FORENSIC BIOCHEMISTRY: SOUTHERN AND WESTERN BLOT TECHNIQUES, DNA PROFILING

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LECTURE CONTENT

- INTRODUCTION
- SOUTHERN BLOTTING
- WESTERN BLOTTING
- DNA PROFILING (DNA FINGERPRINTING)
- PARENTAL TESTING PROCEDURES

INTRODUCTION

- **Then the Lord said to Cain, "Where is your brother Abel?" He said," I do not know; am I my brother's keeper?" And the Lord said "What have you done? Listen; your brother's blood is crying out to me from the ground!"-*Genesis 4:9-10***
- **DNA: a true marker of identity**

FORENSIC SCIENCE

- **Definition – Relating to the use of science or technology in the investigation and establishment of facts or evidence in a court of law.**
- **Source – From the Latin word Forum. The Forum was a public gathering place during Roman times, where judicial activities and public business was conducted.**

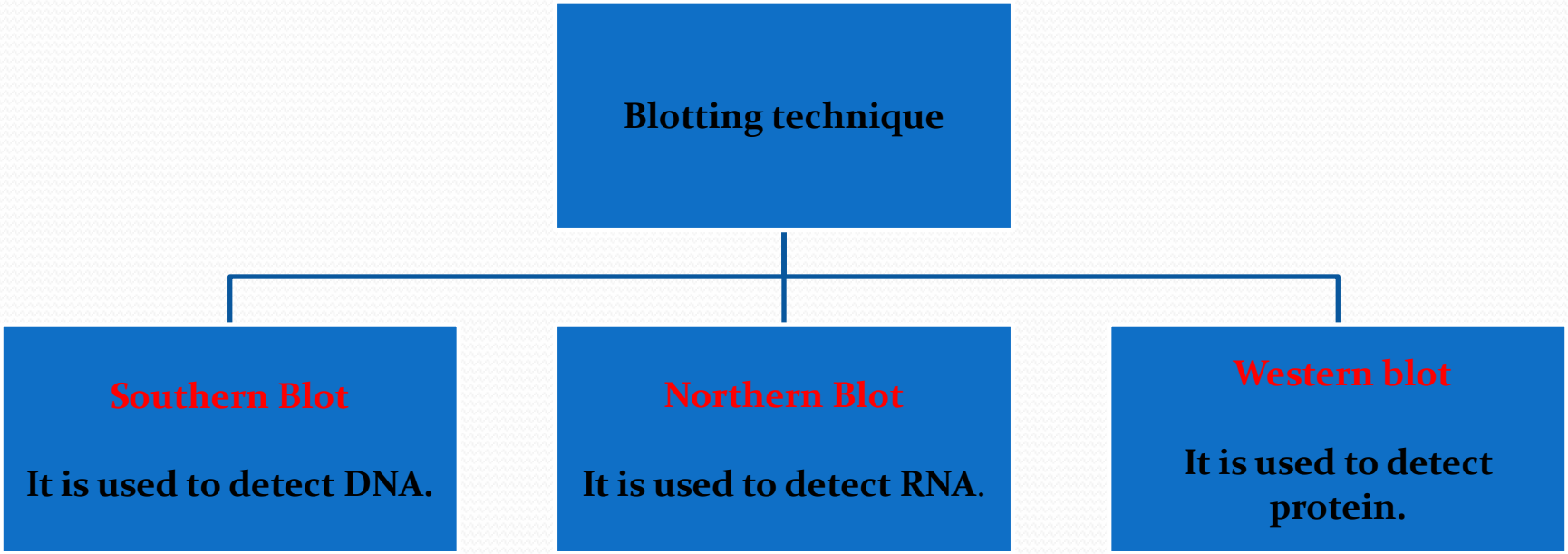
HISTORY OF FORENSIC SCIENCE

- **Many people believe that Arthur Conan Doyle was the first to popularize forensic science with his Sherlock Holmes novels. The first, *A Study in Scarlet*, was published in 1887.**

WHAT IS BLOTTING?

- **Blots are techniques for transferring DNA , RNA and proteins onto a carrier so they can be separated, and often follows the use of a gel electrophoresis. The Southern blot is used for transferring DNA, the Northern blot for RNA and the western blot for PROTEIN.**

TYPES OF BLOTTING TECHNIQUES



SOUTHERN BLOTTING

- **Professor Sir Edwin Southern, Professor of Biochemistry and Fellow of Trinity developed this method in 1975.**
- **Southern won the Lasker Award for Clinical Medical Research prize for the method of finding specific DNA sequences he developed this procedure at Edinburgh University more than 30 years ago. The technique is known as DNA transfer or 'Southern blotting'**

SOURTHERN BLOTTING CONT'D

- **This method Involves separation, transfer and hybridization.**
- **It is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples. The DNA detected can be a single gene, or it can be part of a larger piece of DNA such as a viral genome.**

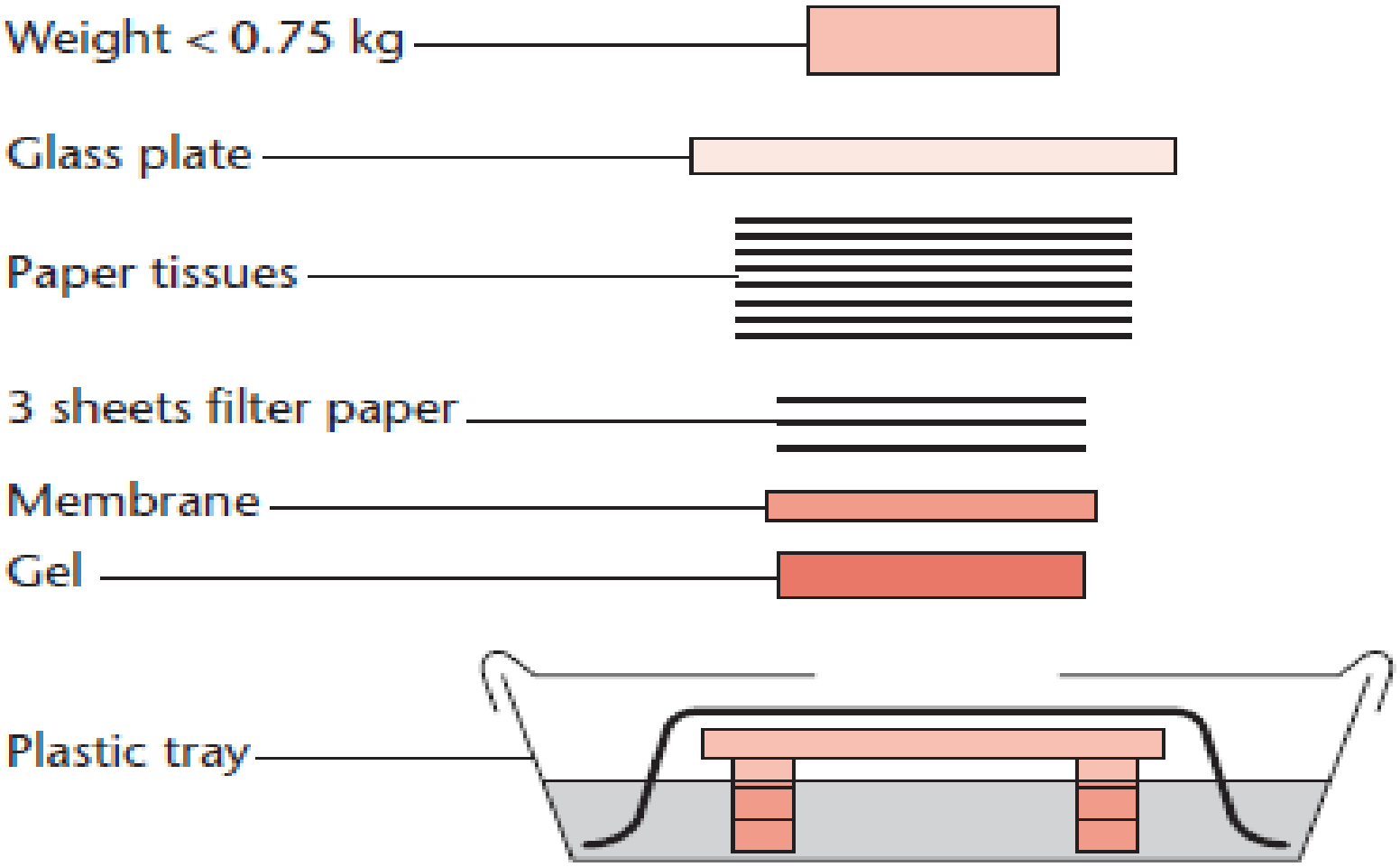
SOURTHERN BLOTTING CONT'D

- **Southern blotting combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size separated DNA to a filter membrane for probe hybridization.**
- **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 OF SOURTHERN BLOTHING

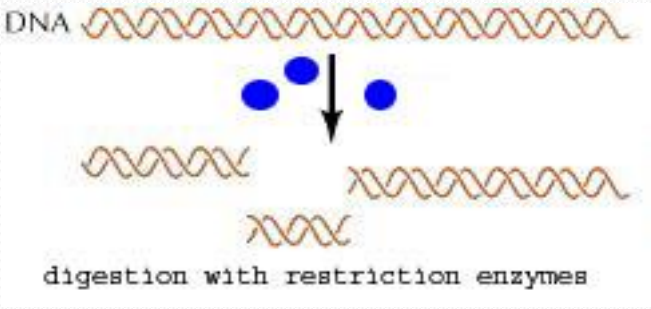
1. The mixture of molecules is separated.
2. The molecules are immobilized on a matrix.
3. The probe is added to the matrix to bind to the molecules.
4. Any unbound probes are then removed.
5. The place where the probe is connected corresponds to the location of the immobilized target molecule

BLOTTING APPARATUS

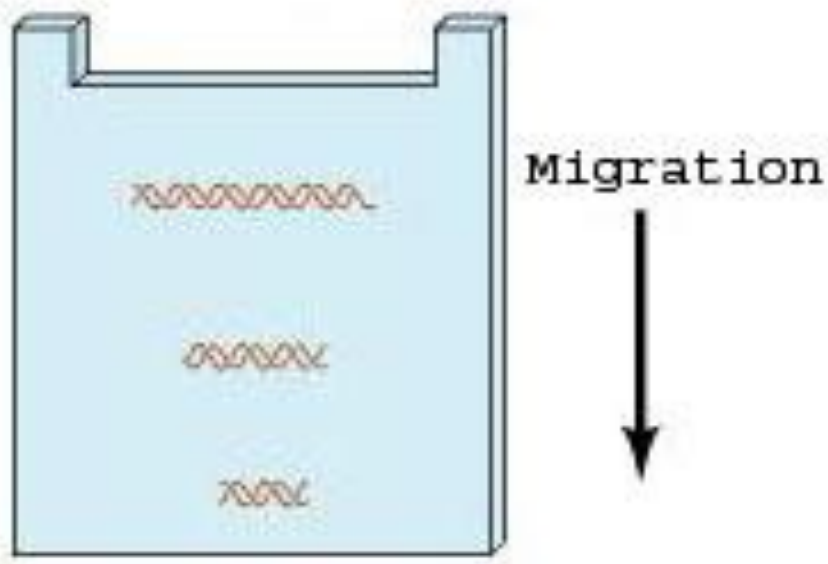


STEPS IN SOURTHERN BLOTTING

1. **Digest the DNA with an appropriate restriction enzyme.**

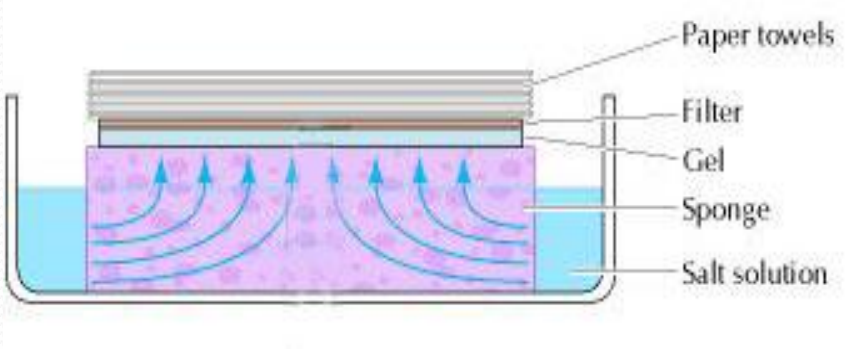


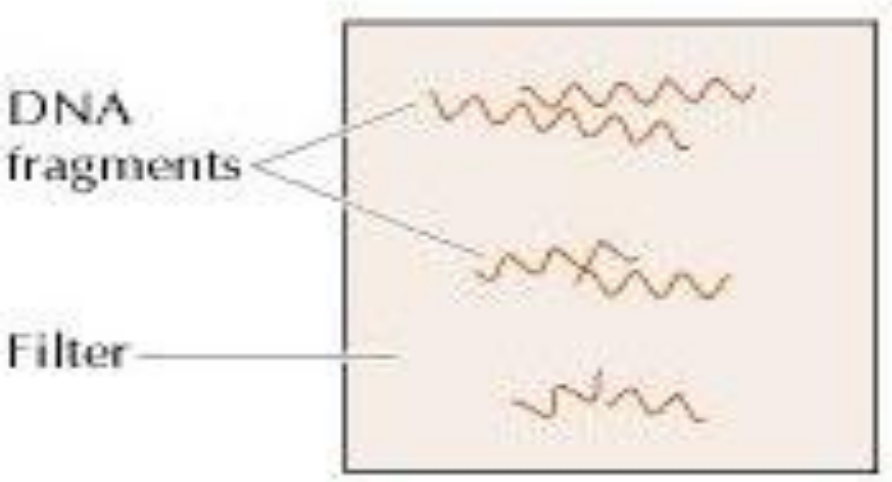
2. **The complex mixture of fragments is subjected to gel electrophoresis to separate the fragments according to size.**



CONT'D

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

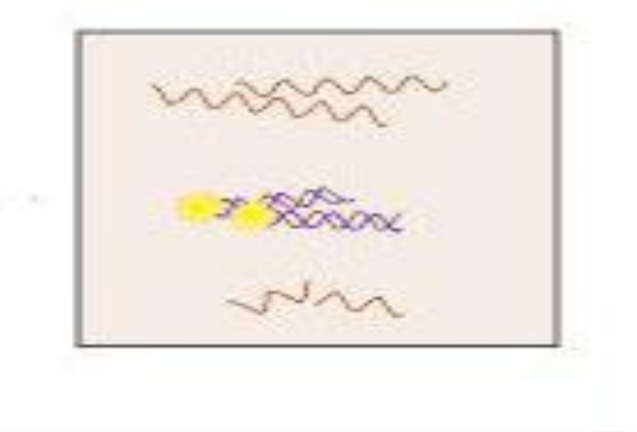




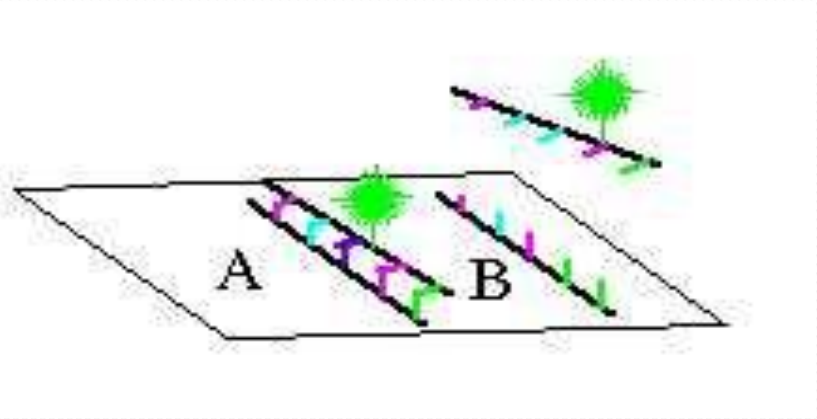
4. This procedure preserves the distribution of the fragments in the gel, creating a replica of the gel on the filter.

CONT'D

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

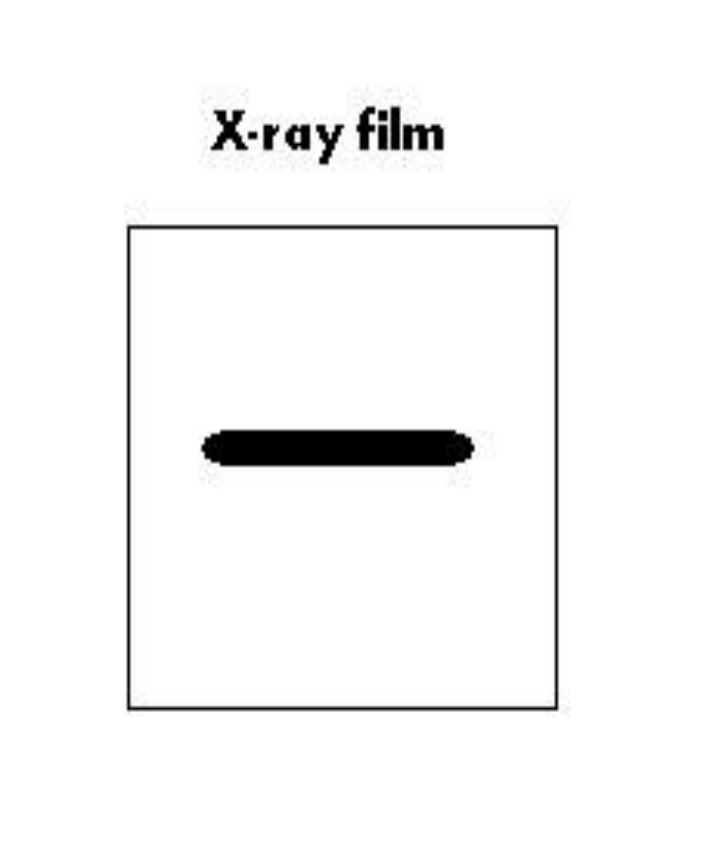


- The probe hybridizes to the complementary DNA restriction fragment.



CONT'D

6. 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.



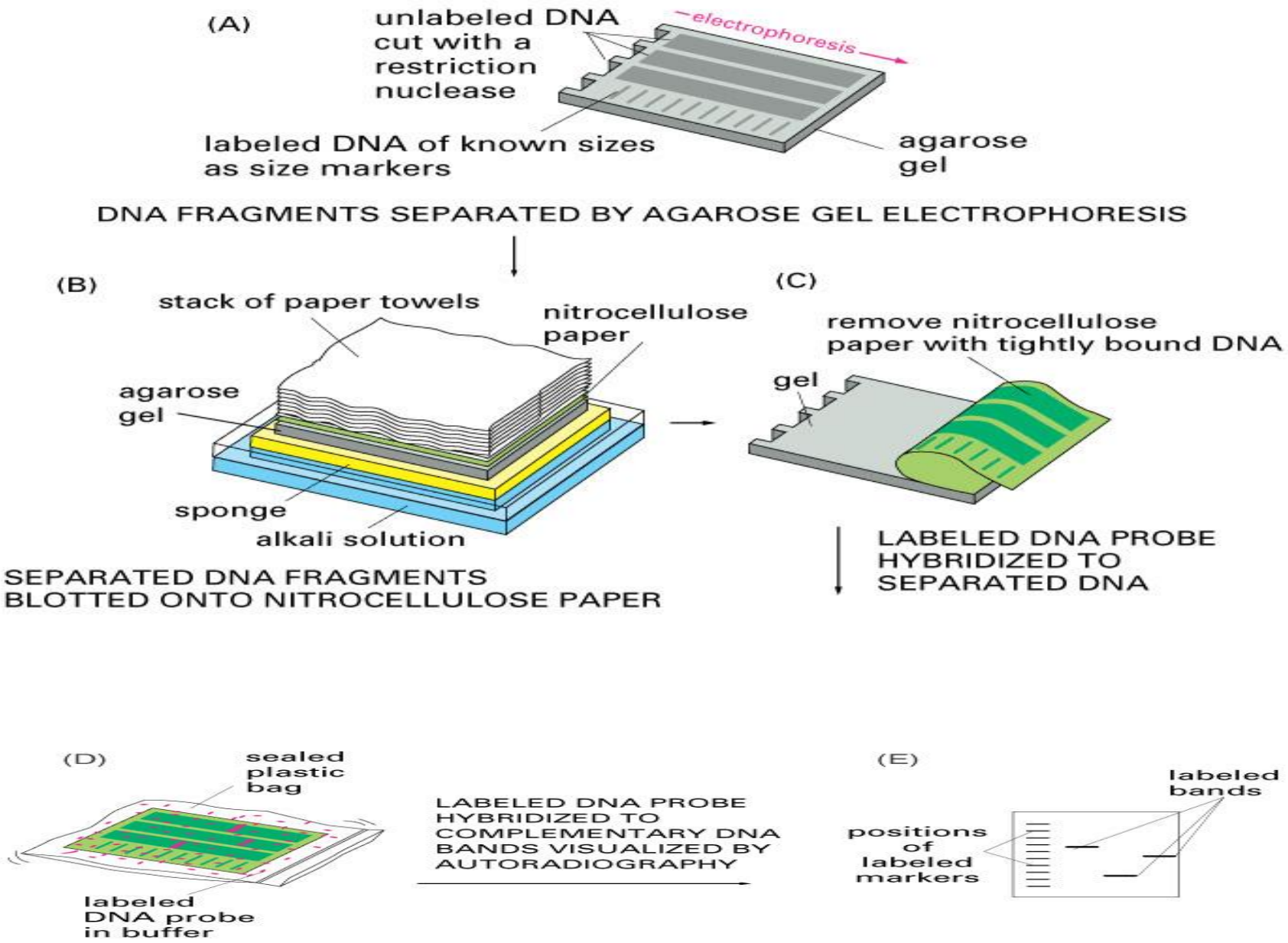


Figure 10-14 part 2 of 2 **Essential Cell Biology, 2/e. (© 2004 Garland Science)**

APPLICATIONS

- Southern blots are used in gene discovery , mapping, evolution and development studies, diagnostics and forensics (It is used for DNA fingerprinting, preparation of RFLP maps)
- identification of the transferred genes in transgenic individuals, etc
- Southern blots allow investigators to determine the molecular weight of a restriction fragment and to measure relative amounts in different samples.
- Southern blot is used to detect the presence of a particular bit of DNA in a sample
- analyze the genetic patterns which appear in a person's DNA.

WESTERN BLOTTING

- Western blotting, also known as immunoblotting or protein blotting, is a technique used to detect the presence of a specific protein in a complex protein mixture
- It is a core technique in cell biology, molecular biology, virology and others
- Western blots have become one of the most common analytical tools for the
  - Detection of viral proteins
  - characterization of monoclonal and Polyclonal antibody preparations
  - and in determining the specificity of the immune response to viral antigens

WESTERN BLOTTING

- The Western blotting procedure relies upon three key elements to accomplish this task:
  - The separation of protein mixtures by size using gel electrophoresis
  - The efficient transfer of separated proteins to a solid support;
  - and the specific detection of a target protein by appropriately matched antibodies
- Once detected, the target protein will be visualized as a band on a blotting membrane, X-ray film, or an imaging system

STEPS INVOLVED IN WESTERN BLOTTING

1. Sample preparation
2. Gel Electrophoresis
3. Blotting (or transfer)
4. Blocking
5. Antibody Probing
6. Detection

1- Sample Preparation

- All sources of protein, from single cells to whole tissues, biological fluids and proteins secreted in vitro, are open to analysis by Western blotting
- In most cases, the cells are harvested, washed, and lysed to release the target protein
- For best results, all these steps should be carried out on ice
- This will minimize proteolysis, dephosphorylation, and denaturation, since all begin to occur once the cells are disrupted

1- Sample Preparation

- **Numerous methods are available for disrupting cells and preparing their contents for analysis by Western blotting**

Detergent lysis	The membranes are solubilized, lysing cells and liberating their contents
Ultrasonication	The sound waves generate a region of low pressure, causing disruption of the membranes of cells
Freeze/thaw lysis	Cells are disrupted by the repeated formation of ice crystals and the method is usually combined with enzymatic lysis
Enzymatic digestion	The enzymes dissolve cell walls, coats, capsules, capsids, or other structures

- **To ensure that samples are in the proper range of detection for the assay, and so they can be compared on an equivalent basis, it is important to know the concentration of total protein in each sample**

2- Gel Electrophoresis- Gel Preparation

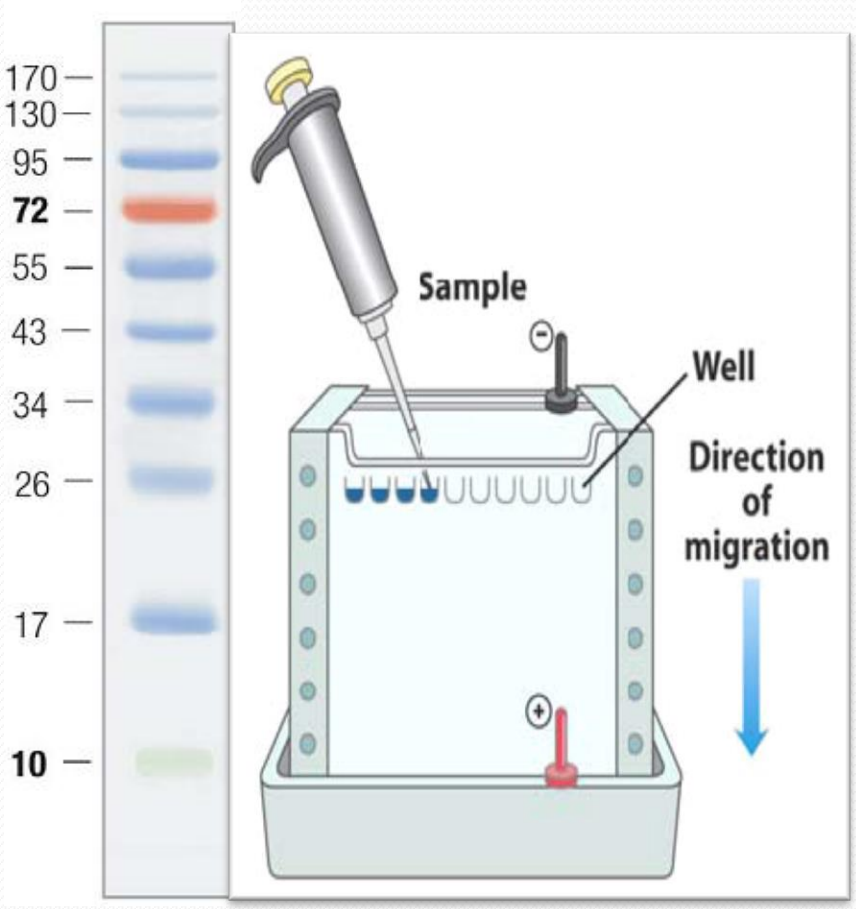
Reagent	8% (Running Gel)	5% (Stacking Gel)
Acrylamide/ Bisacrylamide (40%) *	4.0 mls	2.5 mls
1 M Tris-HCl	7.5 mls	7.5 mls
Distilled water	8.2 mls	9.7 mls
10% SDS	200 µl	200 µl
10% Ammonium Persulfate	100 µl	100 µl
TEMED (added last)	10 µl	10 µl
* = 19:1 w:w ratio of acrylamide to N,N'-methylene bis-acrylamide		

2- Gel Electrophoresis- Sample Buffer

- A sample of protein, is boiled in sample buffer (at 95°C for 5 minutes) which contains:
  - The β-mercaptoethanol reduces disulfide bonds
  - SDS disrupts protein secondary and tertiary structure
  - Glycerol to make samples sink into wells
  - Bromophenol Blue dye to visualize samples

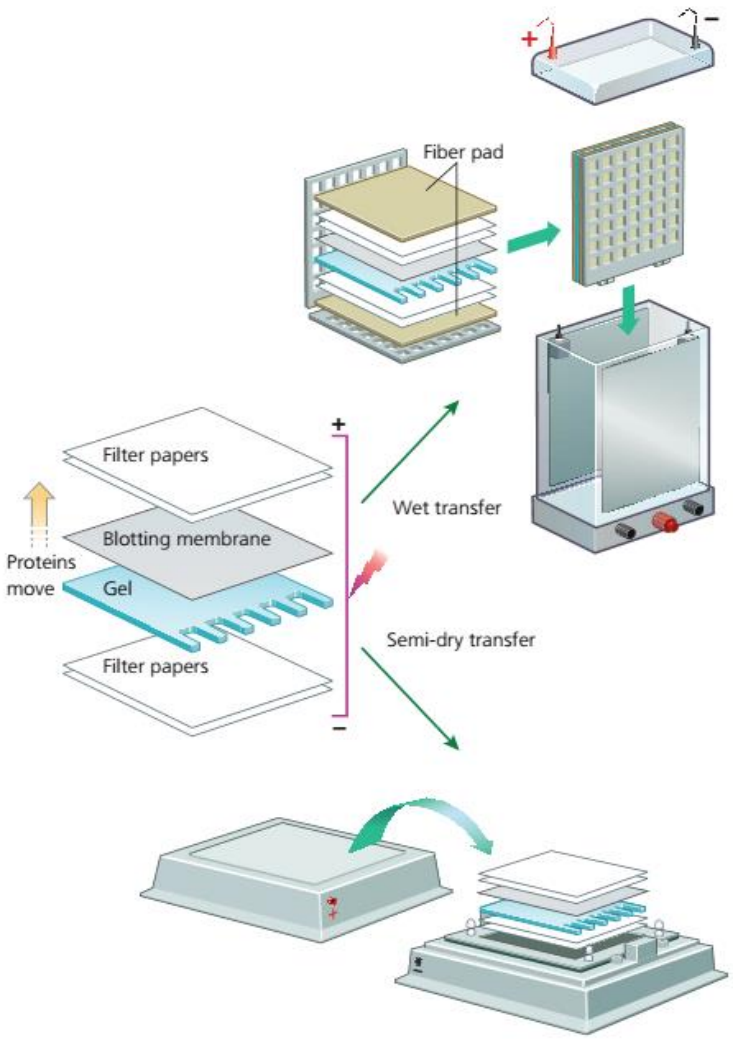
2- Gel Electrophoresis- Sample Buffer

- The end result has two important features:
  1. All proteins contain only primary structure and
  2. All proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.
- They migrate through a gel towards the positive pole at a rate proportional to their linear size



3- Blotting

- Following gel electrophoresis, the separated protein mixtures are transferred to a solid support for further analysis
- Transfer can be done in wet or semi-dry conditions
- Semi-dry transfer is generally faster
- Wet transfer is recommended for large proteins, >100 kD
- For both kinds of transfer, the membrane is placed next to the gel
- The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane

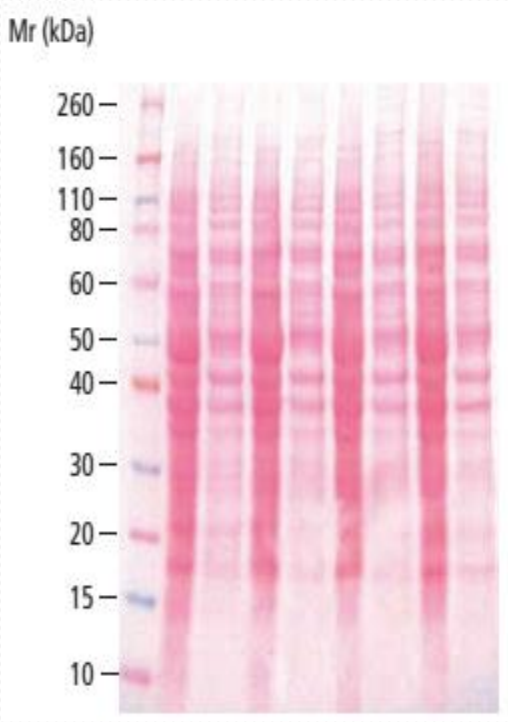


3- Blotting- Blotting Membranes

- The solid support onto which the separated proteins are transferred is usually of two types, both of which bind proteins with high affinity:
  - Nitrocellulose membrane
    - has excellent protein binding and retention capabilities
    - is brittle and thus it is usually less effective when blots need to be reused
  - Polyvinylidene fluoride (PVDF) membrane
    - PVDF demonstrates superior mechanical strength making it suitable for stripping/reprobing

3- Blotting- Visualization of proteins in membranes: Ponceau Red stain

- Ponceau Red is a reversible stain with poor sensitivity
- Ponceau S is compatible with both nitrocellulose and PVDF membranes
- This is a quick and easy way to visualize proteins transferred to membranes
- Ponceau S is easily removed with water and is regarded as a “gentle” treatment that does not interfere with subsequent immunological detection steps



4- Blocking

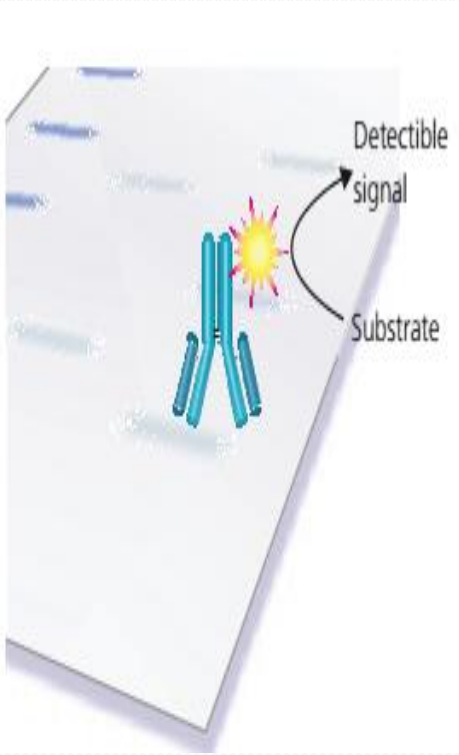
- Blocking is a very important step in the immunodetection phase of Western blotting because it prevents non-specific binding of antibody to the blotting membrane
- The most commonly used blocking solutions contain 3-5% BSA or non-fat dried milk in a solution of PBS (phosphate buffered saline) or TBS (tris buffered saline)
- Often, a small amount of Tween 20 detergent is added to blocking and washing solutions to reduce background staining, and the buffer is known as PBST or TBST

5- Antibody Probing

- Once the protein samples are separated and transferred onto a membrane, the protein of interest is detected and localized using a specific antibody
- The blot will be incubated in a dilute solution of antibody, usually for a few hours at room temperature or overnight at 4°C
- The antibody is diluted in wash buffer (PBST or TBST) or in the blocking solution, the choice depends upon the antibody
- Since antibody preparations vary in their levels of purity and specific binding properties, there will be differences in the level of dilution required
- The manufacturer's datasheet should provide dilution recommendations for a particular preparation

6- Detection with Substrate

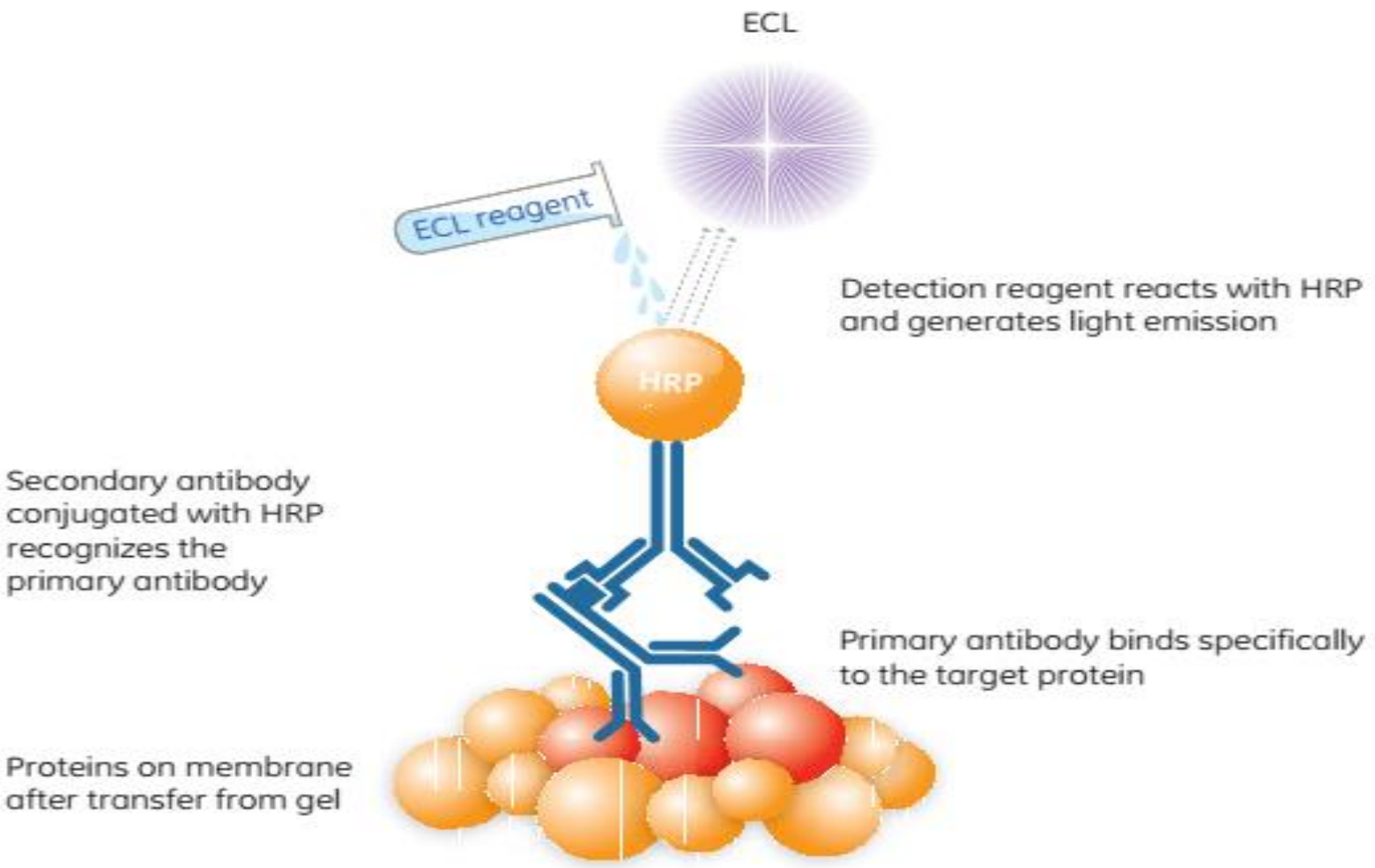
- The most common antibody label used in Western blots is HRP, a small, stable enzyme with high specificity and rapid turnover
- The signal is detected when HRP is exposed to a substrate solution in the final step of the immunodetection procedure
- Substrate solutions for Western blotting are chemical reagents that are acted upon by the enzyme to yield a signal that can be easily measured
- HRP label is typically detected with either colorimetric or chemiluminescent substrates



6- Detection with Substrate

- Colorimetric substrates for HRP {eg. Tetramethylbenzidine(TMB)} produce purple/black bands directly on the surface of the blot
- More routinely, HRP is used with ECL (enhanced chemiluminescence) detection
- For ECL detection, the substrate is luminol which is oxidized by HRP in the presence of H_2O_2 to produce light
- The emitted light is detected by exposing the Western blot to X-ray film, or by using a CCD camera for light capture
- The emitted light forms a band on the film, or on the screen of the imaging system, indicating where the HRP-labeled antibody has bound to the target protein
- ECL detection of HRP is extraordinarily sensitive, allowing for the visualization of picogram to femtogram amounts of target protein

Detection


ECL
ECL reagent
Detection reagent reacts with HRP and generates light emission
HRP
Secondary antibody conjugated with HRP recognizes the primary antibody
Primary antibody binds specifically to the target protein
Proteins on membrane after transfer from gel