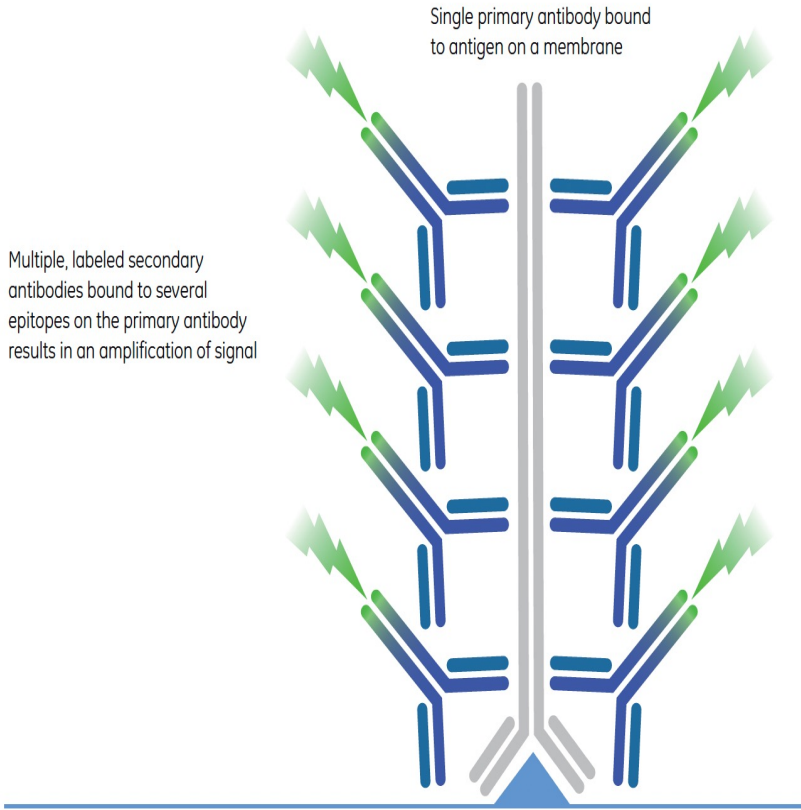
Note: soak the blotted membrane in freshly prepared blocking agent for 30 min to 2 h at room temperature with constant agitation.

V. Antibody probing

- Transferred protein of interest is detected and localized using a specific **antibody**.

- The common practice in Western blotting is to utilize a **non-labeled primary antibody** directed against the target protein and a **species-specific, labeled secondary antibody** directed against the constant region of the primary antibody.

- The secondary antibody help in amplifying the signals, as many secondary antibodies can theoretically bind simultaneously to the primary antibody.



V. Types of Labeling Antibodies

1. Enzyme-conjugated antibodies:
    a) Alkaline phosphatase (AP)
    b) Horseradish peroxidase (HRP)
2. Fluorophores-conjugated antibodies.
3. Gold-conjugated antibodies.
4. Radioisotopes antibodies.

VI. Detection

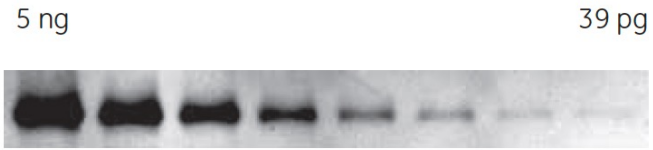
- Detection systems options in western blot:

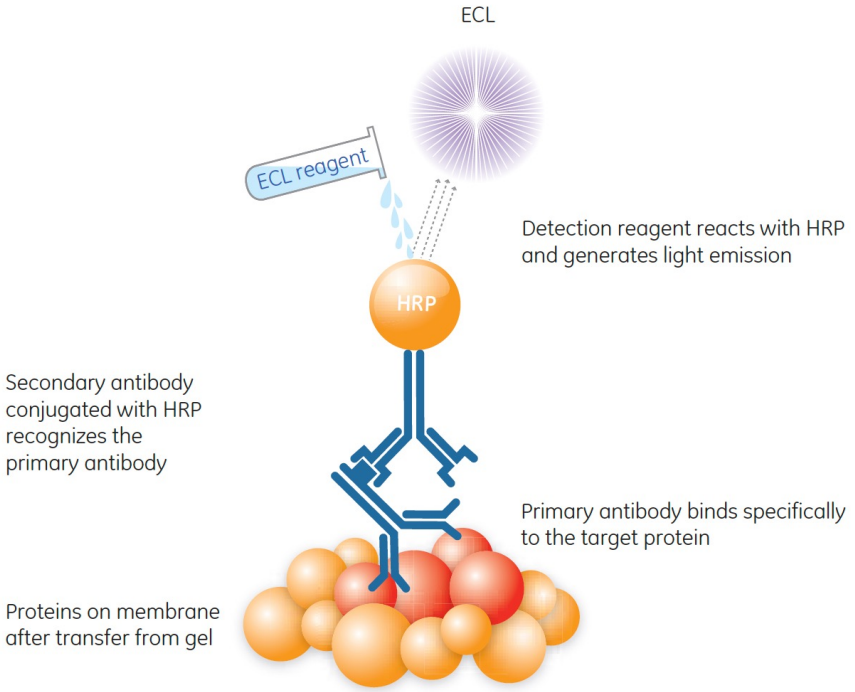
  a. Chemiluminescence
  b. Fluorescence
  c. Chemifluorescence
  d. Chromogenic
  e. Radioisotopic

a. Chemiluminescence

- The **HRP-conjugated secondary antibody** binds to the primary antibody.
- A **luminol peroxide** detection reagent (substrate) is then added which will be catalyzed by the HRP enzyme.

- The **intensity** of the signal is proportional to the amount of antibody, which is related in turn to the **amount** of protein on the blot.



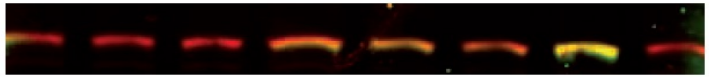


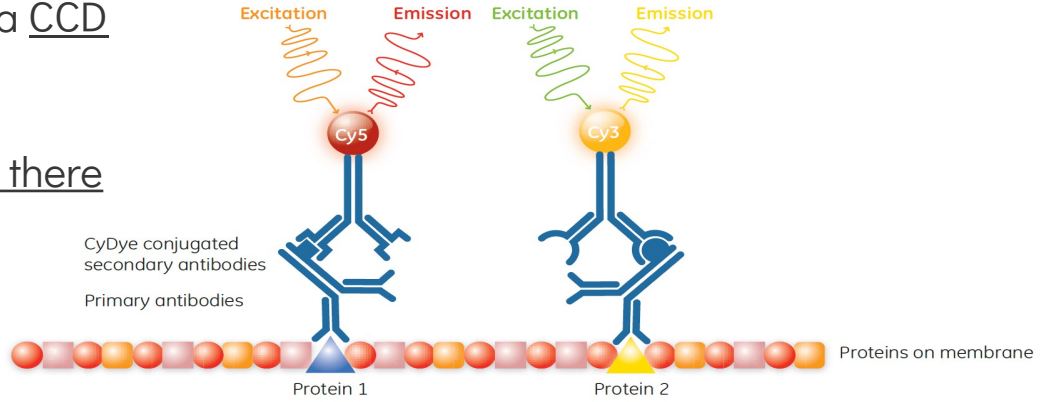
b. fluorescence

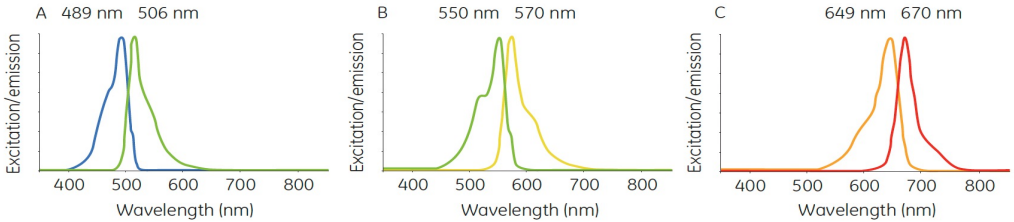
- Fluorophore molecules are excited by laser and a light of higher wavelength (emission) is released, emission signals are captured using a multichannel fluorescent scanner such as a CCD camera-based imager.

- Fluorescence detection is a direct method; there is no need to add substrate reagents.

Cy3/Cy5



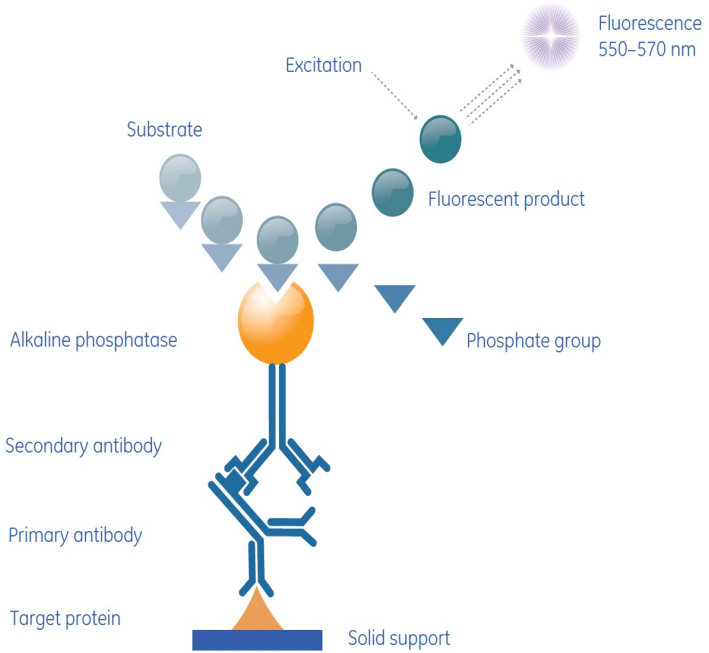




c. Chemifluorescence

- Enzymatic chemifluorescence detection is an indirect method (need to be added substrate).

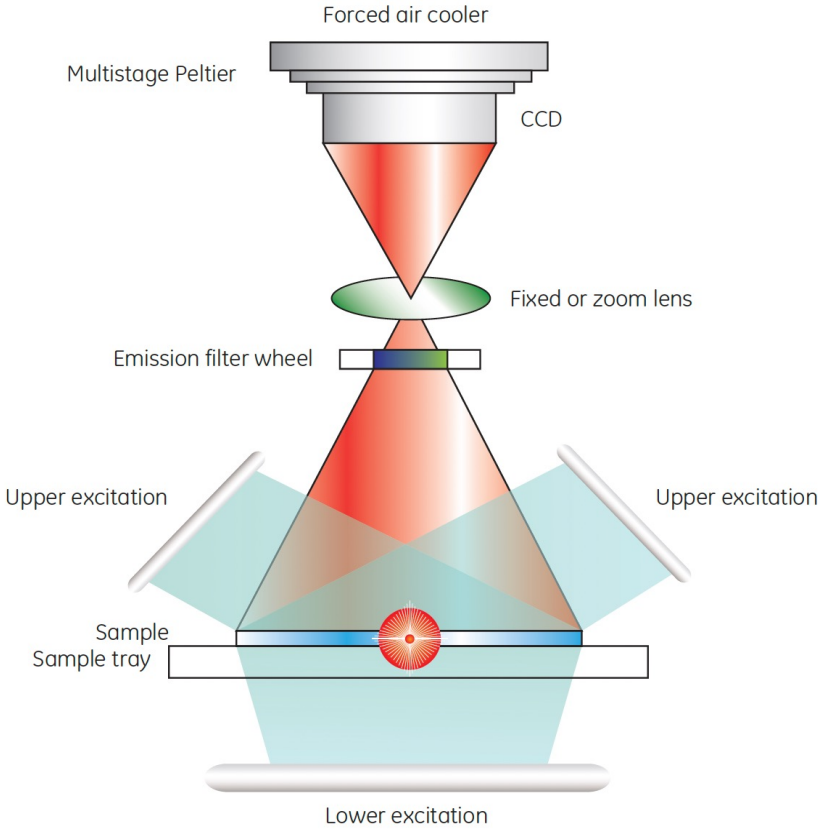
- **Alkaline Phosphatase (AP)-conjugated antibodies** react with a fluorogenic substrate to generate a stable fluorophore.



VII. Imaging

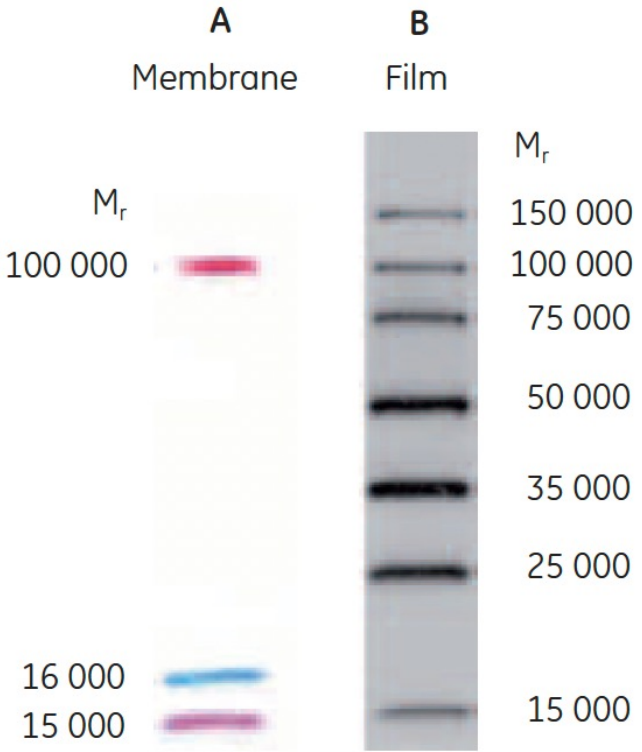
The light signal can be detected by:

- X-ray film
- **Digital imaging** with a charge-coupled device (CCD) camera-based imager

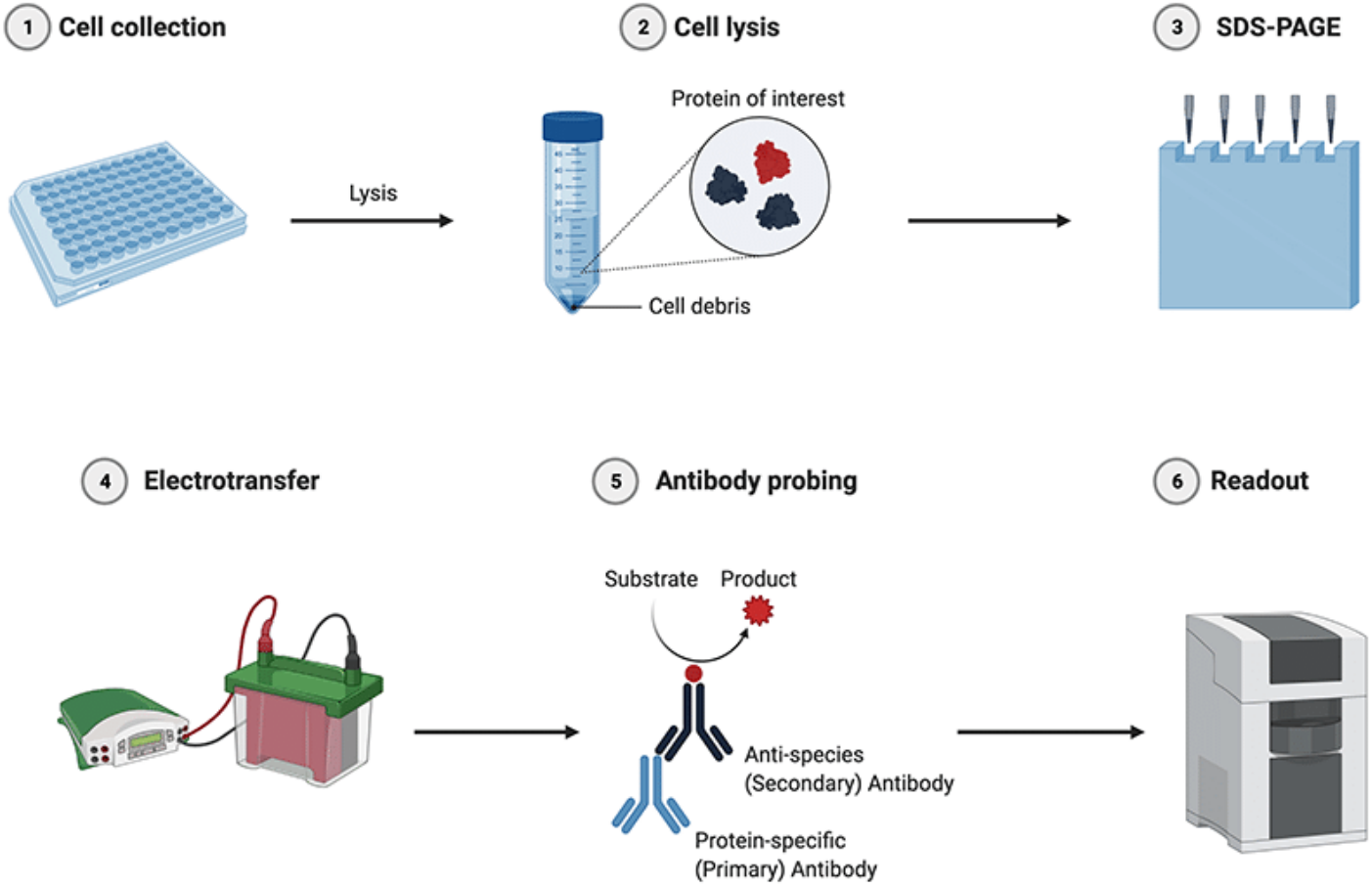


Molecular weight markers

- **Molecular weight markers (Ladder)** are used to define the size of proteins run in a gel.
- **Markers are composed of** different proteins of known size and the distances migrated over the time of the run.
- **Markers** facilitate the estimation of the size of unknown proteins.



Summary of Western Blotting Procedure



1DE Blot

VIII. Application of Western Blotting in clinical immunology

- **Western** blotting is used in clinical immunology labs as confirmatory testing for HIV, HTLV, syphilis, and ANA. However, recently, the use **of line immunoassay** has been implemented for more convenient processing.

Kits used for confirmation in clinical immunology (line immunoassay):

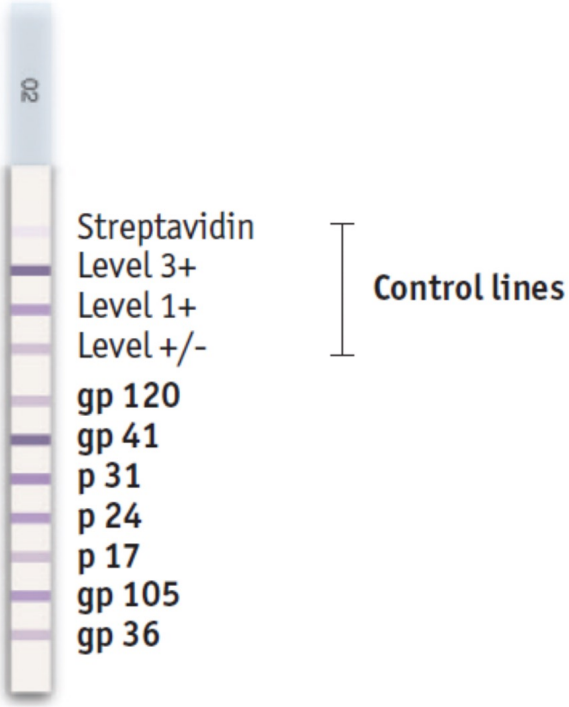
- INNO-LIA HIV I/II Score
- NNO-LIA HTLV I/II Score
- INNO-LIA Syphilis Score
- INNO-LIA ANA

IX. Line blot immunoassay

- Line blots work in a very similar way to Western Blots as far as the assay itself. However, the blot preparation is different.

- Line blots are immunoassays in which purified antigens are deposited as bands on **nitrocellulose membrane strips.**

- Antibody in the patient sample binds to the Ag on the strip, and HRP-conjugated secondary antibodies are used for the detection.



X. HIV I/II Confirmatory Testing

- **The sequence of tests for the diagnosis of HIV infection:**
- Starting with Anti-HIV Ab using ELIZA or CMIA >> A positive result is repeated >> Another sample is collected for confirmatory testing using line blot and qRT-PCR.

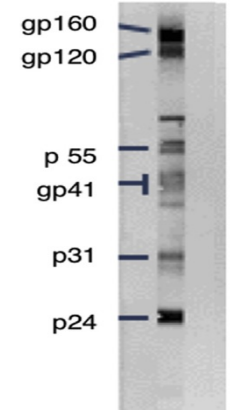
Two Species of HIV:

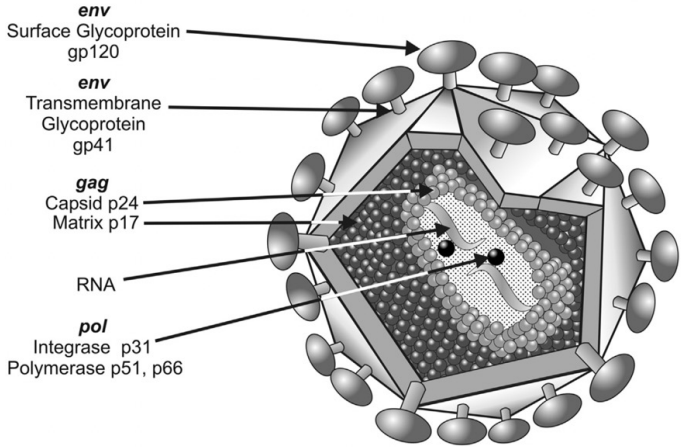
- **HIV type 1 (HIV-1):** most virulent, responsible for most AIDS cases worldwide.
- **HIV-2:** less pathogenic, differs in some of its antigenic components, and has a more limited geographic distribution.
- The nomenclature of HIV viral proteins indicates **"gp" for envelope glycoprotein** or **"p" for other proteins** followed by a number representing its molecular weight.

X. HIV I/II Confirmatory Testing by Line Blot Immunoassay

- The major components of diagnostic utility for HIV-1 include:
  1. Envelope proteins (gp41, gp120, and their precursor, gp160).
  2. The core gene proteins (p55, p24, p17).
  3. The polymerase (pol) gene proteins (p66, p51, p31).

- **HIV-2 proteins** are similar but differ somewhat in the molecular weight of the individual gene products (e.g., p26 corresponds to p24; gp36 and gp105 correspond to gp41 and gp120).





X. HIV I/II Confirmatory Testing by Line Blot Immunoassay

- INNO-LIA HIV I/II Score Kit

- Procedure:

Refer to Insert sheer

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https://www.youtube.com/watch?v=a-B7DMkiFzg