BP 605 T. Pharmaceutical Biotechnology (Theory)

Immunoblotting techniques- ELISA, Western blotting, Southern blotting

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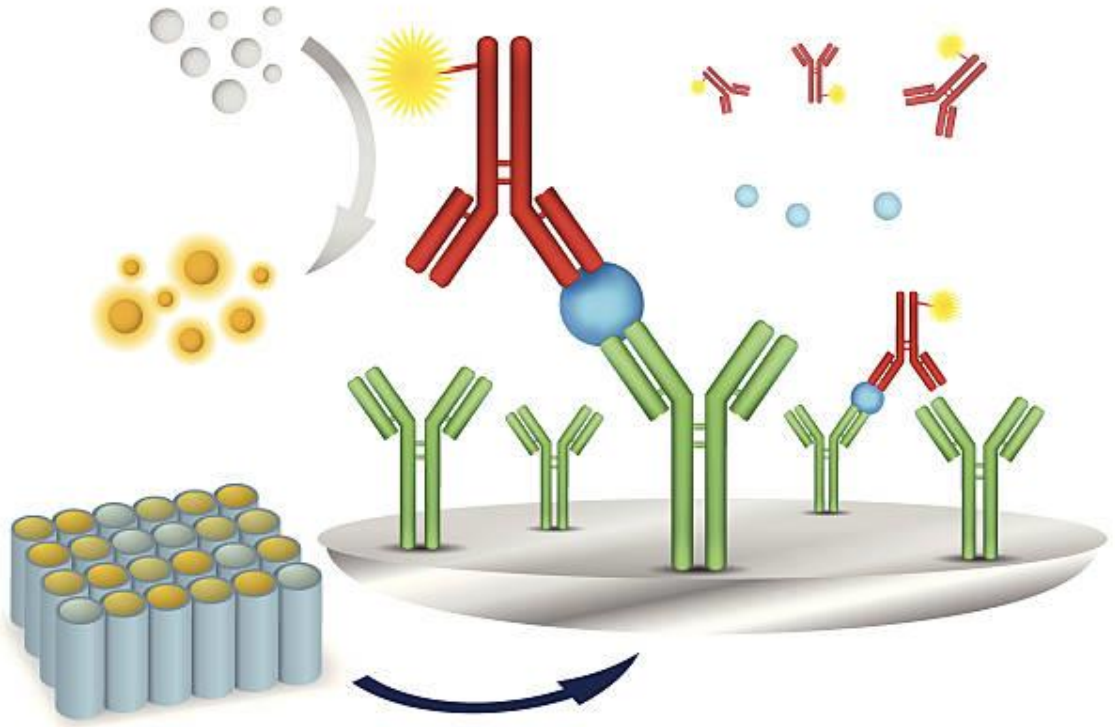
Overview

- Introduction to Immunoblotting techniques
- ELISA – Principle and assay
- Blotting techniques – Western blotting; Southern Blotting

IMMUNOASSAY

- ✓ **Definition:** A technique that utilizes the specificity of binding between an antigen and antibody in order to identify and quantify the specific antigen (or antibody) in a sample
- ✓ For the purpose of quantification, one of the components (Ag or Ab) needs to be labeled
- ✓ The label produces a signal which can be measured
- ✓ Initial assays used radioactive label – RIA
- ✓ Currently non-isotopic labels being used



APPLICATIONS OF IMMUNOASSAYS

✓ *Infections:*	* *Bacterial-*	*M.tuberculosis, H.pylori*
	* *Viral-*	HIV, Hepatitis B Virus
✓ *Analytes:*	* *Hormones-*	TSH, insulin, beta hCG
	* *Vitamins-*	Vitamin D3
	* *Transport proteins-*	Transferrin
✓ *Autoantibodies:*	* *SLE, Rheumatoid arthritis*	
✓ *Cancer biomarkers:*	* *PSA, CA 125, CEA, CA 19.9*	
✓ *Drug monitoring:*	* *Therapeutic-*	Digoxin, Phenytoin
	* *Illicit -*	Heroin, performance enhancers in sports

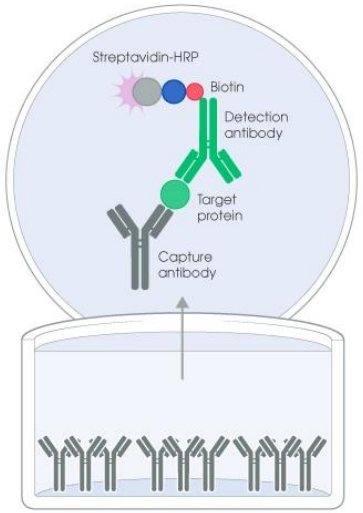
Enzyme Linked Immunosorbent Assay (ELISA)

- ✓ **Definition:** ELISA stands for "enzyme-linked immunosorbent assay." This is a rapid immunochemical test that involves an enzyme (a protein that catalyzes a biochemical reaction). It also involves an antibody or antigen (immunologic molecules). ELISA tests are utilized to detect substances that have antigenic properties, primarily proteins (as opposed to small molecules and ions such as glucose and potassium). Some of these include hormones, bacterial antigens and antibodies
- ✓ Based on specificity of antigen - antibody interaction
- ✓ Antigen of interest is adsorbed on to plastic surface ('**sorbent**')
- ✓ Antigen is recognized by specific antibody ('**immuno**')
- ✓ This antibody is recognized by second antibody ('**immuno**') which has enzyme attached ('**enzyme-linked**')
- ✓ Substrate reacts with enzyme to produce product, usually colored.

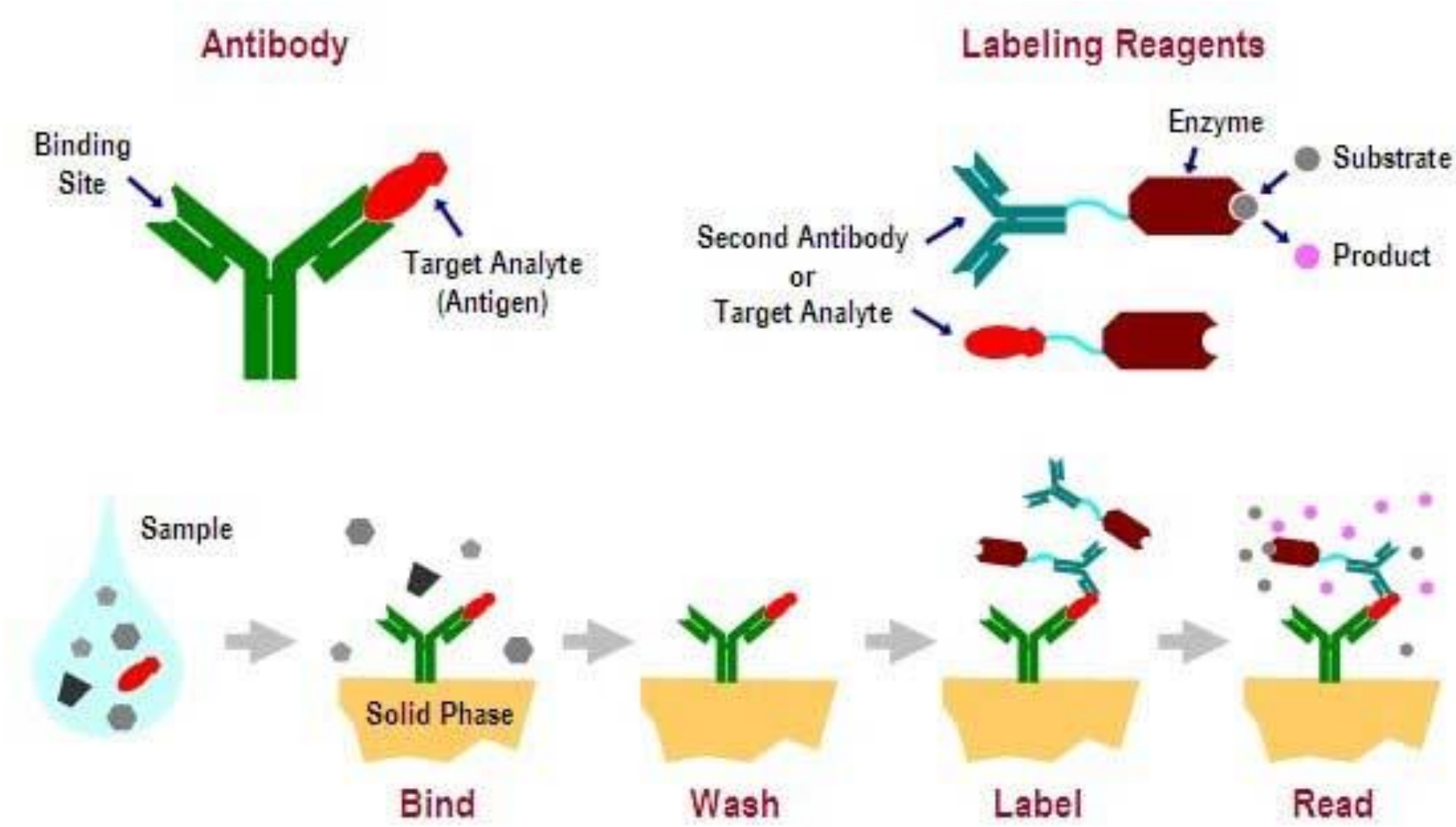
Eva Engvall

Peter Perlmann

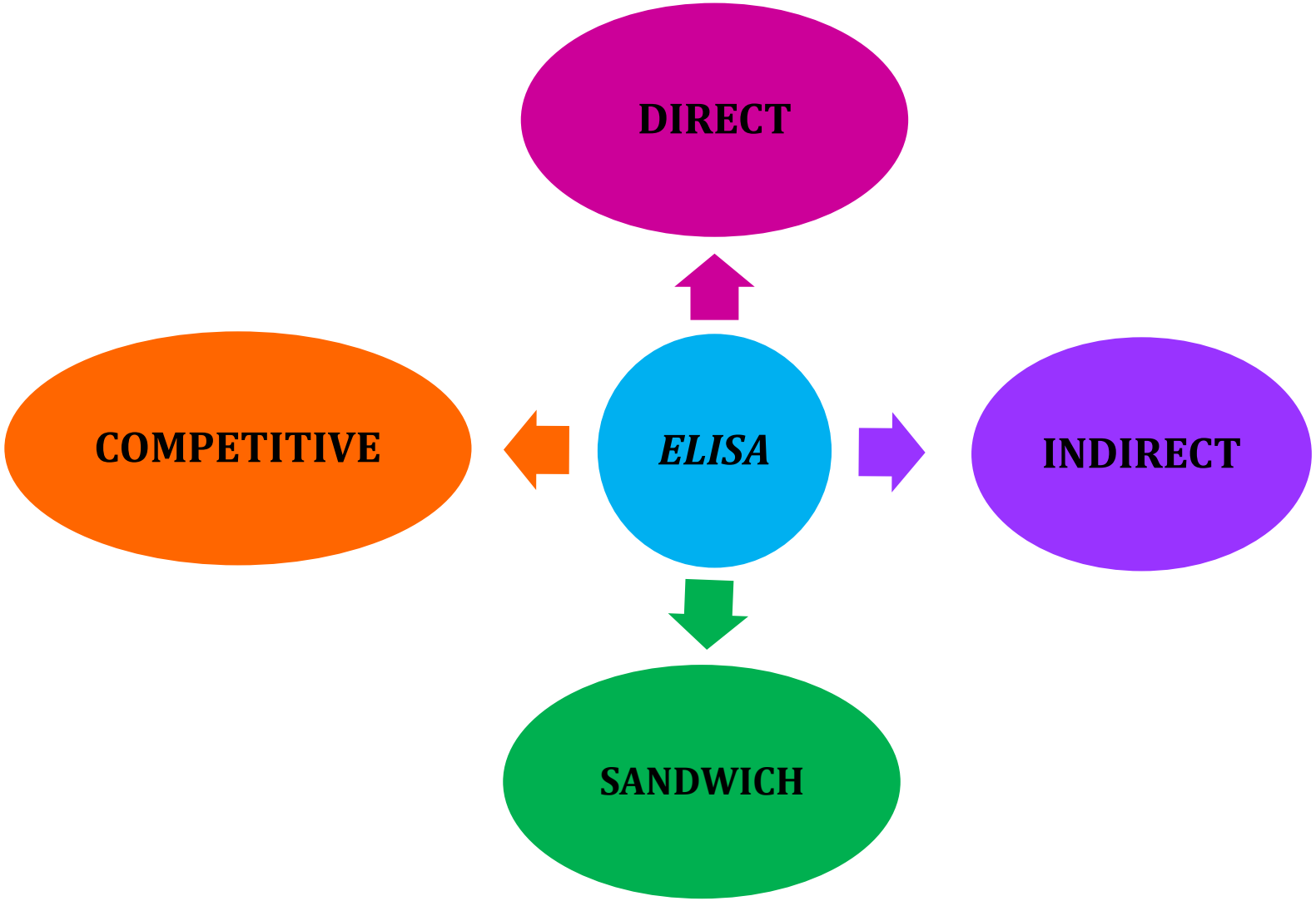
First to develop ELISA in 1971



ELISA

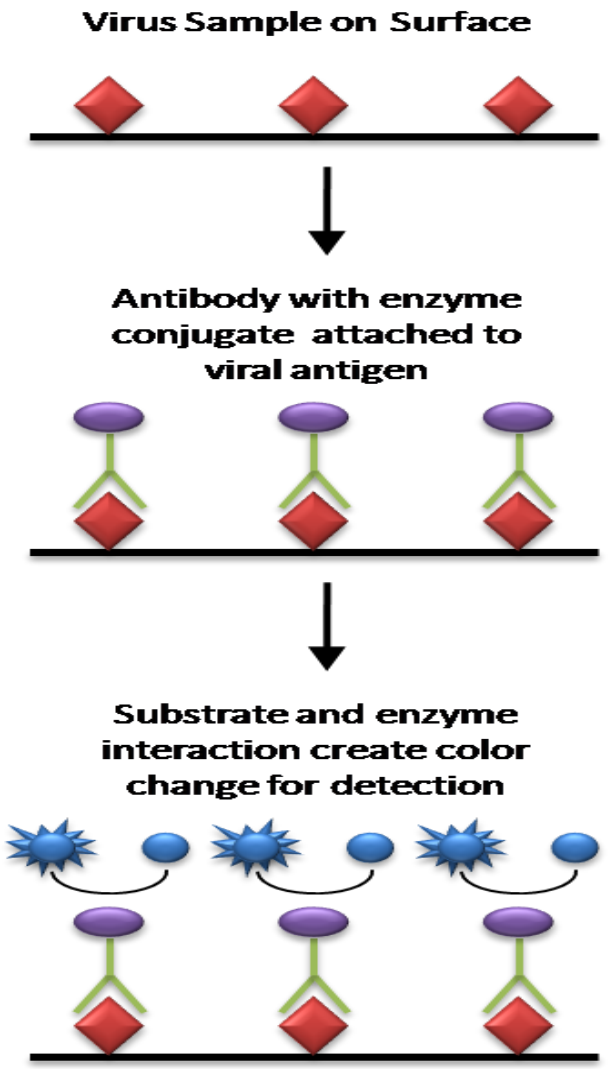

Antibody
Binding Site
Target Analyte (Antigen)
Labeling Reagents
Enzyme
Substrate
Product
Second Antibody or Target Analyte
Sample
Solid Phase
Bind
Wash
Label
Read

TYPES OF ELISA



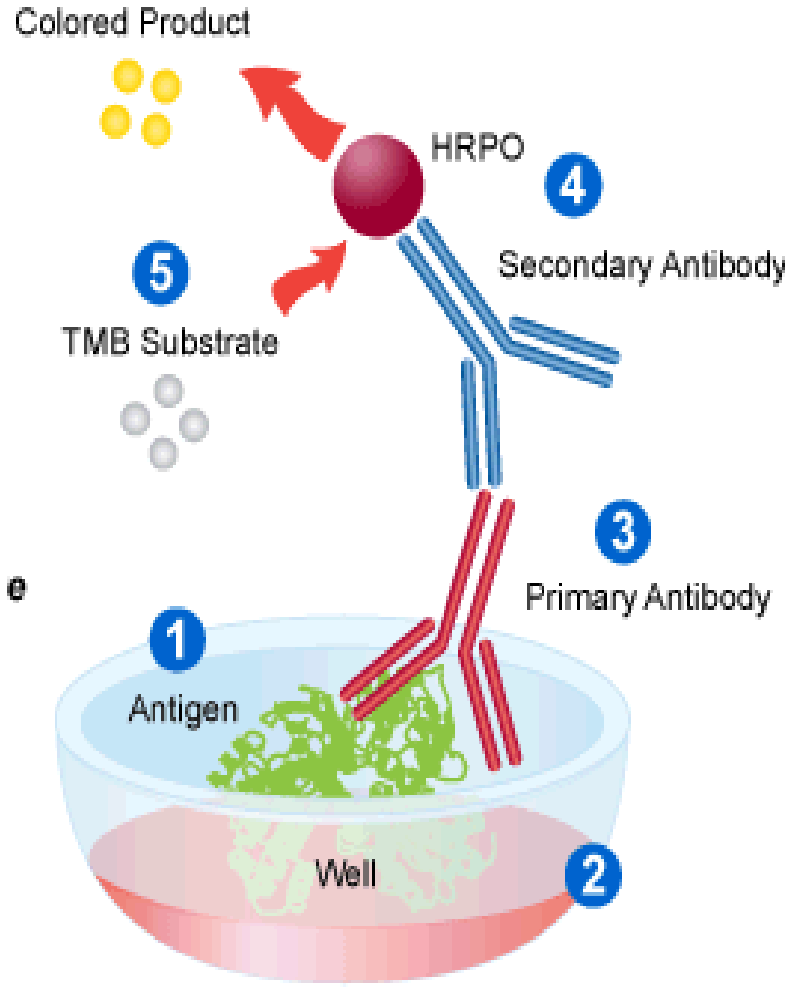
DIRECT ELISA

- ✓ Antigen to be detected is coated directly
- ✓ Non specific sites are blocked
- ✓ Antibody labeled with enzyme is added
- ✓ Wash to remove unbound antibody
- ✓ Substrate for the enzyme is added
- ✓ Color development is proportional to the amount of antigen present
- ✓ E.g. Detecting urinary beta hCG



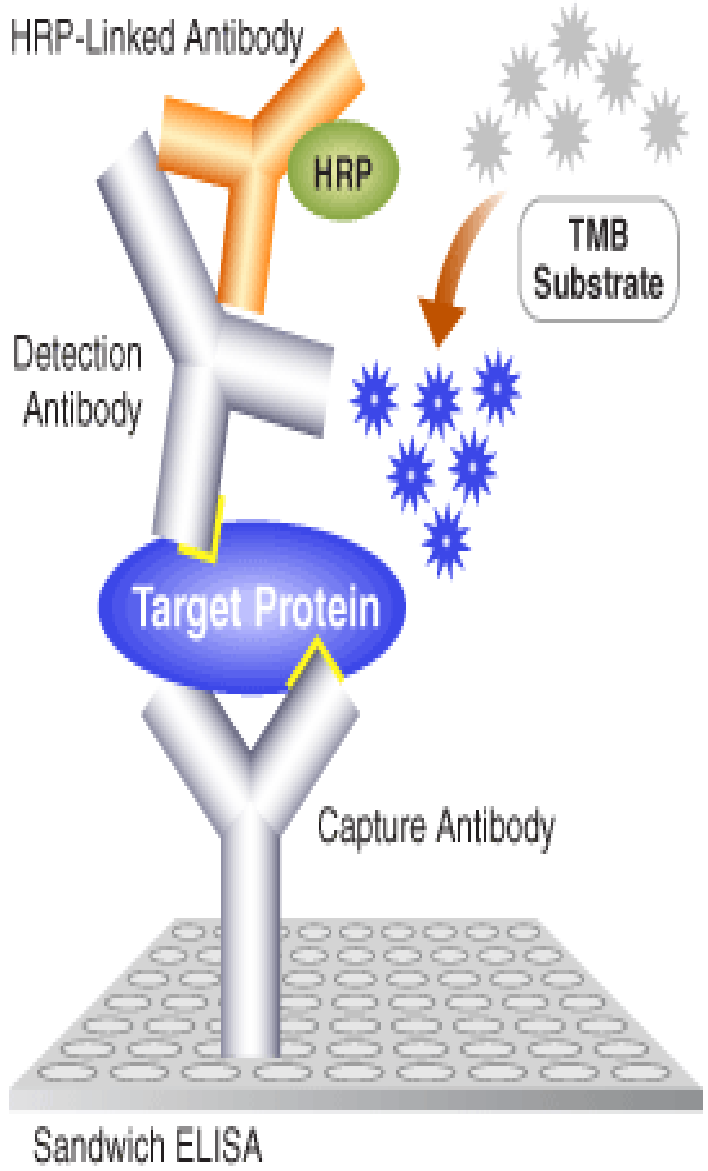
SANDWICH ELISA

- ✓ Used to detect **antibody**
- ✓ **Antigen is coated** in a well
- ✓ Sample containing **specific antibody to be measured**, is added
- ✓ Wash given to remove unbound antibody
- ✓ **Secondary antibody (labeled with enzyme)** against the primary antibody is added
- ✓ **Substrate for the enzyme** is added
- ✓ **Color development is proportional to the amount of antibody** present
- ✓ E.g. Detection of **anti HBV antibody** in serum

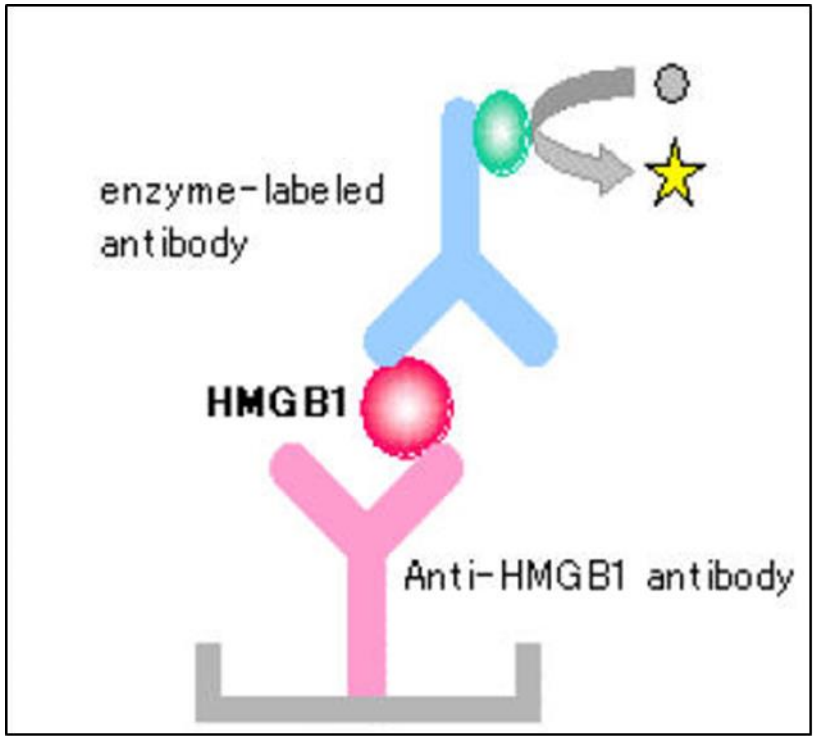


SANDWICH ELISA

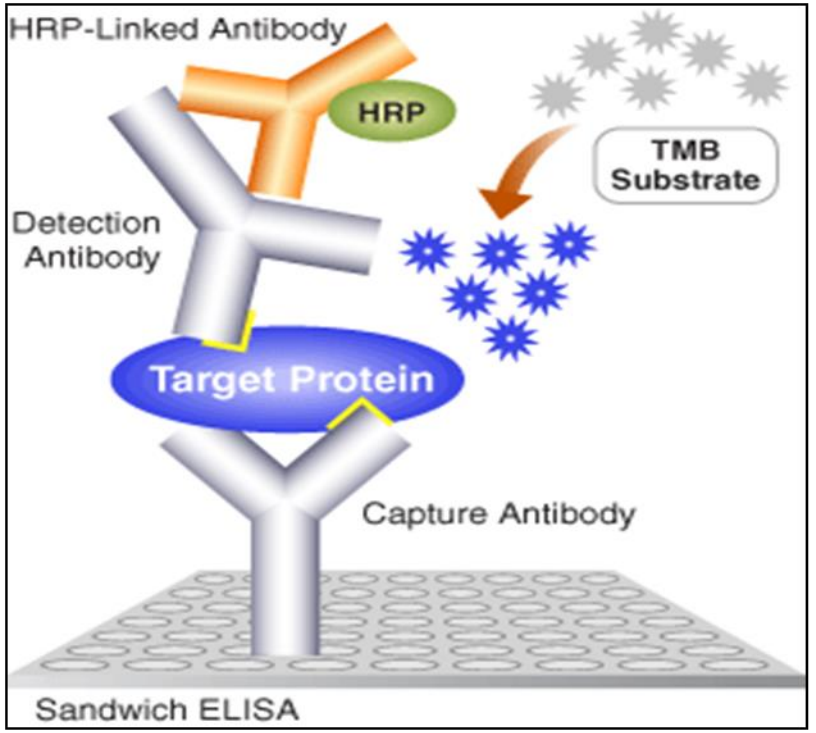
- Used to detect **antigen**
- Capture antibody is coated in the well
- Sample containing Ag to be detected is added
- Wash given to remove unbound antigen
- Detection antibody **(labeled with enzyme),** against a **different epitope** of antigen, is added
- Wash given to remove unbound antibody
- Substrate for the enzyme is added
- Color development is proportional to the amount of antigen present
- Eg. Estimation of serum **insulin**



VARIANTS OF SANDWICH ELISA



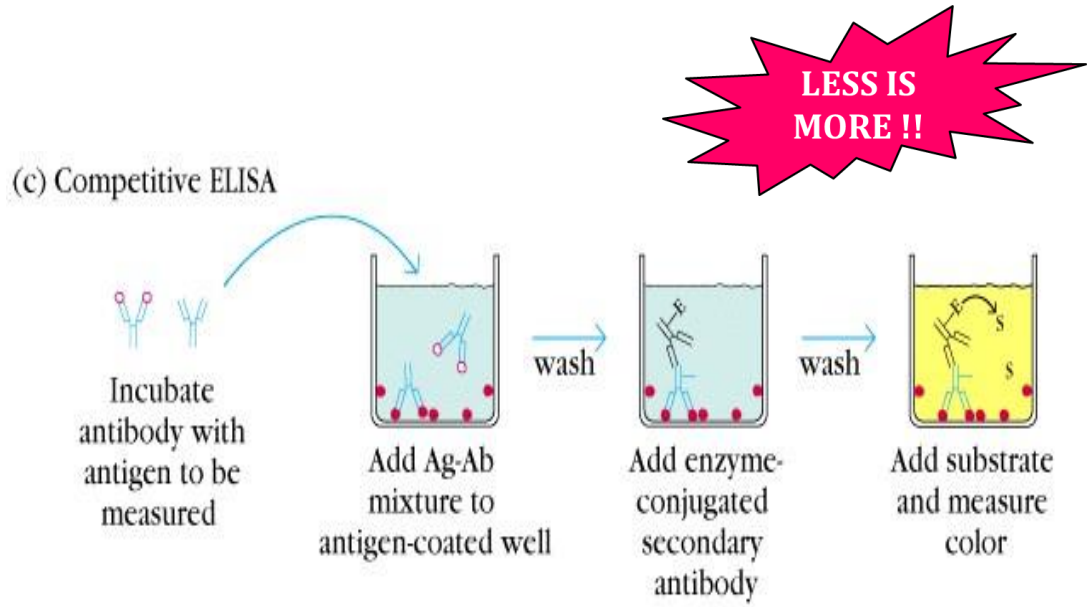
DOUBLE ANTIBODY SANDWICH (DAS) ELISA



TRIPLE ANTIBODY SANDWICH (TAS) ELISA

COMPETITIVE ELISA

- ✓ **Antibody** is first incubated in solution with a sample containing antigen
- ✓ This **Ag-Ab mixture** is then added to an Ag coated well.
- ✓ More the Ag present in the sample, lesser is amount of antibody available to bind to the Ag-coated well
- ✓ Wash given to remove unbound Ag-Ab complex
- ✓ Secondary Ab (labeled with enzyme) against primary Ab is added
- ✓ Substrate for the enzyme is added
- ✓ Color development is **inversely** proportional to the amount of antigen present
- ✓ Used to detect weak immunogens e.g. **hapten**



APPLICATIONS OF ELISA

❖ ***Detection of antigen:***

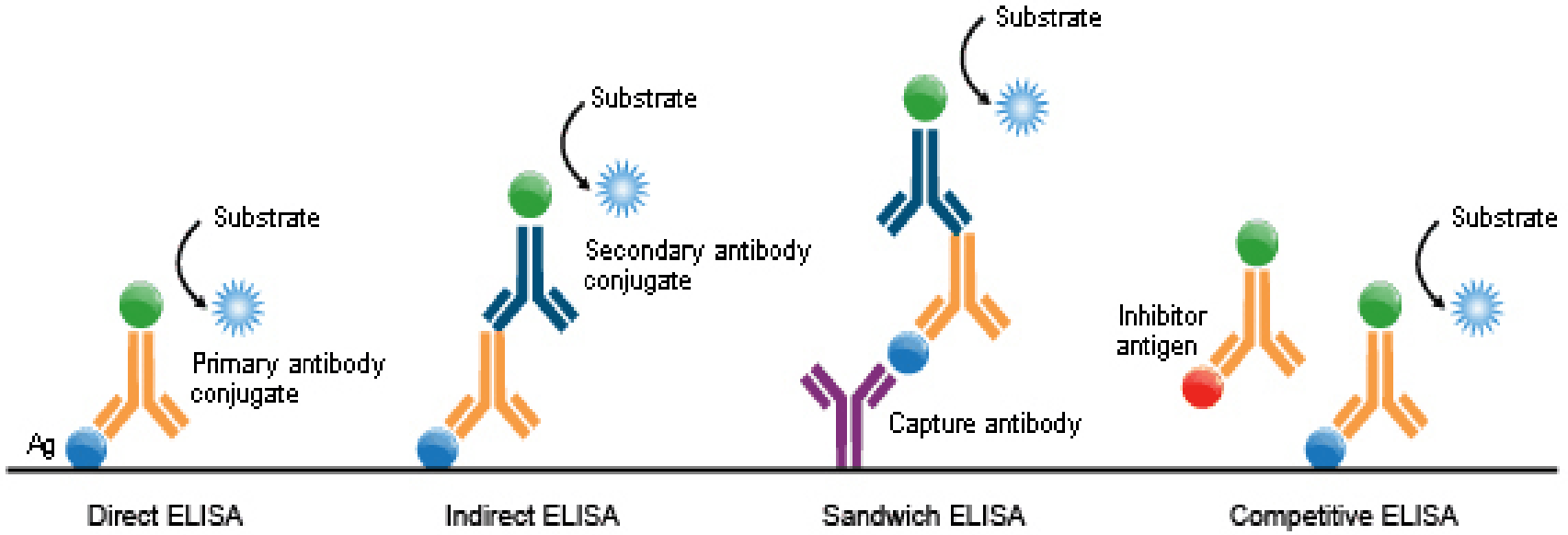
* Infections:	-p24 (HIV) -HBsAg (HBV)
* Hormones:	-β hCG -Insulin
* Oncofetal Ag:	-AFP
* Cytokines:	-IL-6 -TNFα

❖ ***Detection of antibody:***

* Infections:	-Rubella -Toxoplasma -HBV
* Autoimmunity:	-DNA (SLE) -Rheumatoid Factor (Rheumatoid arthritis)

SUMMARY OF ELISA

Type of ELISA	Well coated with	Detects in serum	Example
Direct	Antigen	Antigen	Beta hCG
Indirect	Antigen	Antibody	Anti HBV antibody
Sandwich	Antibody	Antigen	HIV p24 antigen
Competitive	Antigen	Antigen	Hapten detection



BLOTTING TECHNIQUES

- ✓ **Definition:** Visualization of specific DNA , RNA & protein among many thousands of contaminating molecules requires the convergence of number of techniques which are collectively termed BLOT transfer

1. Involves **electrophoretic separation** of a nucleic acid or protein mixture on an **gel**
2. Separation followed by **transfer** of nucleic acid or protein bands onto a support matrix **(nitrocellulose/ nylon membrane)** by electrophoresis
3. Transferred nucleic acid or protein bands **detected** by treating the membrane with **radiolabeled or enzyme-linked probes or monoclonal Abs** specific for the target of interest
4. Finally the target-probe complexes that form on the band containing the nucleic acid or protein is recognized or **visualized** by:

   - **autoradiography**
   - addition of a **chromogenic** substrate
   - addition of a **luxogenic** substrate **(chemiluminescence)**

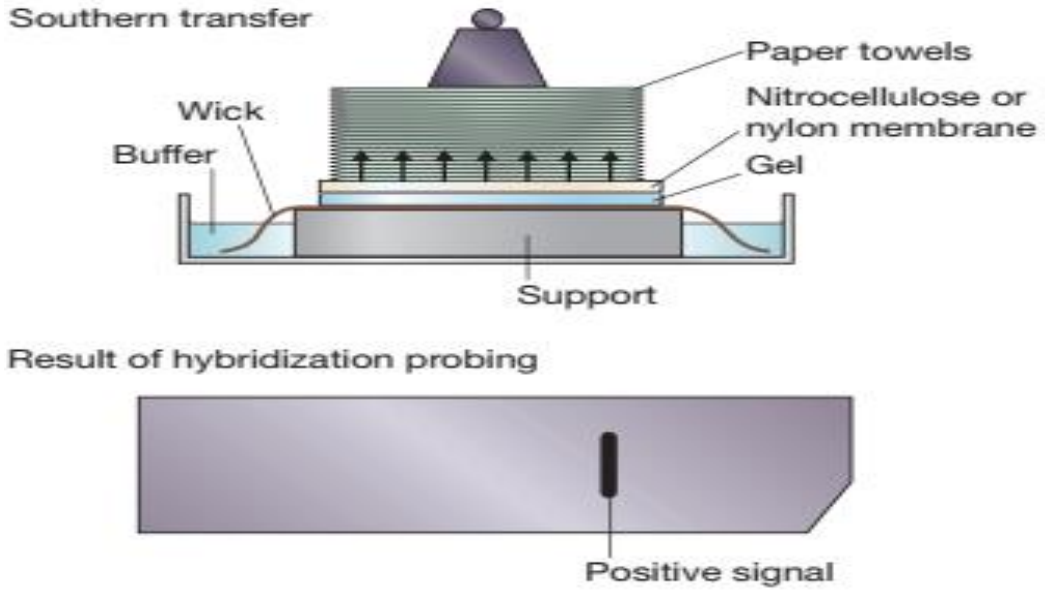
TYPES OF BLOTTING TECHNIQUES

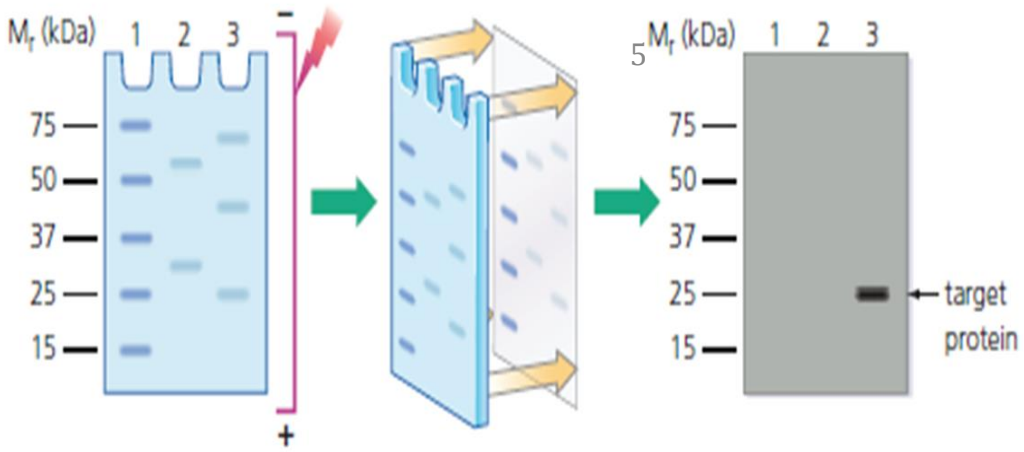
Blotting techniques

Southern Blot
Used for DNA detection

Northern Blot
Used for RNA detection

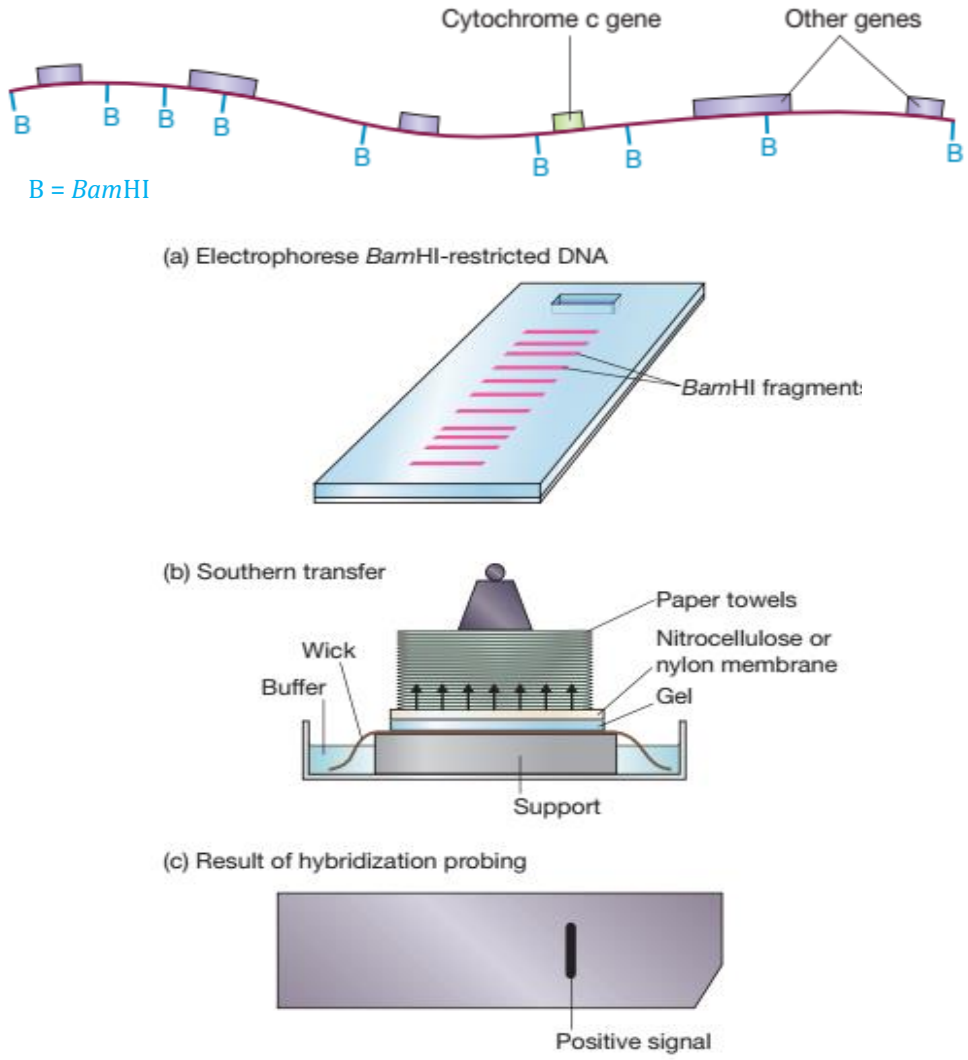
Western Blot
Used for Protein Detection





SOUTHERN BLOT

- ✓ **Transfer of DNA bands from an agarose gel to a membrane** makes use of the technique **perfected in 1975** by **Professor E.M. Southern** and referred to as **Southern transfer**.
- ✓ The membrane is placed on the gel, and buffer allowed to soak through, carrying the DNA from the gel to the membrane where the DNA is bound.
- ✓ The same method can also be used for the **transfer of RNA molecules** ("**Northern**" transfer) or **proteins** ("**Western**" transfer).
  - Southern transfer results in a membrane that carries a replica of the DNA bands from the agarose gel.
  - If the **labeled probe is now applied, hybridization occurs and autoradiography** (or the equivalent detection system for a non-radioactive probe) reveals **which restriction fragment contains the cloned or target gene.**



IMMUNOBLOTTING

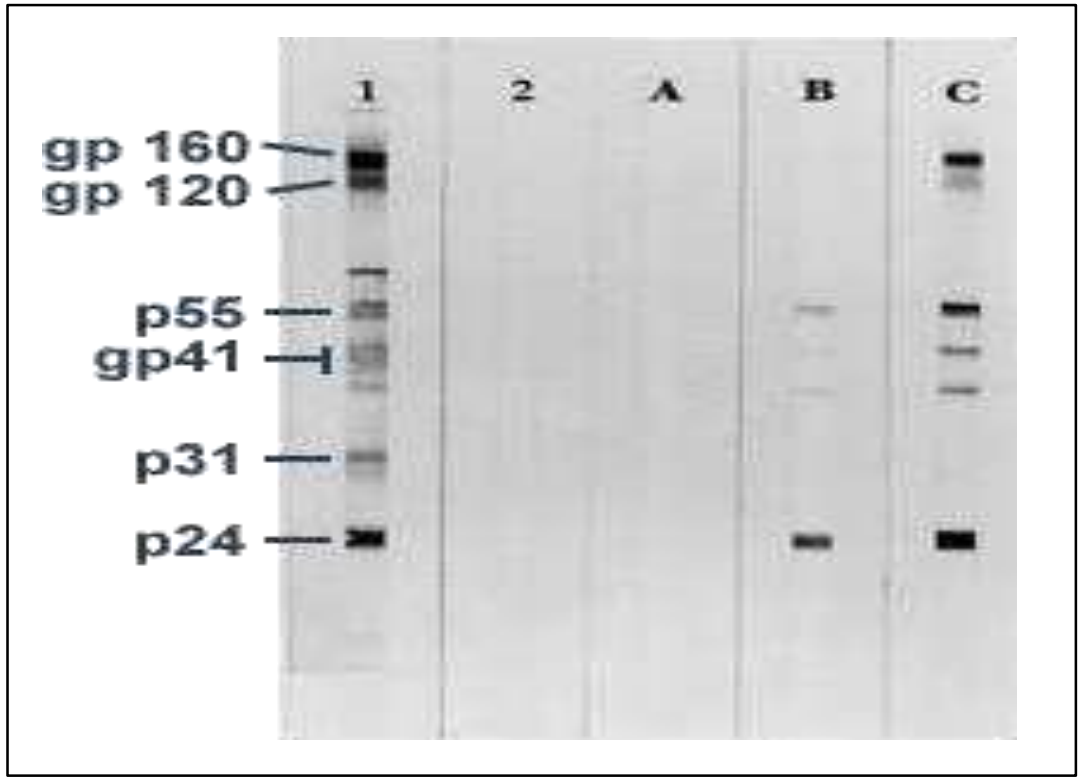
- ✓ Viral antigens are detected with a polyclonal or a MAb onto nitrocellulose paper.
- ✓ After incubation, the protein bands (immune complexes) are visualized with peroxidase-conjugated protein and a colour reagent.
- ✓ A colour develops in the bands where antibody binds to the antigen.
- ✓ Immunoblotting assay mixture of this two technique.

WESTERN BLOTTING

- ✓ Western blotting is based on the principles of immunochromatography where proteins were separated into poly acrylamide gel according to the isoelectric point and molecular weight.
- ✓ A technique for detecting specific proteins separated by electrophoresis by use of labeled antibodies.
- ✓ Immunoblotting is performed chiefly in diagnostic laboratories to identify the desirable protein antigens in complex mixtures.
- ✓ An improved immunoblot method Zestern analysis, is able to address this issue without the electrophoresis step, thus significantly improving the efficiency of protein analysis.
- ✓ Other related techniques include dot blot analysis, zestern analysis, immunohistochemistry where antibodies are used to detect proteins in tissues and cells by immunostaining and enzyme-linked immunosorbent assay (ELISA).

WESTERN BLOTTING

- ✓ A technique to detect the presence of a ***specific protein*** in a complex mixture extracted from cells
- ✓ Three key elements accomplish this task:
  - * ***separation*** of protein mixtures by size using gel electrophoresis
  - * ***efficient transfer*** of separated proteins to a solid support
  - * ***specific detection*** of a target protein by appropriately matched antibodies



Western Blot

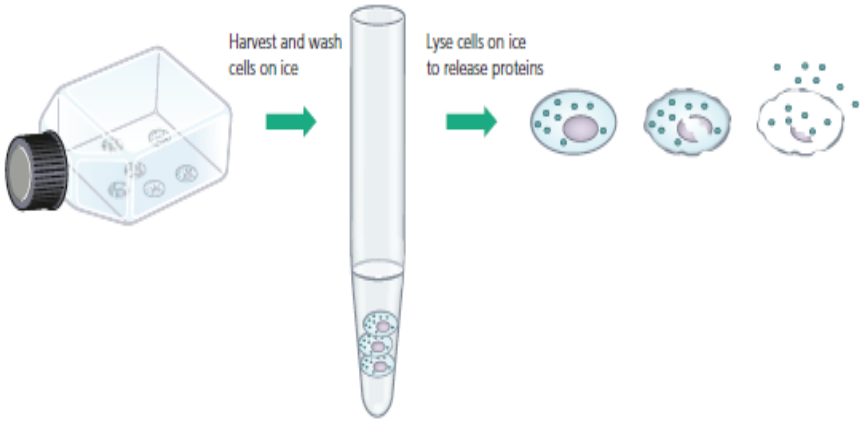
- Lane1: Positive Control
- Lane 2: Negative Control
- Sample A: Negative
- Sample B: Indeterminate
- Sample C: Positive

CONTENTS

- ✓ *Cell / tissue lysis*
- ✓ *Protein quantitation*
- ✓ *Electrophoresis*
- ✓ *Transfer to a support matrix*
- ✓ *Detection*
- ✓ *Data analysis*
- ✓ ***Applications***

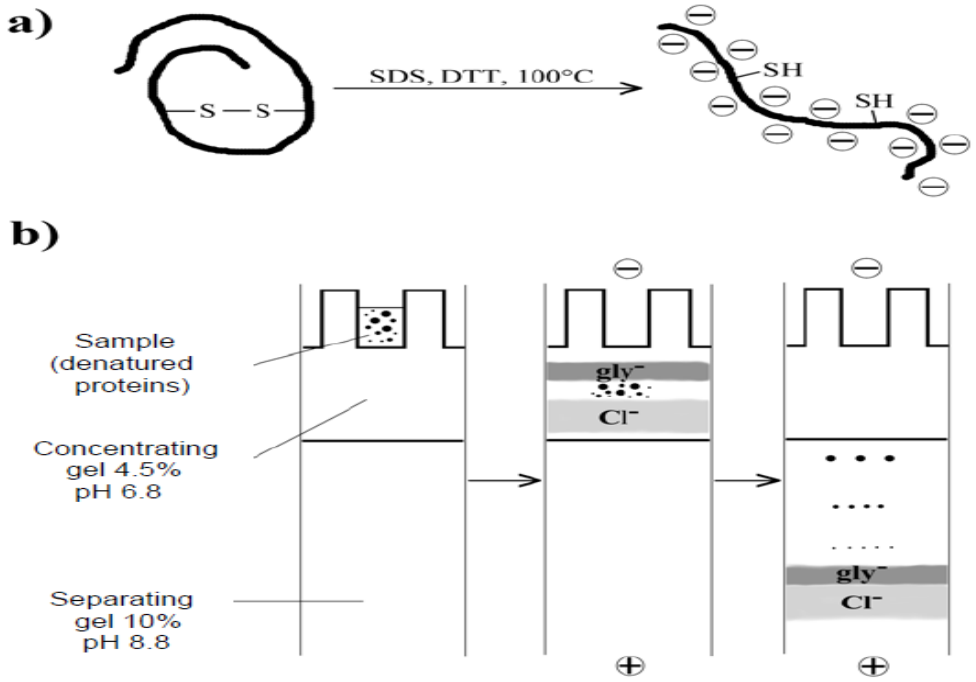
TISSUE PREPARATION

- ✓ Samples may be taken from whole tissue, from cell culture, bacteria, virus or environmental samples.
- ✓ In most cases, samples are solid tissues.
- ✓ First broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication.
- ✓ Cells may also be broken open by one of the above mechanical methods.
- ✓ A combination of biochemical and mechanical techniques, including various types of filtration and centrifugation.
- ✓ To encourage lysis of cells and to solubilize proteins, may be employed : **detergents, salts, and buffers**
- ✓ To prevent the digestion of the sample by its own enzymes **-Anti Protease and phosphatase**
- ✓ To avoid protein denaturing-Tissue preparation is often done at cold temperatures



GEL ELECTROPHORESIS

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point molecular weight, electric charge, or a combination of these factors. Commercially SDS-PAGE gel electrophoresis for protiens.



PAGE

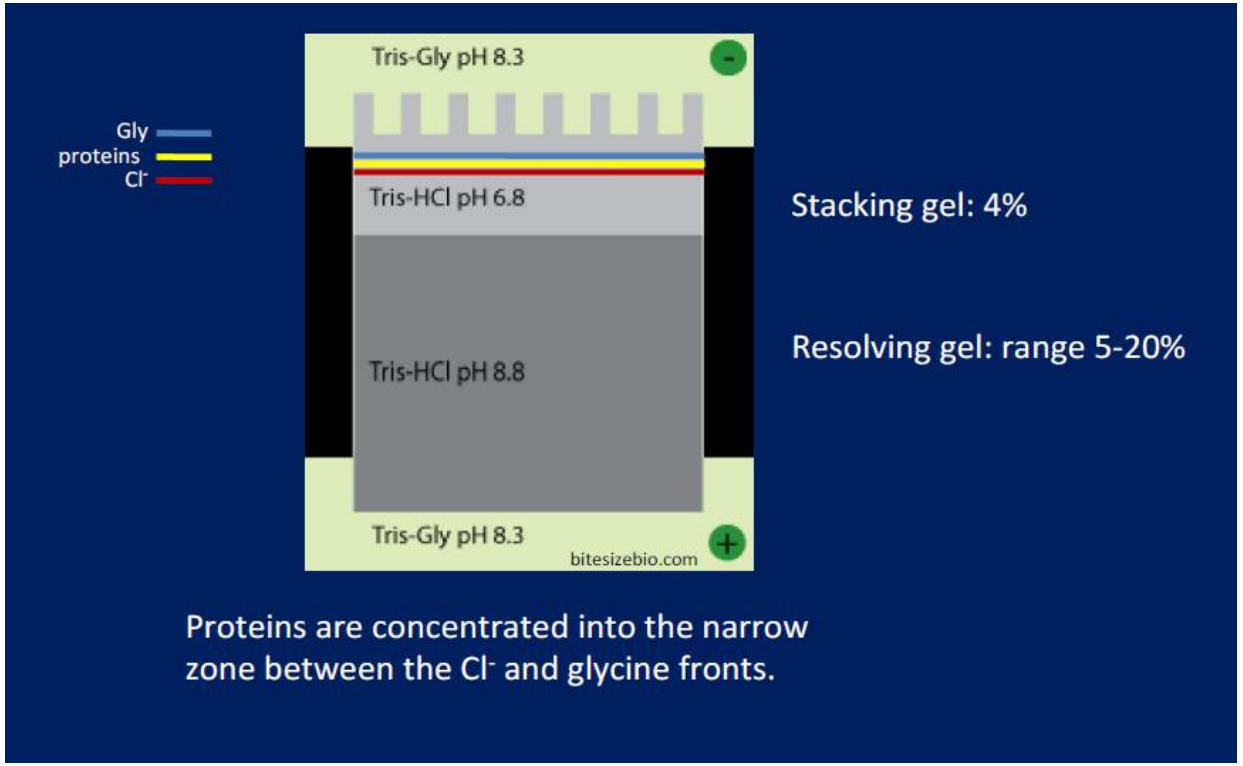
- ✓ If the proteins are denatured and put into an electric field (only), they will all move towards the positive pole at the same rate, with no separation by size.
- ✓ However, if the proteins are put into an environment that will allow different sized proteins to move at different rates.
- ✓ The environment is polyacrylamide.
  - The entire process is called polyacrylamide gel (PAGE) electrophoresis.
  - Small molecules move through the polyacrylamide forest faster than big molecules, big molecules stays near the well.

POLYACRYLAMIDE GEL

- ✓ **Polymerized gel:**

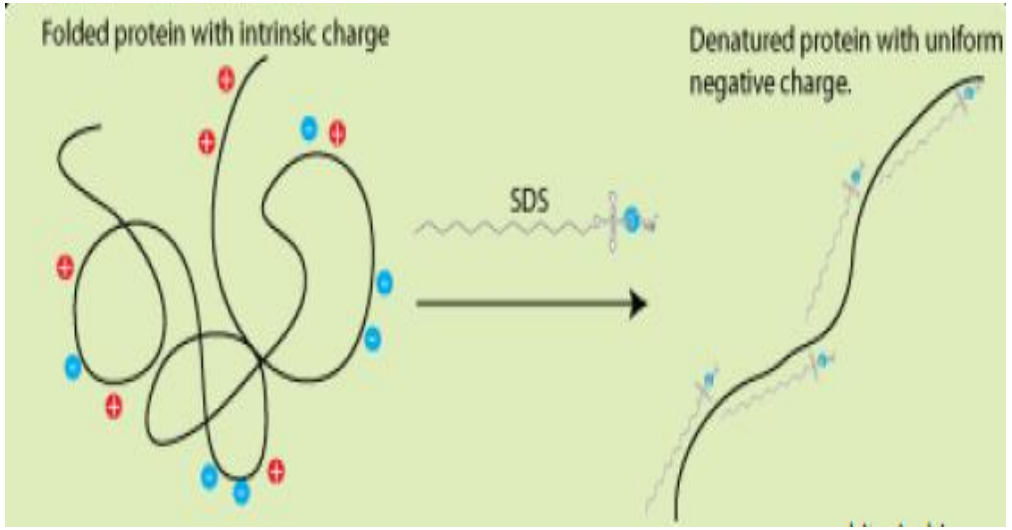
1. **Resolving gels** made in 6%, 10%, 12%, 18%.
2. **Stacking Gel** up to 4 - 5% was added to gel and then the wells are created.

- ✓ The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample.
- ✓ The smaller it is the bigger the percentage.



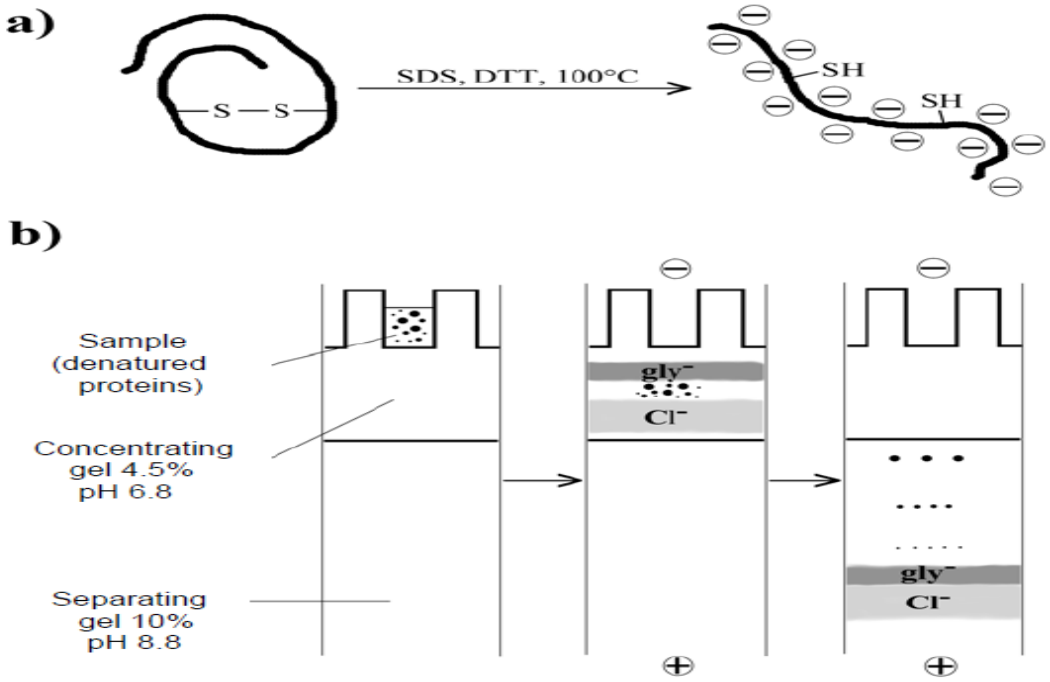
SDS-PAGE (POLYACRYLAMIDE GEL ELECTROPHORESIS)

- ✓ SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used in biochemistry, forensics, genetics and molecular biology
  - to separate proteins according to their electrophoretic mobility.
  - to separate proteins according to their size, and no other physical feature.
- ✓ SDS (the detergent soap) breaks up hydrophobic areas and coats proteins with negative charges thus overwhelming positive charges in the protein.
- ✓ Therefore, if a cell is incubated with SDS, the membranes will be dissolved, all the proteins will be solubilized by the detergent and all the proteins will be covered with many negative charges.



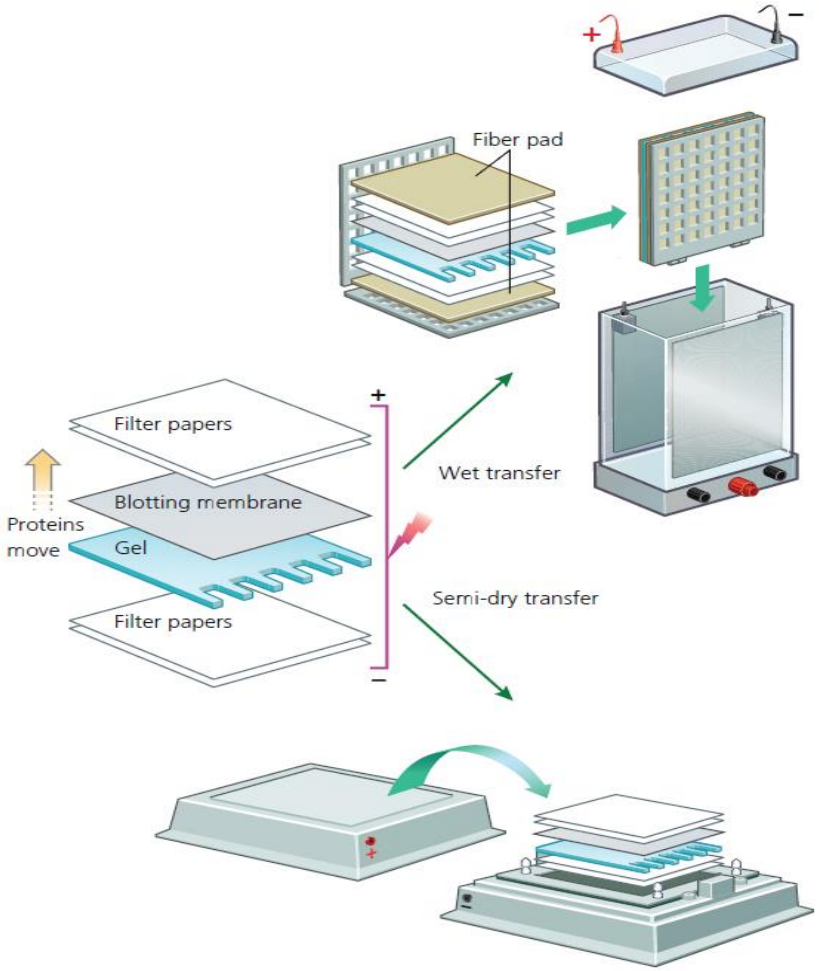
SDS PAGE

- ✓ The denatured Proteins covered negatively charged in the SDS .
- ✓ Are traveling to the positive since they have negative charge.
- ✓ These strands go through the tunnel and are separated by their size.



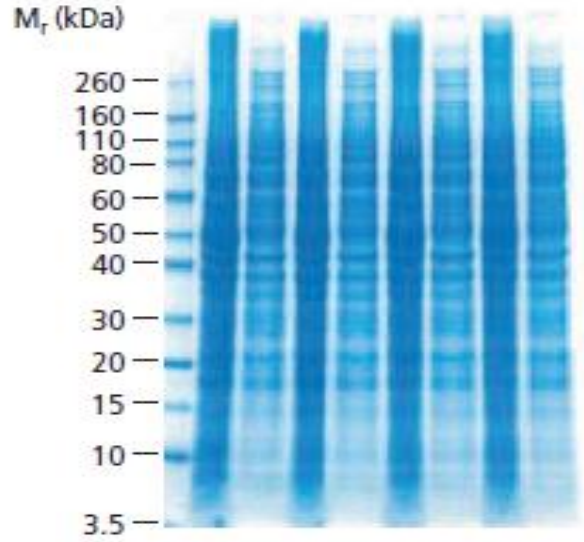
TRANSFER

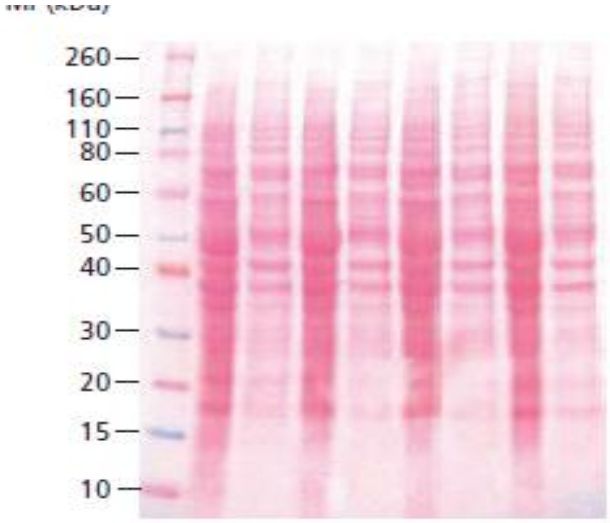
- ✓ In order to make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of **nitrocellulose or polyvinylidene difluoride (PVDF) .** The membrane is placed on top of the gel, and a stack of filter papers placed on top of that.
- ✓ The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.
- ✓ Another method for transferring the proteins is called **electrobloting** and uses an electric current to pull proteins from the gel into membrane.



WESTERN BLOTTING

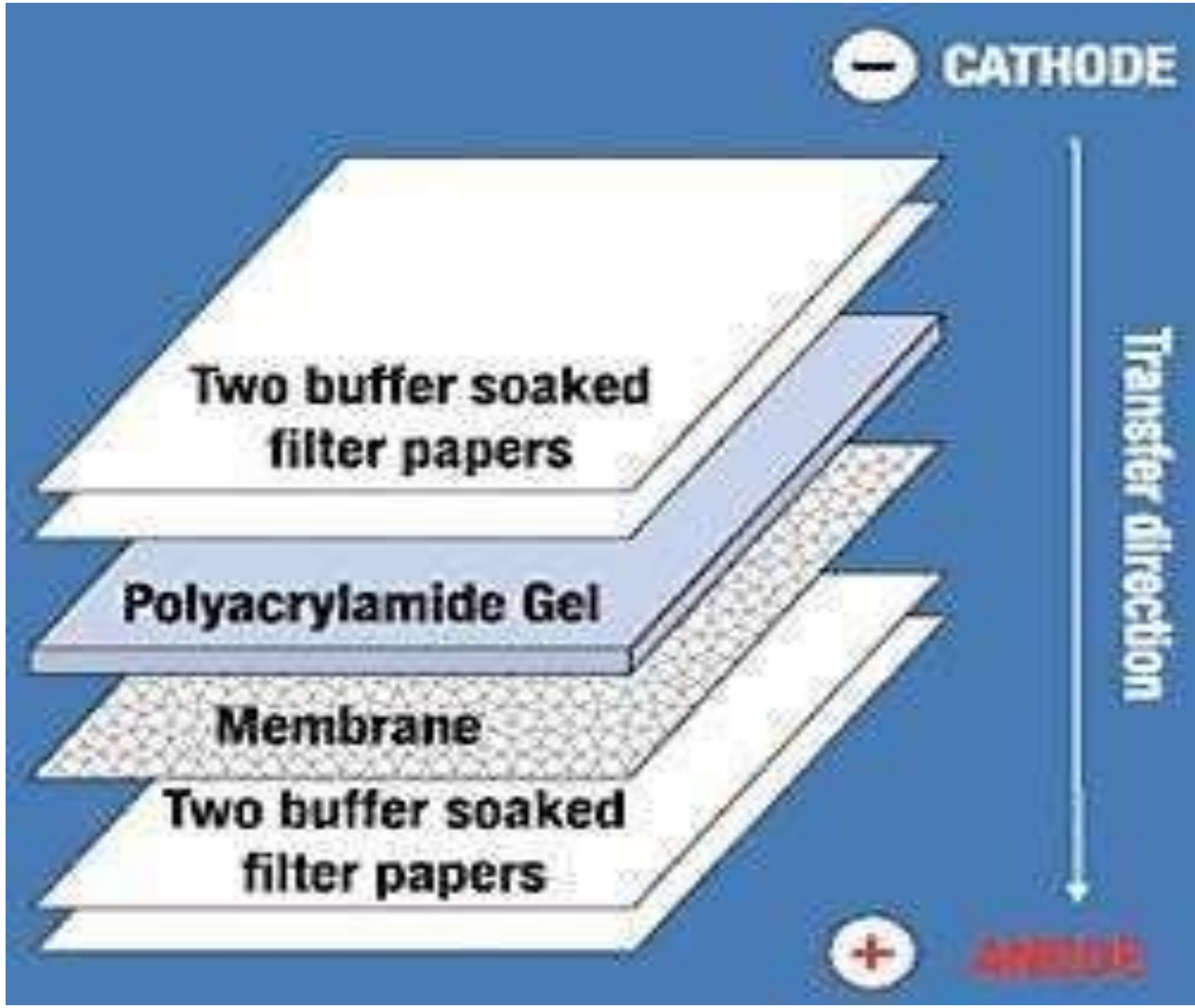
- ✓ Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well).
- ✓ Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.
- ✓ The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with **coomassie or ponceau S** dyes.
- ✓ **Nitrocellulose membranes are cheaper than PVDF**, but are far more fragile and do not stand up well to repeated probings.





BLOTTING

- ✓ Blotting used to transfer the samples from the gel on to a membrane such as a **nylon membrane** or **nitrocellulose membrane**.
- ✓ Analyzed through probing with nucleic acid probes or antibody probes.



BLOCKING

- ✓ Steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein (since the antibody is a protein itself).
- ✓ Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein.
- ✓ Typically **Bovin Serum Albumin (BSA)** or **non-fat dry milk** (both are inexpensive), with a minute percentage of detergent such as **Tween20**.
  - The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached.
  - This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

DETECTION

- The membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour.
- - Two step
- Primary antibody {Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof) }.
- A dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) is incubated with the membrane under gentle agitation for anywhere from 30 minutes to overnight at different temperatures.
- The solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA.

DETECTION

- Secondary antibody {After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody }.
- The secondary antibody is usually linked to biotin or to a reporter enzyms such as alkalin phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.
- Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein.
- A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% horseradish peroxidase; reaction of peroxide radicals with 4-chloronaphthol produces a dark brown stain that can be photographed without using specialized photographic film.

❖ **ONE STEP**

- Historically, the probing process was performed in two steps because of the relative ease of producing

  primary and secondary antibodies in separate processes.
- One-step probing systems that would allow the process to occur faster and with less consumables.
- This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known proteins tags.

ANALYSIS

- In practical terms, not all Westerns reveal protein only at one band in a membrane.
- Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis.
- The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples.
- This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

COLORIMETRIC DETECTION :

- This method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody.
- This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane.
- Protein levels are evaluated through densitometry or spectrophotometry.

DETECTION

RADIOACTIVE DETECTION

- ✓ Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest.
- ✓ Very expensive, health and safety risks are high.

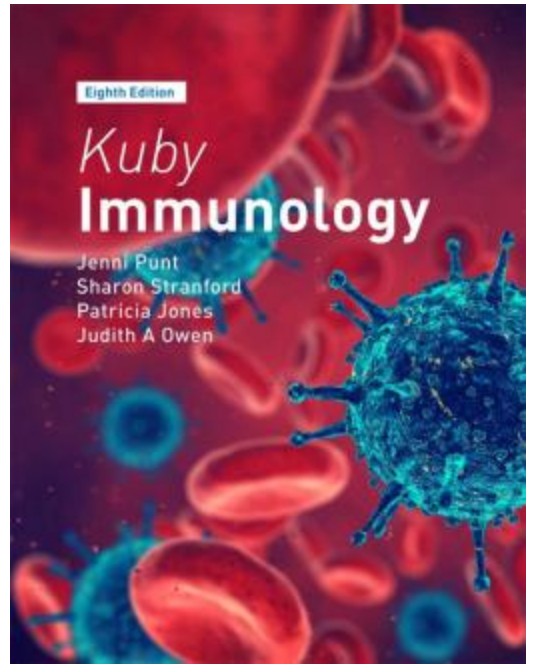
FLUORESCENT DETECTION

- ✓ The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as CCD camera.
- ✓ Allows further data analysis such as molecular weight analysis and a quantitative western blot analysis.
- ✓ The most sensitive detection methods for blotting analysis.

APPLICATIONS

- ✓ The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody.
- ✓ A western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').
- ✓ Some forms of Lyme disease testing employ western blotting.
- ✓ Western blot can also be used as a confirmatory test for Hepatitis B infection.
- ✓ In veterinary medicine, western blot is sometimes used to confirm FIV+ status in cats.

Acknowledgement



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