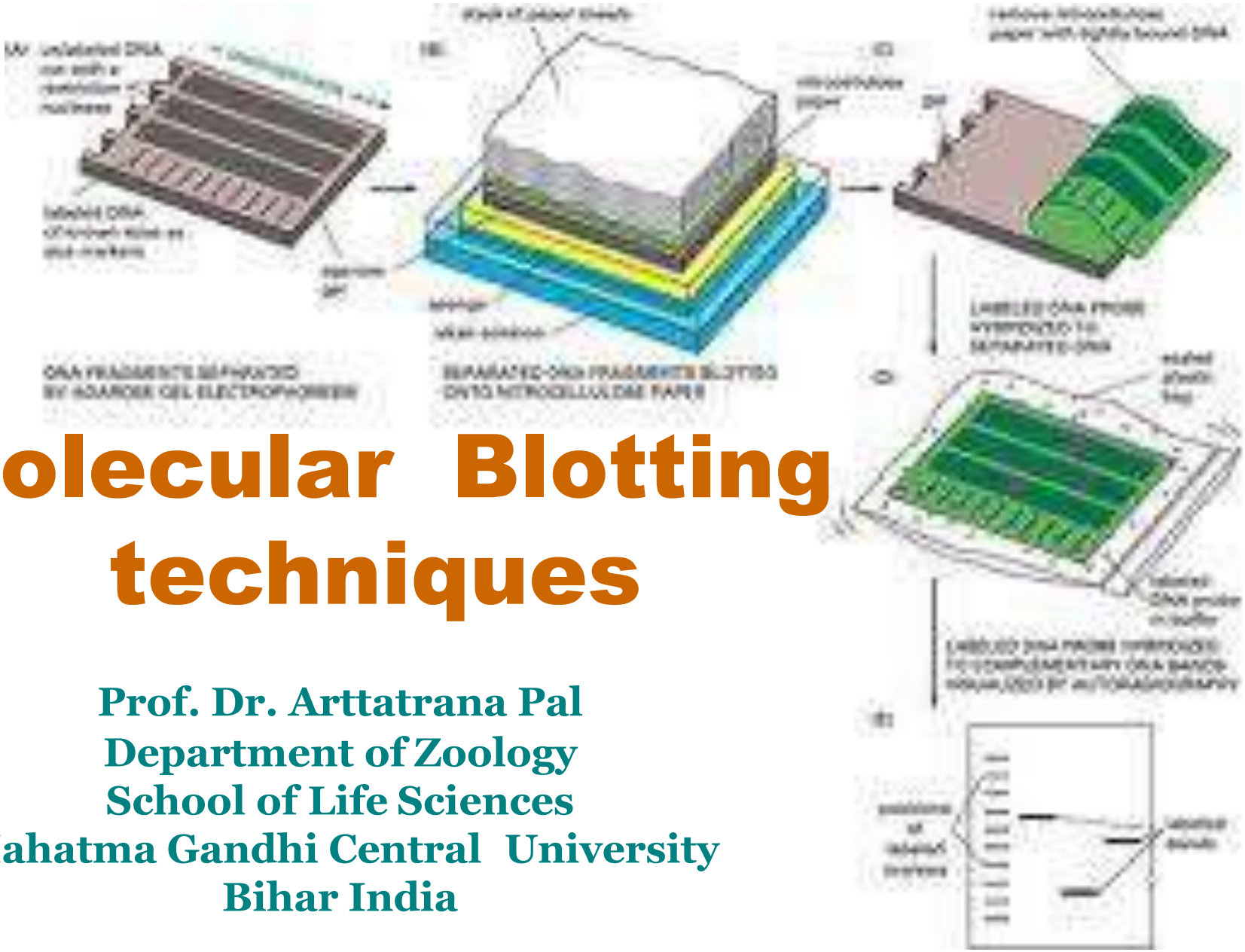


Molecular Blotting techniques

Prof. Dr. Arttatrana Pal
Department of Zoology
School of Life Sciences
Mahatma Gandhi Central University
Bihar India

What is blotting?

Blots are techniques for transferrin DNA, RNA and proteins into a carrier so they can be separated, and often follows the use of a gel electrophoresis

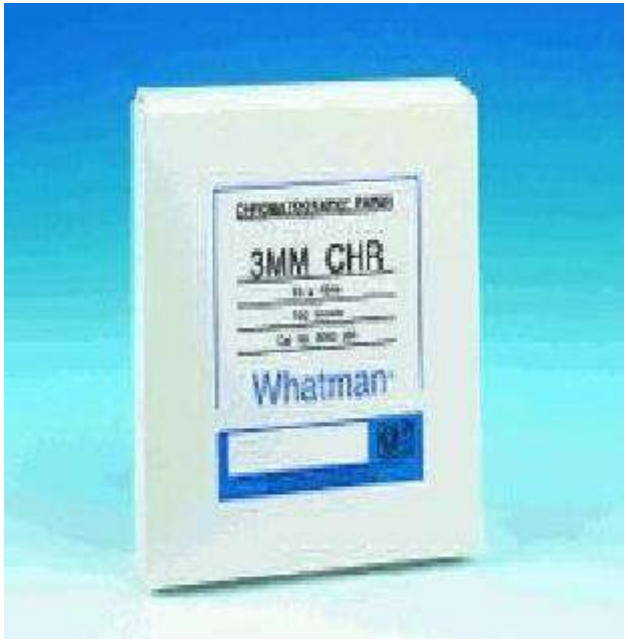
Types of blotting Techniques

Southern Blot: It is used to detect DNA.
Northern Blot: It is used to detect RNA.
Western blot: It is used to detect protein.
Southwestern blotting: detects DNA-binding proteins, since DNA detection is by Southern blotting and protein detection is by western blotting

Whatman paper

- Whatman 3MM paper is the world's most widely used blotting paper.

- This acceptance and usage is due to the high quality, purity and consistency that are relied upon by researchers doing Southern, Northern and Western transfers.



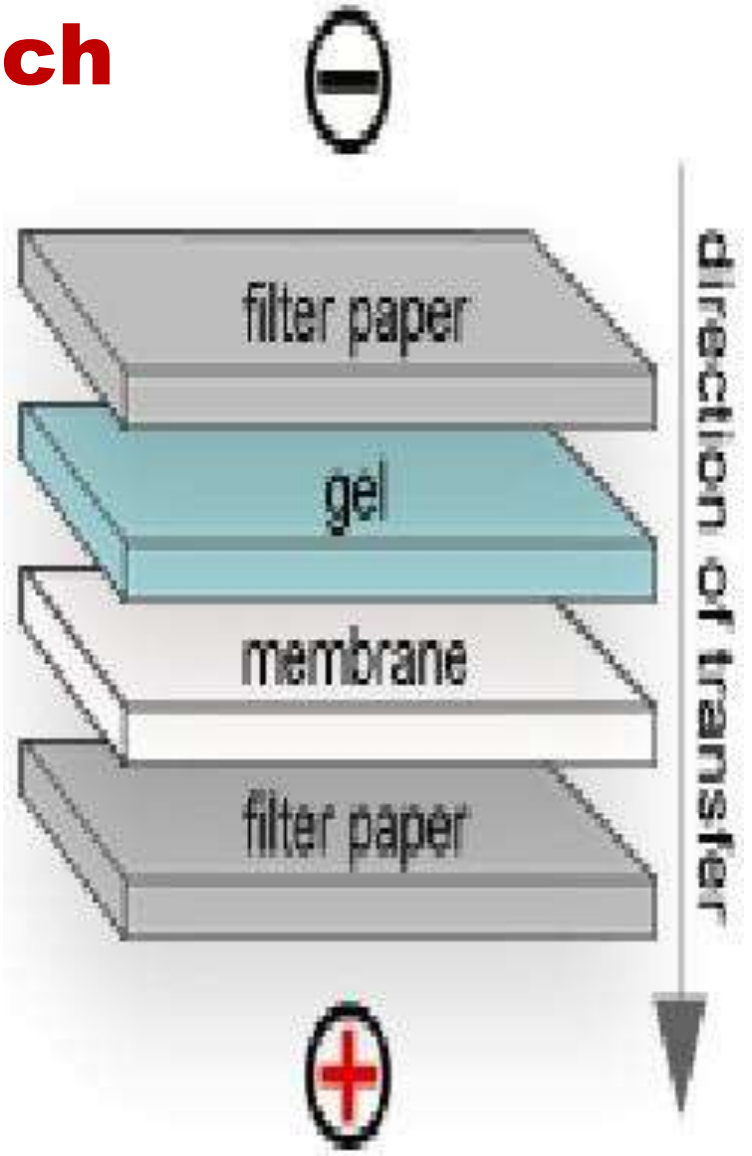
- 3MM paper is now available in the most widely used sizes.

- A medium thickness paper (0.34 mm) is used extensively in electrophoresis for lifting of sequencing gels

Creating the Sandwich

The sandwich consists of :

- filter paper
- Nitrocellulose membrane,
- gel matrix
- another piece of filter paper



Cut two pieces of filter paper and a piece of nitrocellulose membrane to an appropriate size, and soak them in transfer buffer.

- Place a piece of buffer soaked filter paper over the gel.

- Flip the gel over, place the buffer soaked nitrocellulose membrane against the exposed gel.

- Place the second piece of buffer soaked filter paper on the nitrocellulose membrane

- Check to see that there are no bubbles between the membrane and the gel.

SOUTHERN BLOTTING
(detect DNA)

This method Involves separation, transfer and hybridization.

- **The Southern blot is used to detect the presence of a particular piece of DNA in a sample.**

- **The DNA detected can be a single gene, or it can be part of a larger piece of DNA such as a viral genome.**

- **The key to this method is Hybridization.**

- **Hybridization - Process of forming a double-stranded DNA molecule between a single-stranded DNA probe and a single-stranded target patient DNA**

PRINCIPLE

1. The mixture of molecules is separated.

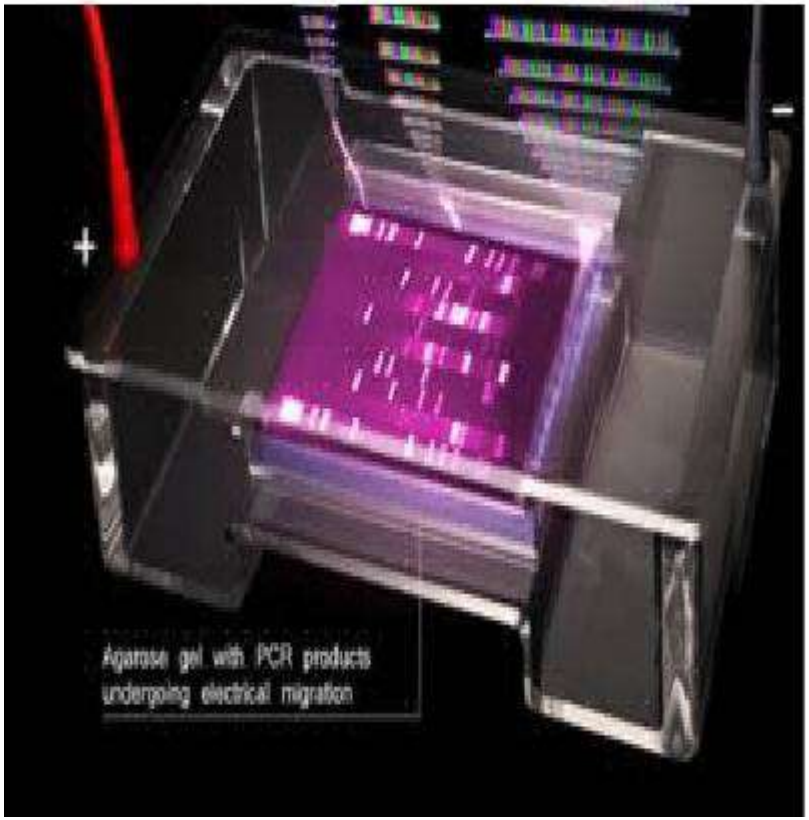
2. The molecules are immobilized on a matrix.

3.The probe is added to the matrix to bind to the molecules.

4. Any unbound probes are then removed.

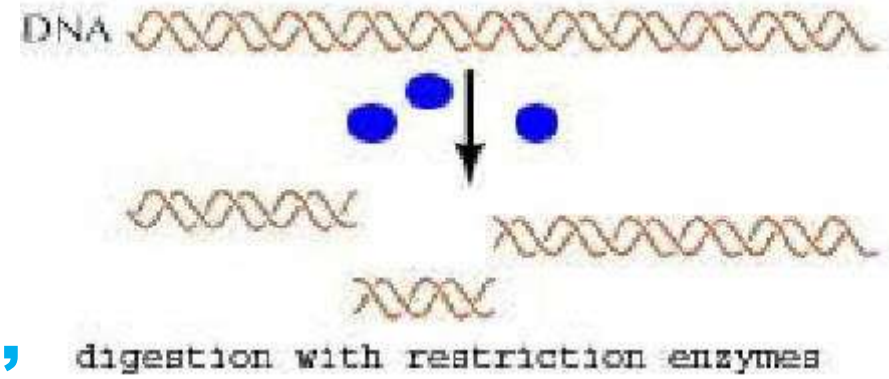
5.The place where the probe is connected corresponds to the location of the immobilized target molecule

APPARATUS

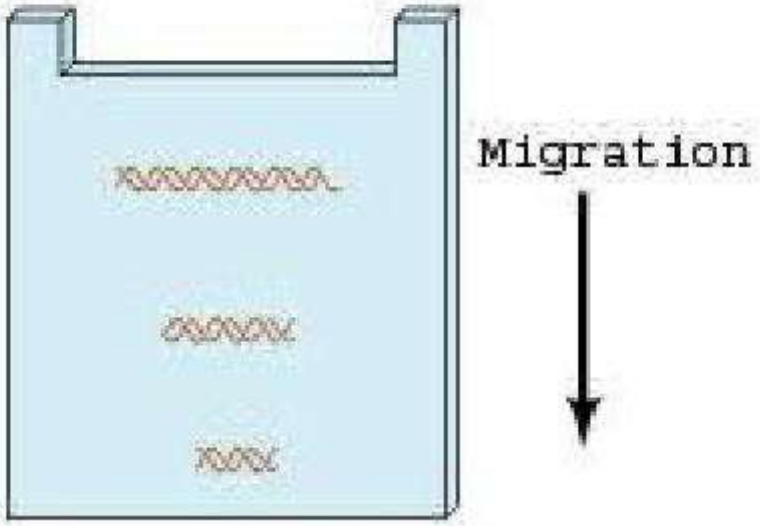

+
−
Agarose gel with PCR products
undergoing electrical migration

Steps in southern blotting

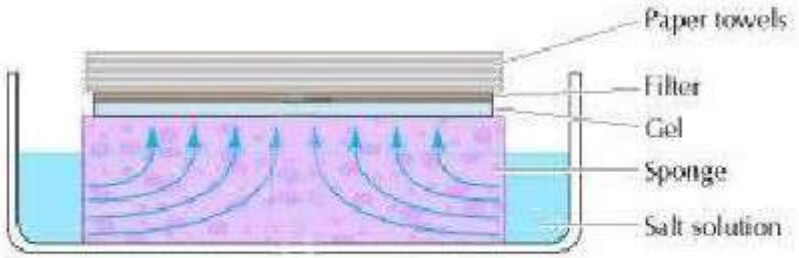
1.The DNA to be analyzed, such as the total DNA of an organism, is digested to completion with a **restriction enzyme.**



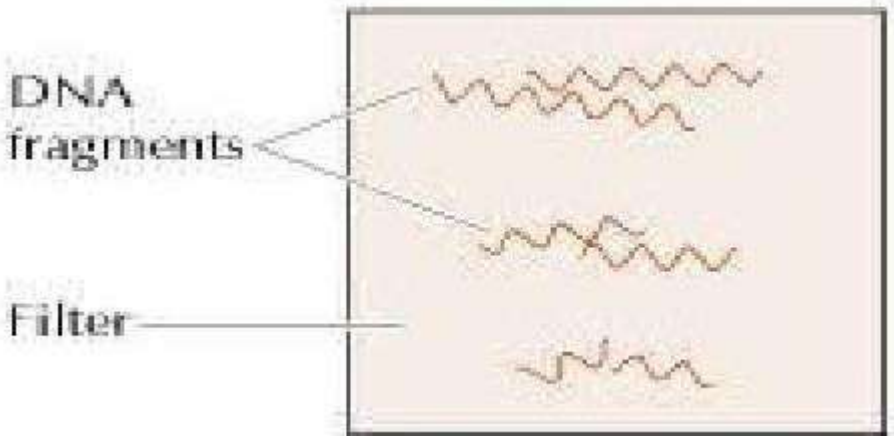
2.The complex mixture of fragments is subjected to gel electrophoresis to separate the fragments according to size



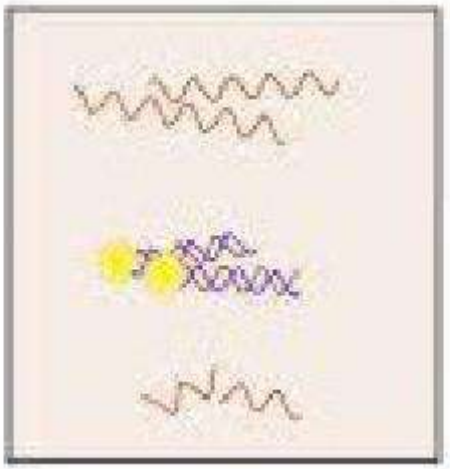
3.The restriction fragments present in the gel are denatured with alkali and transferred onto a nitrocellulose filter or nylon membrane by blotting.



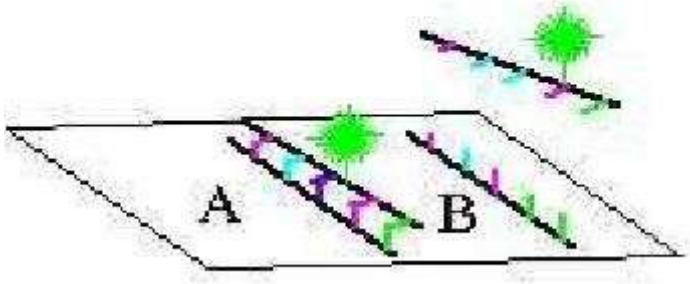
4.This procedure preserves the distribution of the fragments in the gel, creating a replica of the gel on the filter



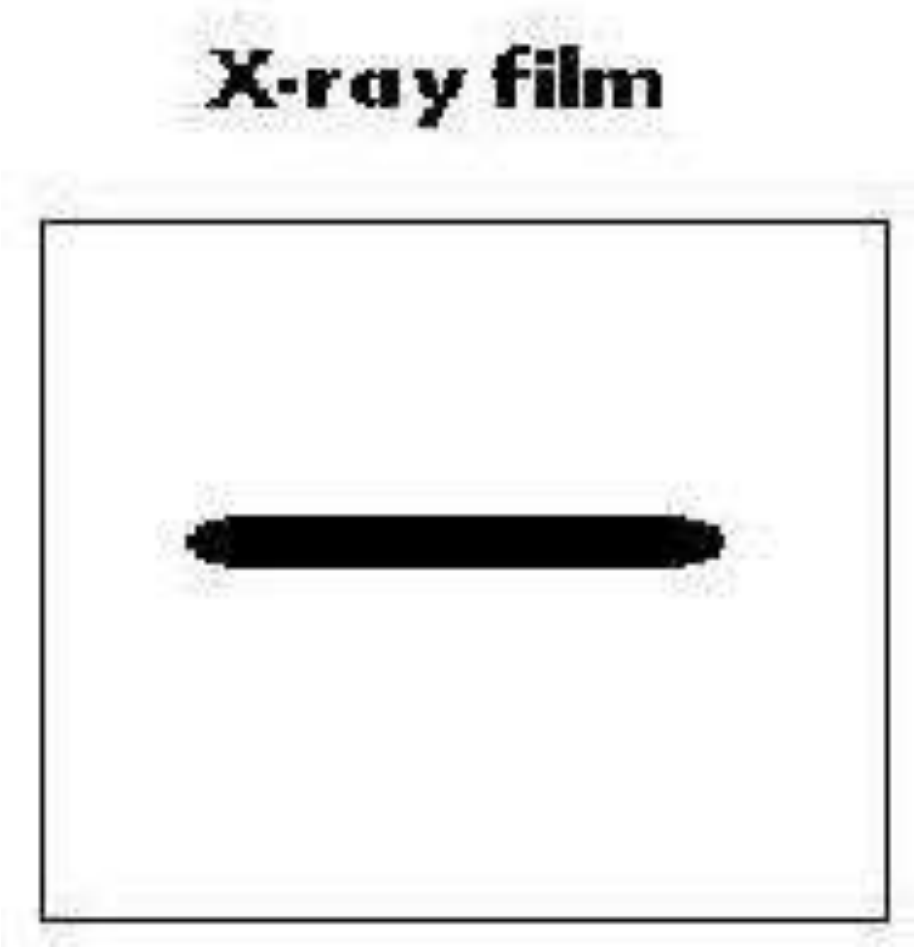
5.The filter is incubated under hybridization conditions with a specific radiolabeled DNA probe.

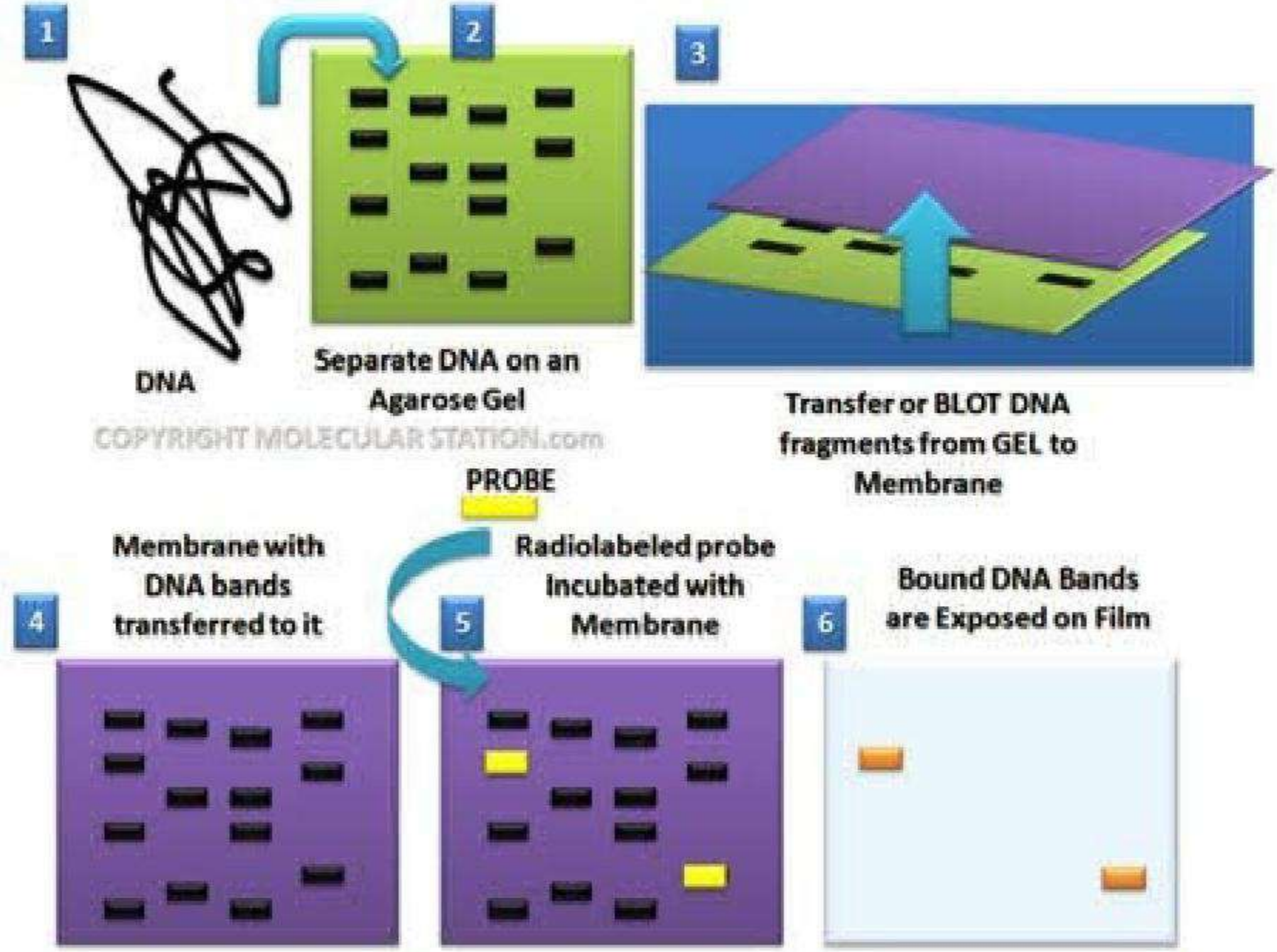


6.The probe hybridizes to the complementary DNA restriction fragment.

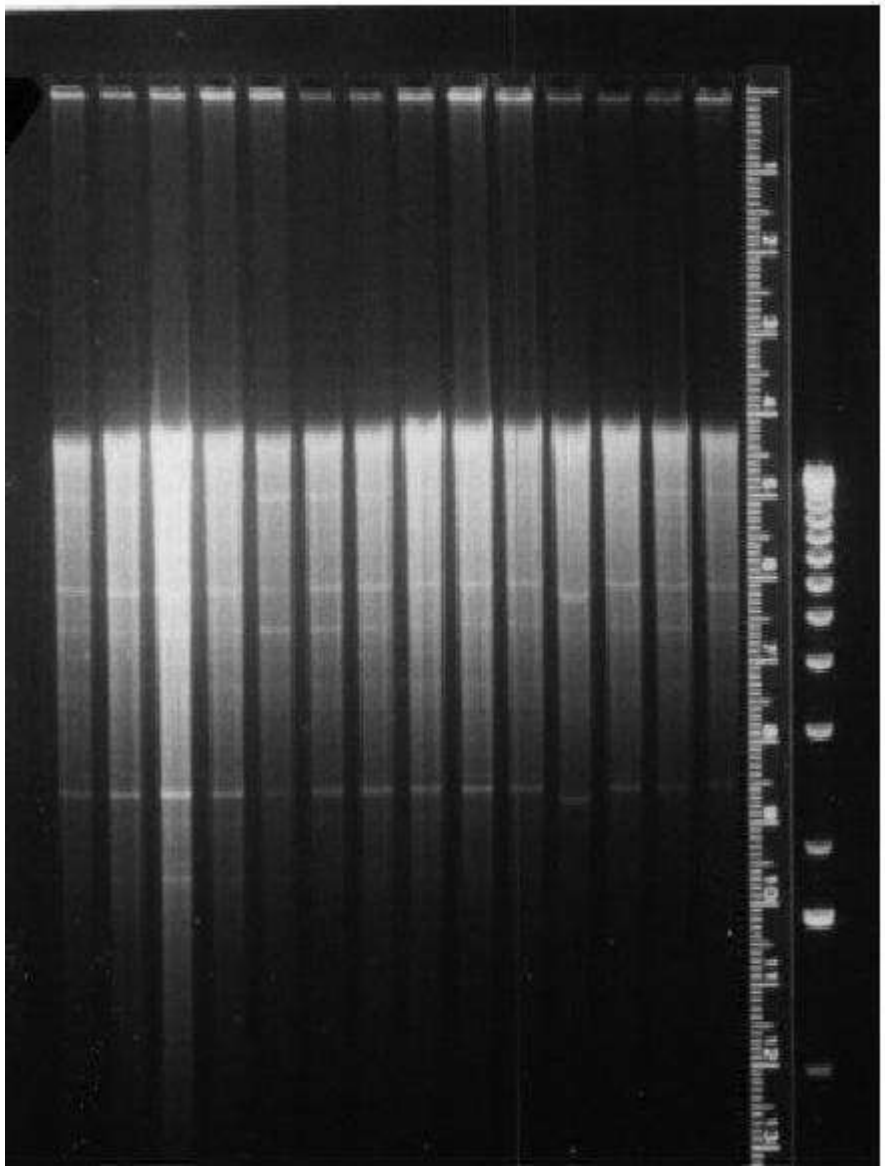


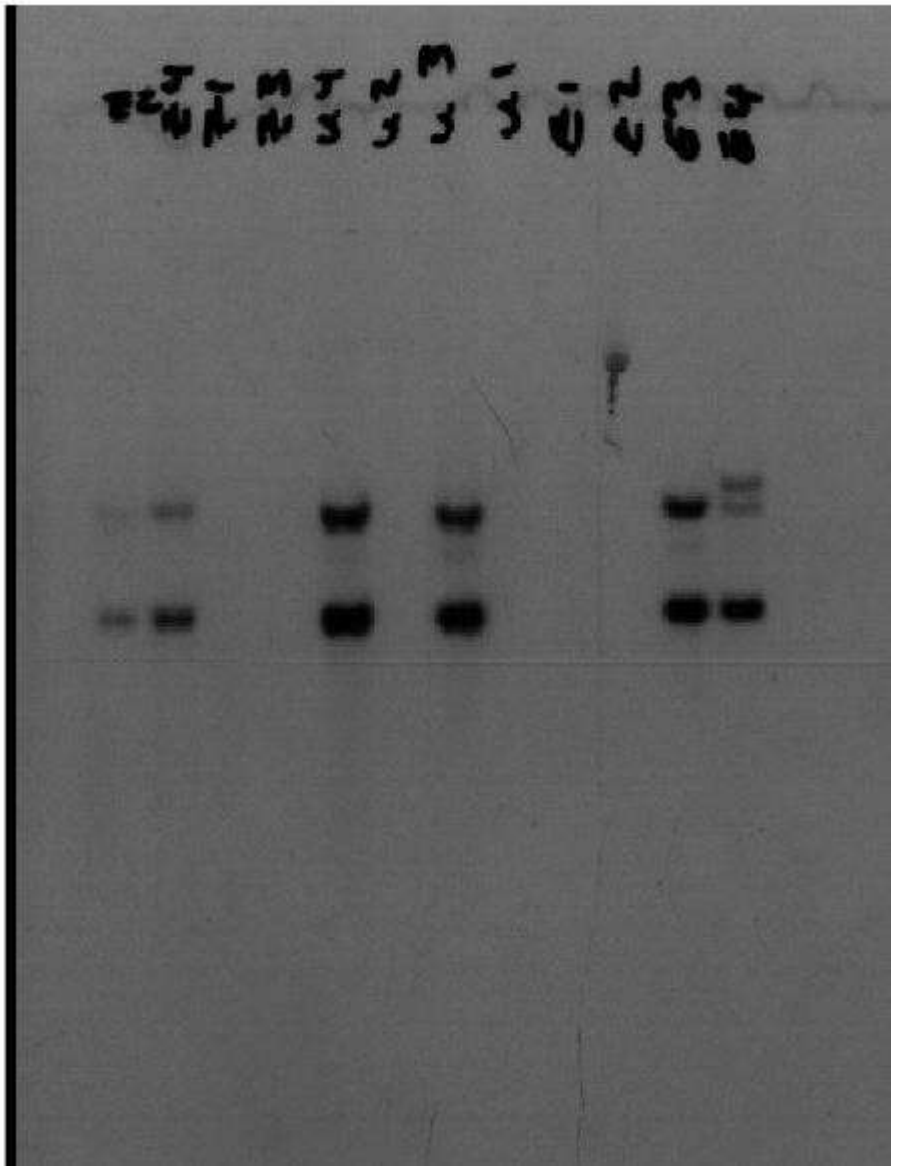
7. Excess probe is washed away and the probe bound to the filter is detected by **autoradiography**, which reveals the DNA fragment to which the probe hybridized




1
2
3
DNA
Separate DNA on an Agarose Gel
Transfer or BLOT DNA fragments from GEL to Membrane
COPYRIGHT MOLECULAR STATION.com
PROBE
Membrane with DNA bands transferred to it
Radiolabeled probe Incubated with Membrane
Bound DNA Bands are Exposed on Film
4
5
6

An Example





Applications

- Southern blots are used in
  - gene discovery
  - Mapping
  - Evolution and development studies
  - Diagnostics
  - Forensics etc.

- In regards to genetically modified organisms, Southern blotting is used for testing to ensure that a particular section of DNA of known genetic sequence has been successfully incorporated into the genome of the host organism

Applications

- Southern blots allow investigators to determine the
  - molecular weight of a restriction fragment
  - to measure relative amounts in different samples.

- Southern blot is used to detect the presence of a particular bit of DNA in a sample

- Aanalyze restriction digestion fragmentation of DNA or a biological sample

Northern Blotting

Northern blotting is a technique for detection of specific RNA sequences.

Northern blotting was developed by James Alwine and George Stark at Stanford University and was named such by analogy to Southern blotting

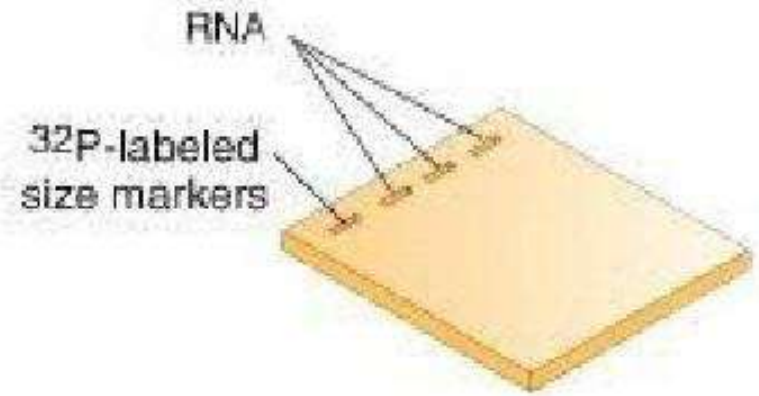
Steps involved in Northern blotting

- RNA is isolated from several biological samples (e.g. Various cell lines, Various tissues, various developmental stages of same tissue etc.)

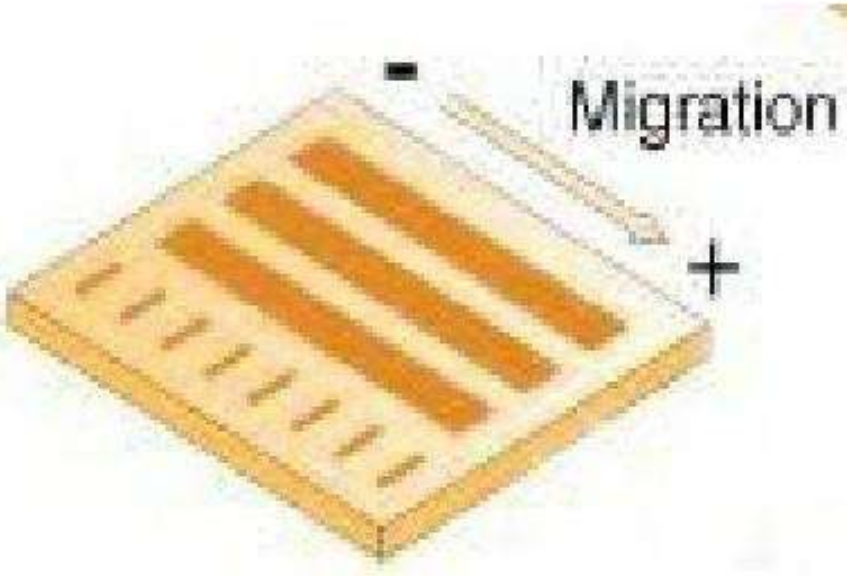
N.B.: RNA is more susceptible to degradation than DNA.

Steps involved in Northern blotting

- Sample's are loaded on gel and the RNA samples are separated according to their size on an agarose gel

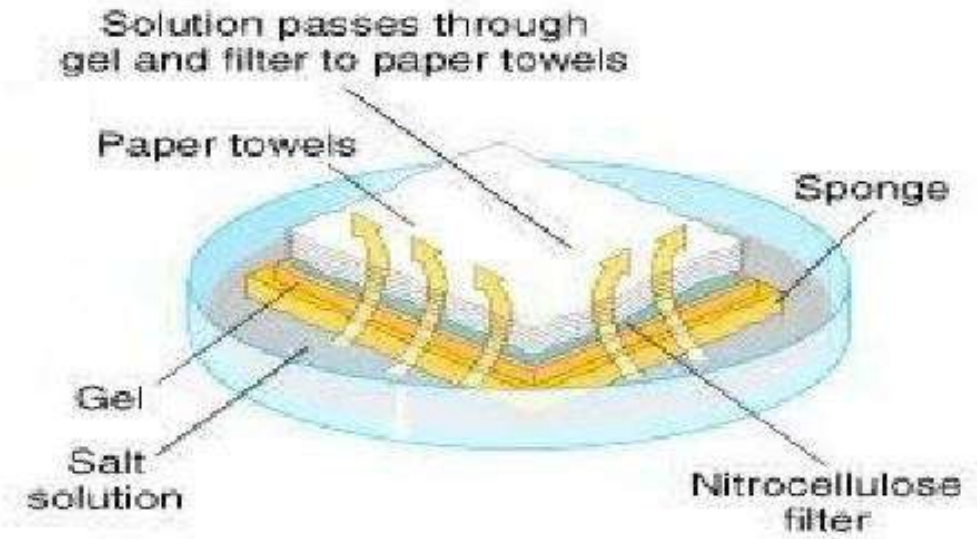


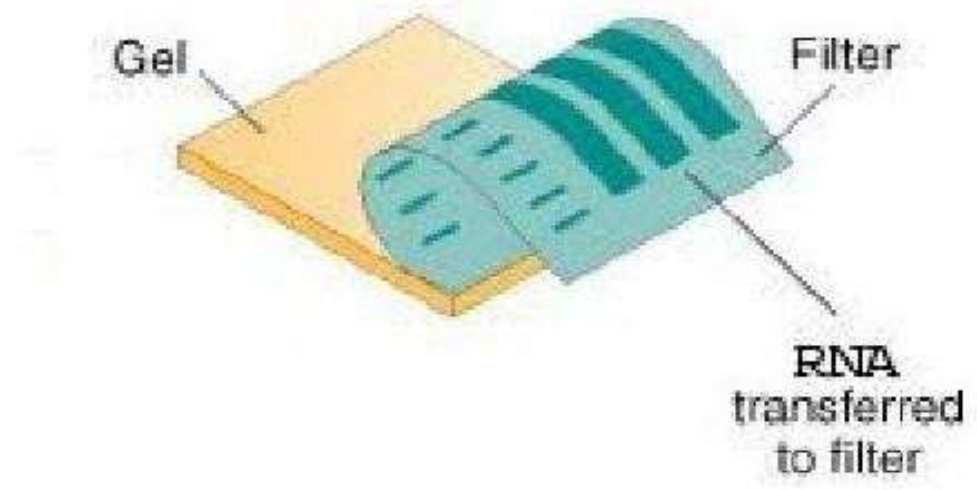
- The resulting gel following after the electrophoresis run



Steps involved in Northern blotting

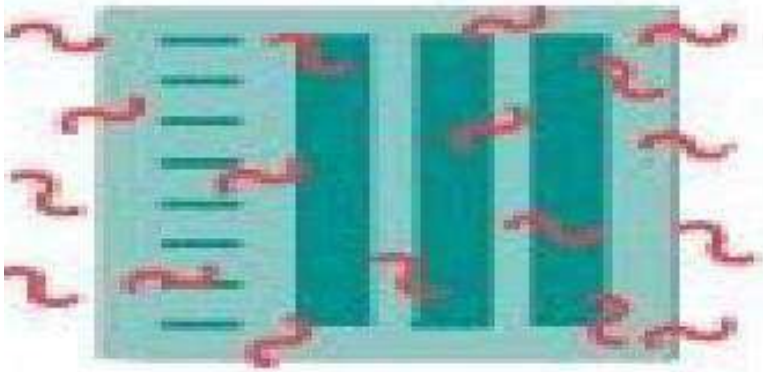
- The gel is then blotted on a nylon membrane or a nitrocellulose filter paper by creating the sandwich arrangement



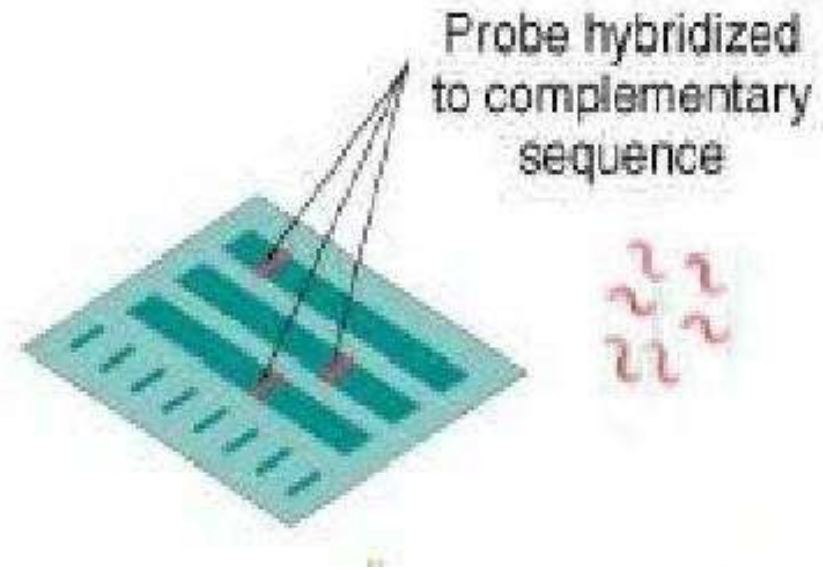


Steps involved in Northern blotting

- The membrane is placed in a dish containing hybridization buffer with a labeled probe



- Thus, it will hybridize to the RNA on the blot that corresponds to the sequence of interest



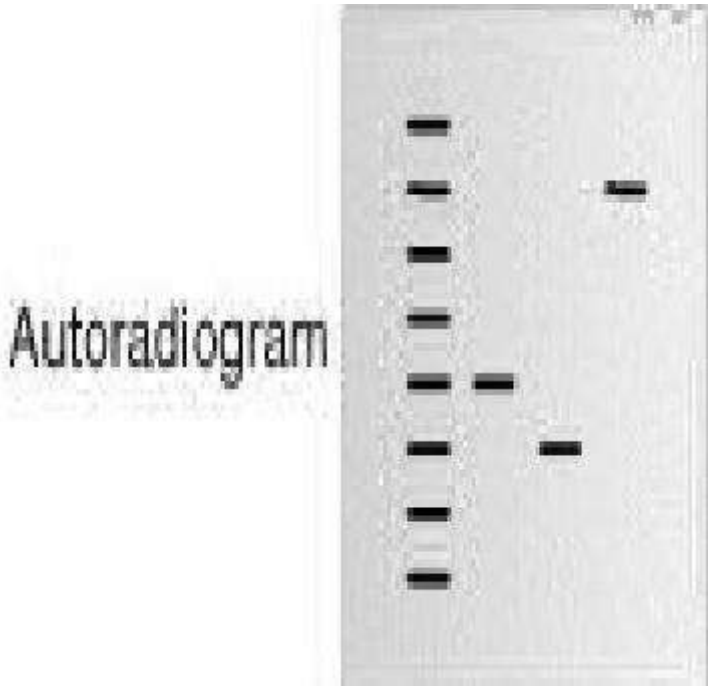
- The membrane is washed to remove unbound probe

Steps involved in Northern blotting

- The labeled probe is detected via autoradiography or via a chemiluminescence reaction (if a chemically labeled probe is used)

- In both cases this results in the formation of a dark band on an X-ray film

- Now the expression patterns of the sequence of interest in the different samples can be compared



APPLICATIONS

A standard for the direct study of gene expression at the level of mRNA (messenger RNA transcripts)

- Detection of mRNA transcript size
- Study RNA degradation
- Study RNA splicing can detect alternatively

spliced transcripts

- Study RNA half-life
- Study IRES (internal ribosomal entry site) – to remove possibility of RNA digestion vs. 2nd cistron translation.
- Often used to confirm and check transgenic / knockout mice (animals)

Disadvantages of Northern Blot

- Often radioactivity is used. This prevents ease of performing it, use and disposal
- New methods of non-radioactive detection have been generated allowing non-radioactive detection
- The whole process of northern blotting takes a long time usually, from sample preparation through to detection
- If RNA samples are even slightly degraded by RNases, the quality of the data and quantitation of expression is quite negatively affected

Disadvantages of Northern blot

- The standard northern blot method is relatively less sensitive than nuclease protection assays and RT-PCR.

- The sensitivity of northern blots may be increased with the use of nylon positively-charged membranes, use of a highly specific antisense probe.

- Detection with multiple probes is a problem.

- Often, the membranes must be stripped before hybridization and detection with a second probe. This is a problem as harsh conditions are required to strip off probes from the blot and is also time consuming. Also, there is a limit to the amount of times a blot may be stripped.

Western blotting

- Western blotting is an Immunoblotting technique which rely on the specificity of binding between a molecule of interest and a probe to allow detection of the molecule of interest in a mixture of many other similar molecules.

- In Western blotting, the molecule of interest is a protein and the probe is typically an antibody raised against that particular protein.

- The SDS PAGE technique is a prerequisite for Western blotting

SDS PAGE

Principle

- **SDS PAGE uses an anionic detergent (SDS) to denature proteins.**

- **The protein molecules become linearized. One SDS molecule binds to 2 amino acids.**

- **Due to this, the charge to mass ratio of all the denatured proteins in the mixture becomes constant.**

- **These protein molecules move in the gel (towards the anode) on the basis of their molecular weights only & are separated.**

- **The charge to mass ratio varies for each protein (in its native or partially denatured form). Estimation of molecular weight would then be complex. Hence, SDS denaturation is used.**

- The gel matrix is formed of polyacrylamide. The polyacrylamide chains are crosslinked by N,N-methylene bisacrylamide comonomers. Polymerisation is initiated by ammonium persulfate (radical source) and catalysed by TEMED (a free radical donor and acceptor).

- The resolution & focus of the protein bands is increased by using discontinuous gels- the stacking gel (pH 6.8, %T=3 to 5 %) & the resolving gel (pH 8.8, %T= 5 to 20 %). %T represents acrylamide percentage.

- These gels are usually run at constant current.

- As such, bulk of the current is carried by the denatured, negatively charged, SDS-coated protein molecules. At this stage, the glycine ions lag behind the proteins. The order is as follows- chloride ions, denatured proteins, glycine ions.

- Upon entering the resolving gel (pH=8.8), the glycine zwitterions deprotonate to the anionic form.

- The proportion of these ions increase from 0.0015% to 15.8%.

- The carrying of the current is now shared by the ions such that protein molecules have a greater freedom to separate on the basis of molecular weights.

- Due to their small size, the glycine anions also tend to overtake the protein band, thus providing a sandwiching effect & greater resolution in the gel.

Western blotting

- Detects the presence of (and gives us the size of) a specific protein in a crude protein extract.

- Can be used to determine if a gene is expressed (transcribed and translated) or not.

Making Protein Extract

Tissue or Cell line or Bateria

↓

Tissue homogenate/ Cell homogenate

↓

Centrifugation/ Freeze Thaw Cycle

↓

Cytoplasmic extract or Nuclear Extract

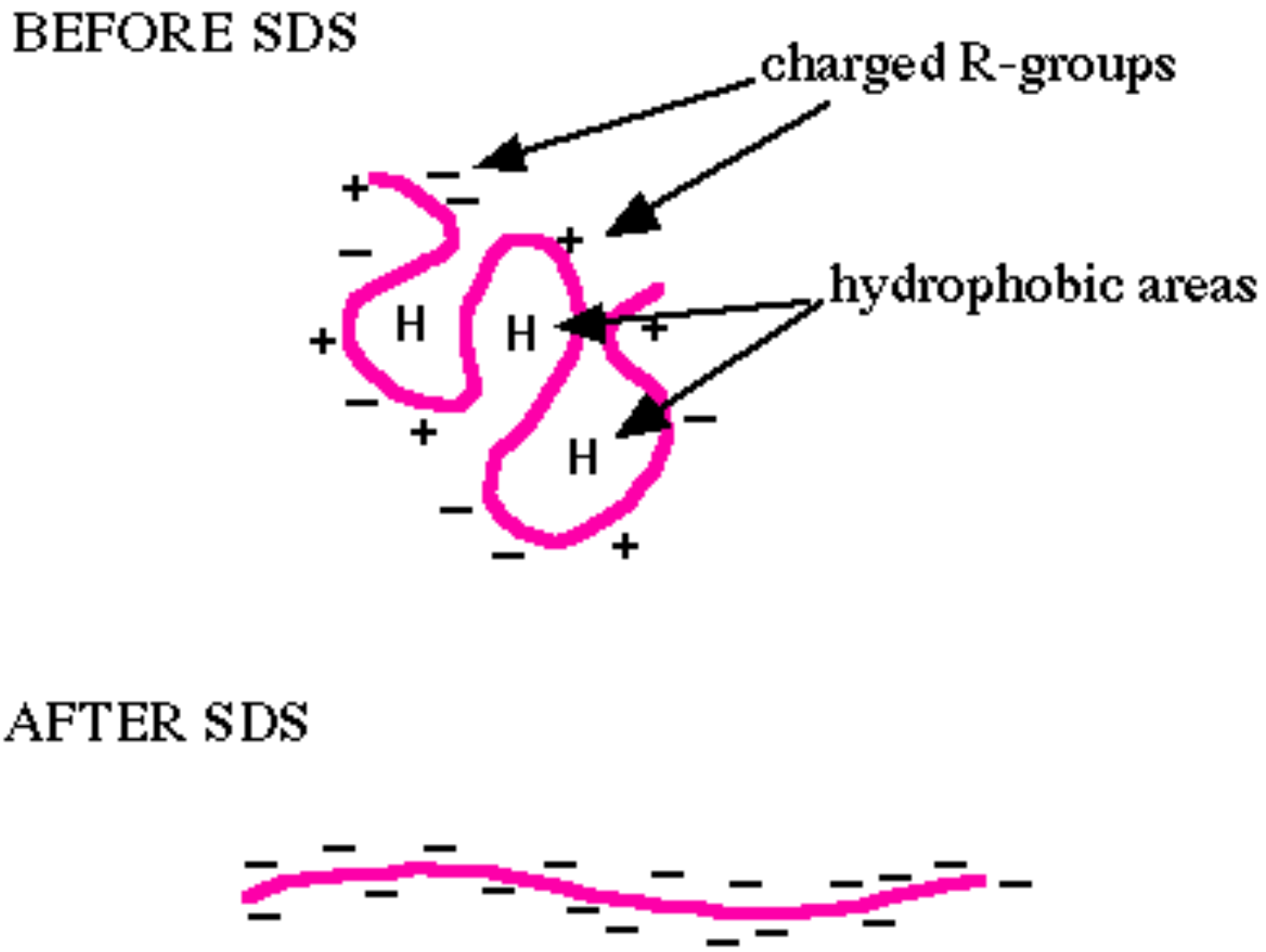
↓

Protein Quantitation

↓

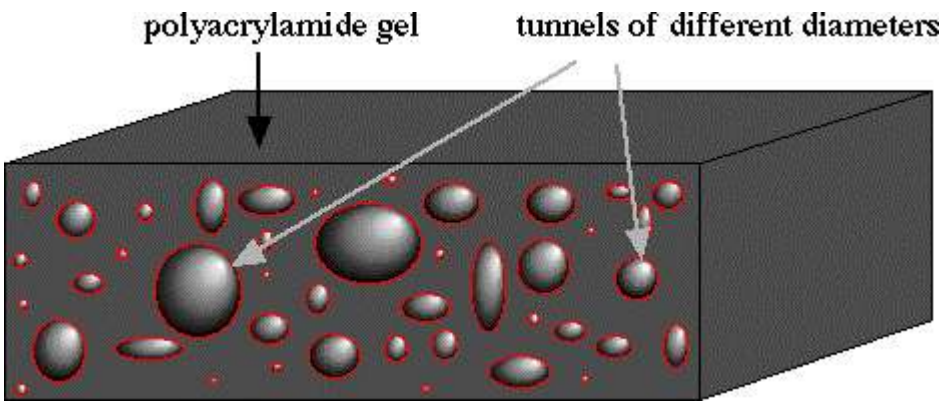
Protein Separation by PAGE

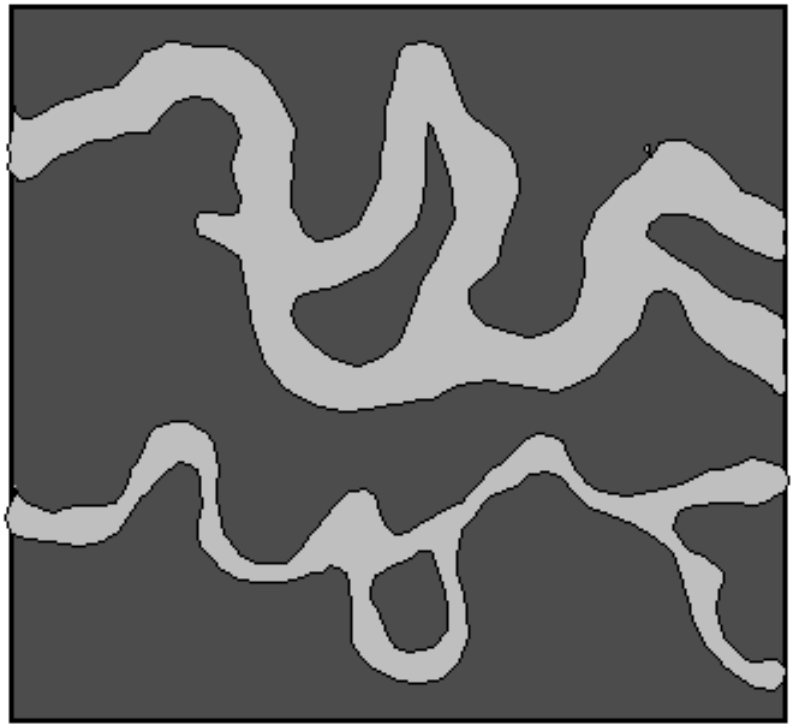
Separating proteins by size



Based on size of primary

Acrylamide gel

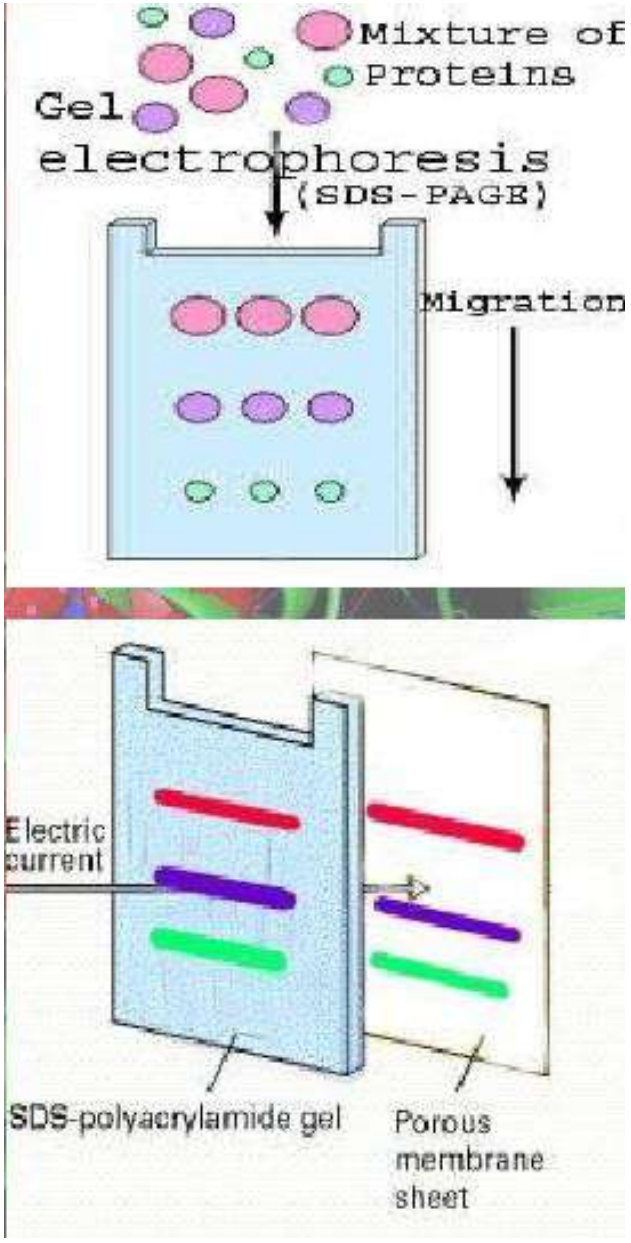




Steps in western blotting

A protein sample is subjected to electrophoresis on an SDS-polyacrylamide gel

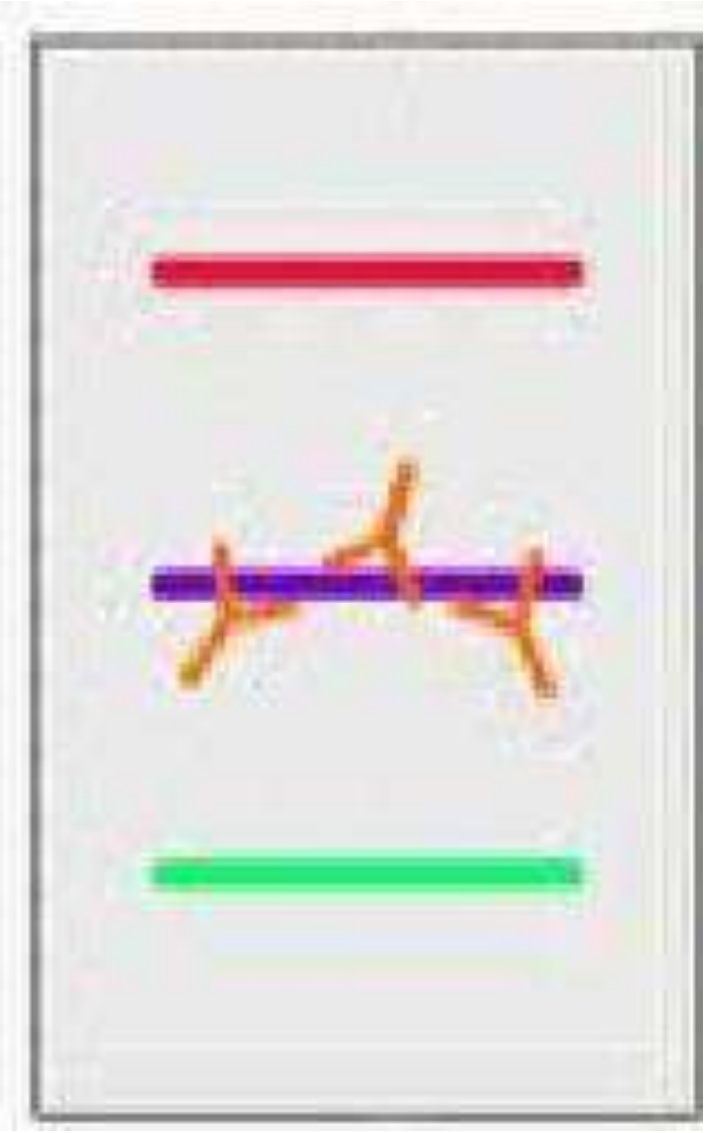
- **Electroblotting transfers the separated proteins from the gel to the surface of a <u>nitrocellulose membrane</u>**



The blot is incubated with a generic protein (such as milk proteins or BSA) which binds to any remaining sticky places on the nitrocellulose.

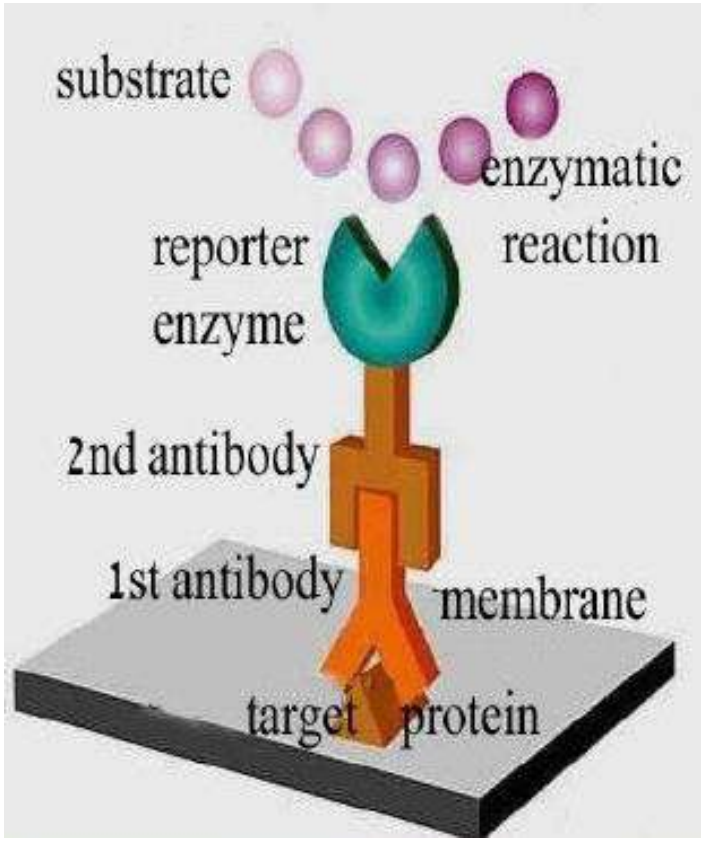
An antibody that is specific for the protein of interest (primary antibody: Ab1) is added to the nitrocellulose sheet and reacts with the antigen.

Only the band containing the protein of interest binds the antibody, forming a layer of antibody molecules

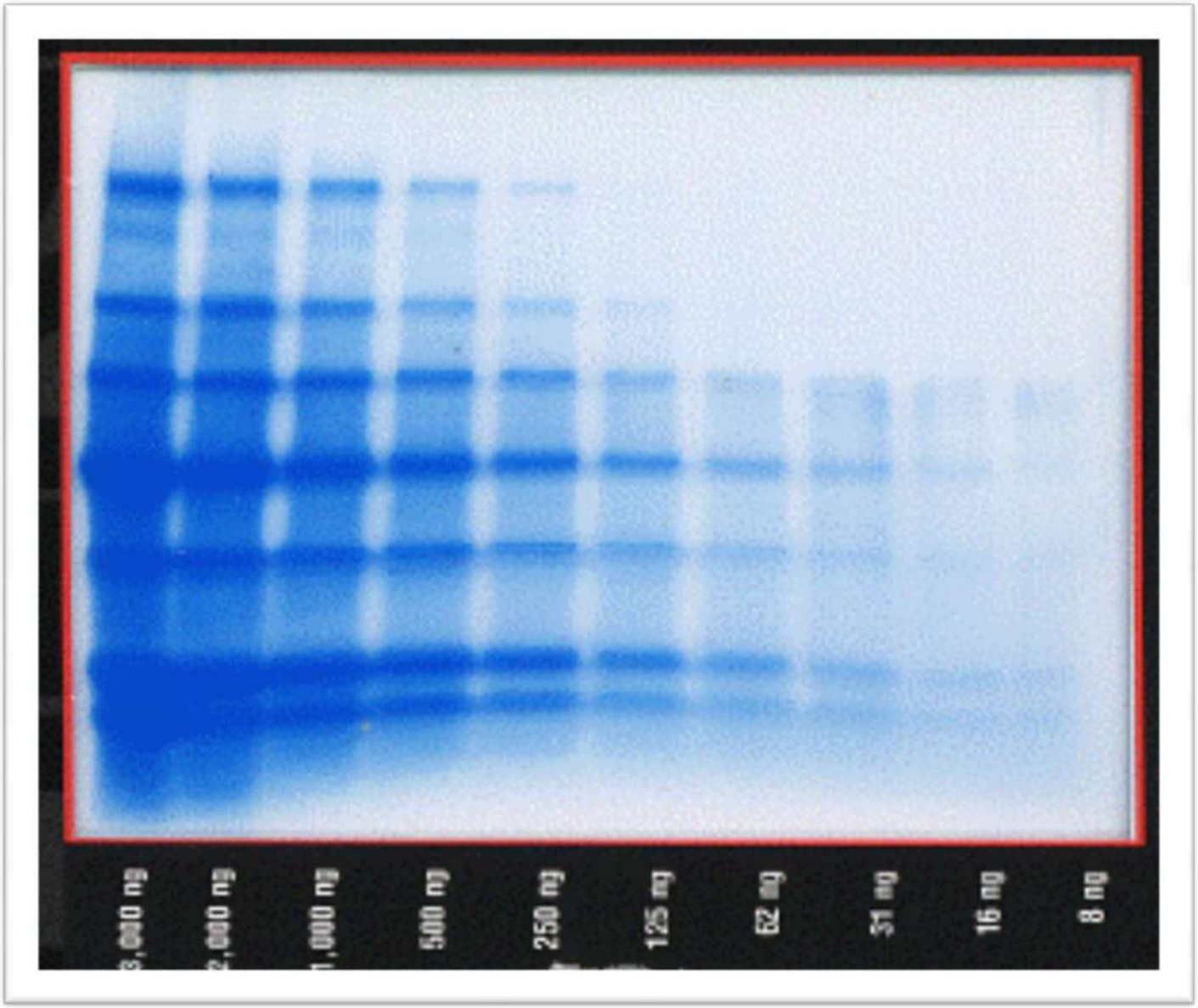


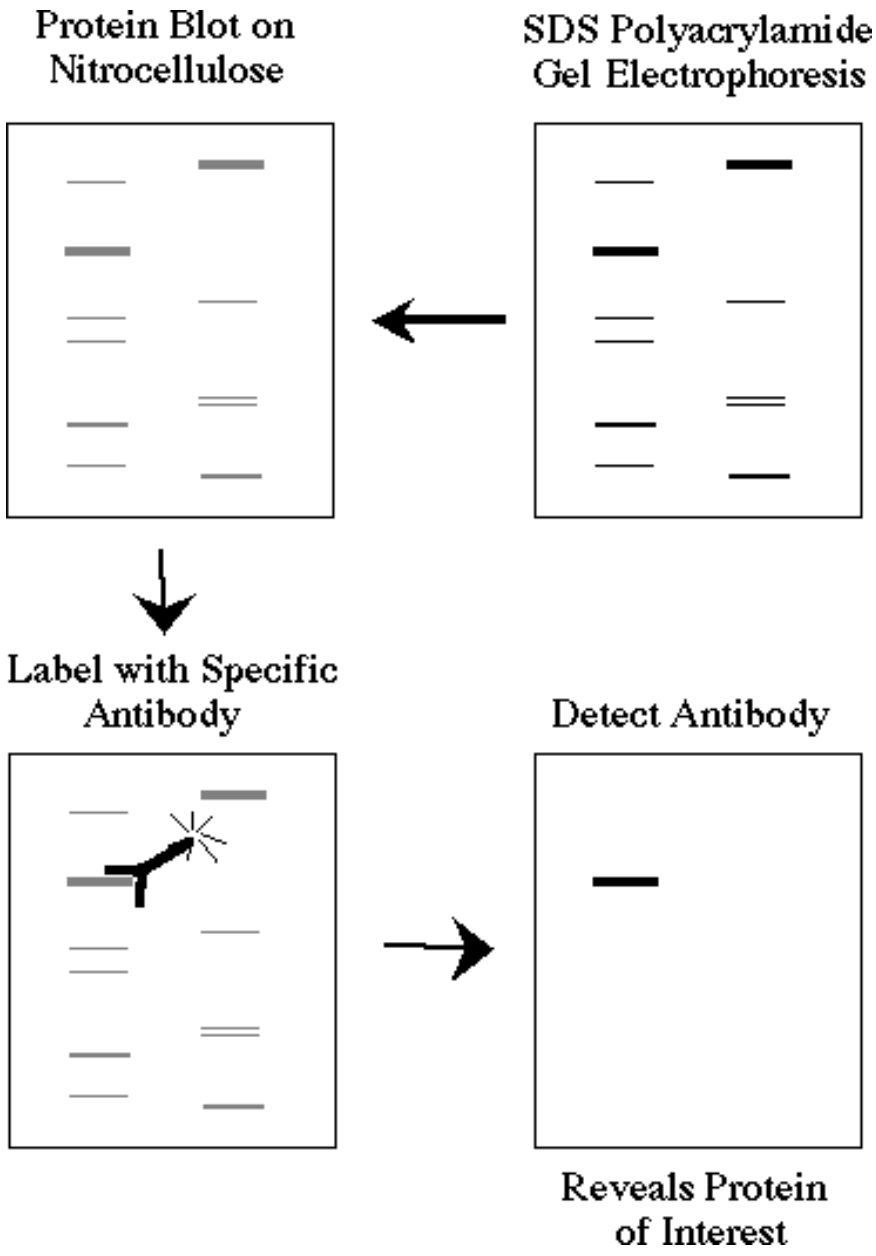
Following several rinses for removal of non-specifically bound Ab1, the Ab1-antigen complex on the nitrocellulose sheet is incubated with a **second antibody (Ab2)**, which specifically recognizes the **Fc domain of the primary antibody** and binds it.

Ab2 is radioactively labeled, or is covalently linked to a reporter enzyme, which allows to visualize the **protein-Ab1-Ab2 complex**



SDS PAGE




Protein Blot on Nitrocellulose
SDS Polyacrylamide Gel Electrophoresis
Label with Specific Antibody
Detect Antibody
Reveals Protein of Interest

markers
sample 1 aduas
sample 2 aduas
sample 3 aduas
pure sample
sample 1 aduas
sample 2 aduas
sample 3 aduas
pure sample
markers