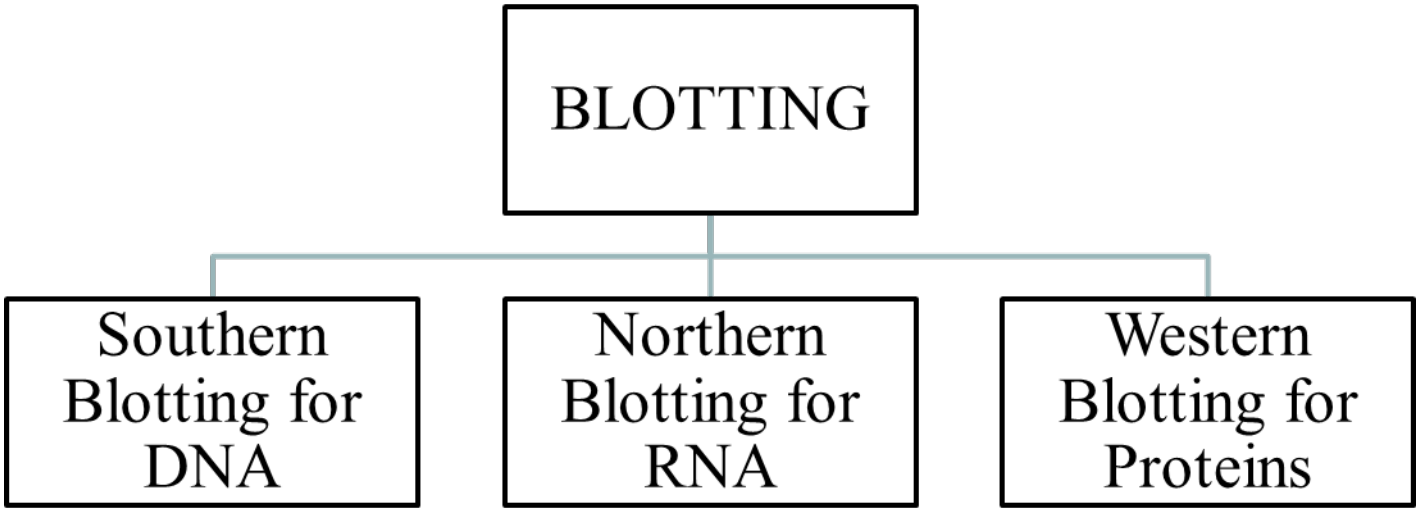
BLOTTING TECHNIQUES

AGENDA

- Introduction to Blotting techniques
- Transfer techniques
- Blotting Membranes
- Methods & Applications
  - Southern Blotting
  - Northern Blotting
  - Western Blotting

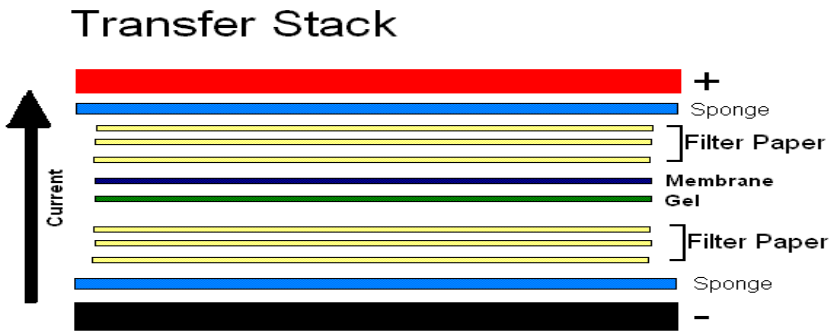
BLOTTING

- A molecular technique of transferring proteins, DNA or RNA, onto a carrier or membrane. Done after a gel electrophoresis, transferring the molecules from the gel onto the blotting membrane or adding the samples directly onto the membrane.



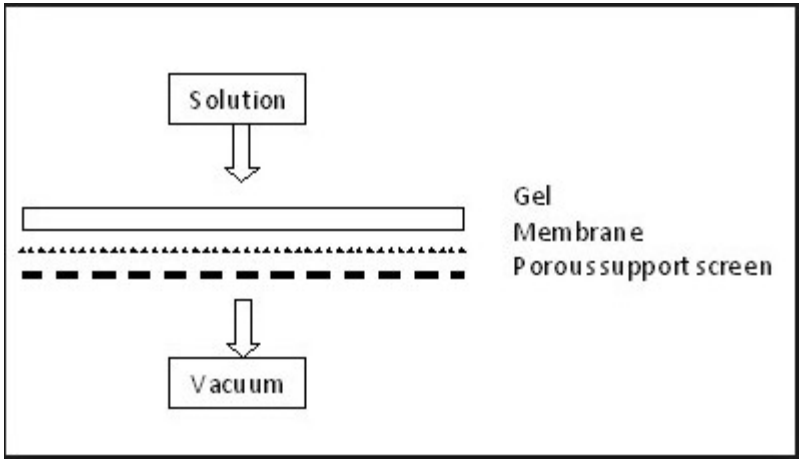
BLOTTING

- TRANSFER PROCESS:
  - **Capillary Method (Wet transfer method):** The biomolecules are transferred from the gel to the membrane using the capillary transfer method. The buffer moves from a high potential area to low potential area, carrying the biomolecules along with it. This facilitate movement from the gel to membrane.
  - **Electro-blotting:** Relies upon current and a transfer buffer solution to drive proteins or nucleic acids onto a membrane. In the electrophoretic or electro-blotting transfer technique, the gel and transfer paper are disposed between two electrodes which serve to produce an electric field there between. The proteins, nucleic acids, and like specimens are driven out of the gel and onto the transfer sheet by means of the electric field.



BLOTTING

- TRANSFER PROCESS:
  - **Vacuum blot Method:** In a vacuum blot, instead of using capillary forces, the force that will transfer the DNA is a vacuum. Vacuum blotting can be performed in two hours. It is therefore a much faster method of transferring DNA. The membrane can then be used in hybridization experiments.



BLOTTING MEMBRANES

- TYPES OF MEMBRANES USED IN SOUTHERN & NORTHERN BLOTTING:
  - **Nitrocellulose: Nitrocellulose membrane is a high quality membrane ideal for blotting of proteins and nucleic acids.**
    - **Composed of 100% pure nitrocellulose to provide high-quality transfer**
    - **Contains no support fabric or detergents**
    - **Compatible with commonly used transfer conditions and detection methods such as staining, immunodetection, fluorescence, or radiolabeling**
    - **Provides high-sensitivity with low background**
    - **Supplied in a pre-cut, pre-assembled membrane/filter paper sandwich for convenience**
  - **Nylon Membranes: particularly suitable for fast, high resolution electrotransfer of nucleic acids. The major features of Nylon membrane are:**
    - **Greater tensile strength and that DNA can be bound covalently by UV cross-linking. Nylon membranes can therefore be reprobed up to about 12 times without becoming broken or losing their bound DNA.**
    - **High sensitivity for superior detection of nucleic acids**
    - **High binding capacity for nucleic acids**
    - **Intrinsic hydrophobicity for rapid wetting**

BLOTTING MEMBRANES

- TYPES OF MEMBRANES USED IN SOUTHERN & NORTHERN BLOTTING:
  - **Aminobenzyloxymethyl Cellulosic Membranes: Modified cellulosic paper substituted with m-aminobenzyloxymethyl groups, ideal for blotting of RNA.**
    - **The amino moieties in the groups are activated prior to use by conventional diazotization techniques. The heterocyclic bases on nucleic acids and nitrogenous heterocyclic bases on proteins form covalent bonds with the phenyl ring containing the diazo group.**
    - **Most preffered used for Northern Blotting. RNA binding with Nitrocellulose membrane moiety is unstable.**
    - **Most stable binding of RNA in amino benzyloxymethyl filter paper.**

BLOTTING MEMBRANES

- TYPES OF MEMBRANES USED IN WESTERN BLOTTING:
  - **Nitrocellulose and Supported Nitrocellulose**: Mostly used for western blotting (Protein) and is still a popular membrane for this procedure. Protein binding to nitrocellulose is instantaneous, nearly irreversible. Nitrocellulose is easily wetted in water or transfer buffer and is compatible with a wide range of protein detection systems. Unsupported nitrocellulose is innately fragile and is not recommended for stripping and reprobing. Supported nitrocellulose is an inert support structure with nitrocellulose applied to it. The support structure gives the membrane increased strength and resilience. Supported nitrocellulose can withstand reprobing and autoclaving (121°C) and retains the ease of wetting and protein binding features of nitrocellulose.
  - **Polyvinylidene Difluoride (PVDF) Membranes:** ideal support for N-terminal sequencing, amino acid analysis, and immunoassay of blotted proteins (western blotting). PVDF retains proteins during exposure to acidic or basic conditions and in the presence of organic solvents. Exhibit better binding efficiency of electroblotted material in the presence of SDS in the transfer buffer. PVDF membranes must be wetted in 100% methanol prior to use, but once wet may be used with a transfer buffer that contains no methanol.

Southern Blotting

- This method Involves separation, transfer and hybridization. Technique developed by E.M. Southern in 1975
- 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 DNA

Methods in Southern Blotting

- Restriction endonucleases cut high-molecular-weight DNA strands into smaller fragments.
- The mixture of fragmented DNAs is separated in agarose get electrophoresis.
- The restriction fragments present in the gel are denatured with alkali and transferred onto a nitrocellulose filter or nylon membrane by blotting.

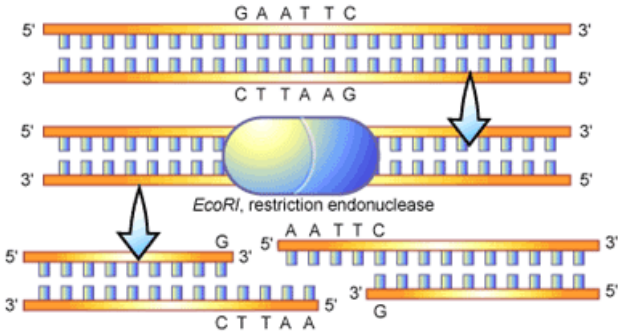


Fig 1: EcoRI Restriction Endonuclease

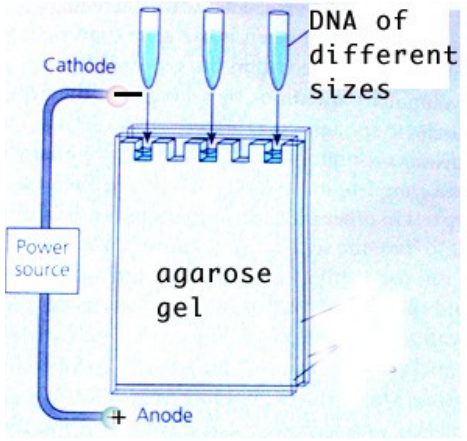


Fig 2: Agarose gel electrophoresis

Methods in Southern Blotting (contd…)

- This procedure preserves the distribution of the fragments in the gel creating a replica of the gel on the filter.
- A sheet of nitrocellulose or nylon membrane is placed on top of the gel. Pressure is applied evenly to the gel to ensure good and even contact between gel and membrane.

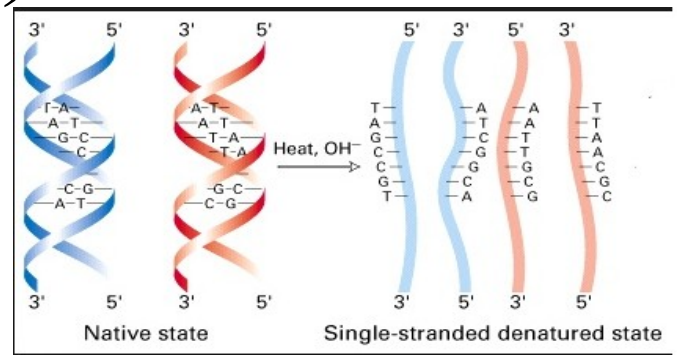
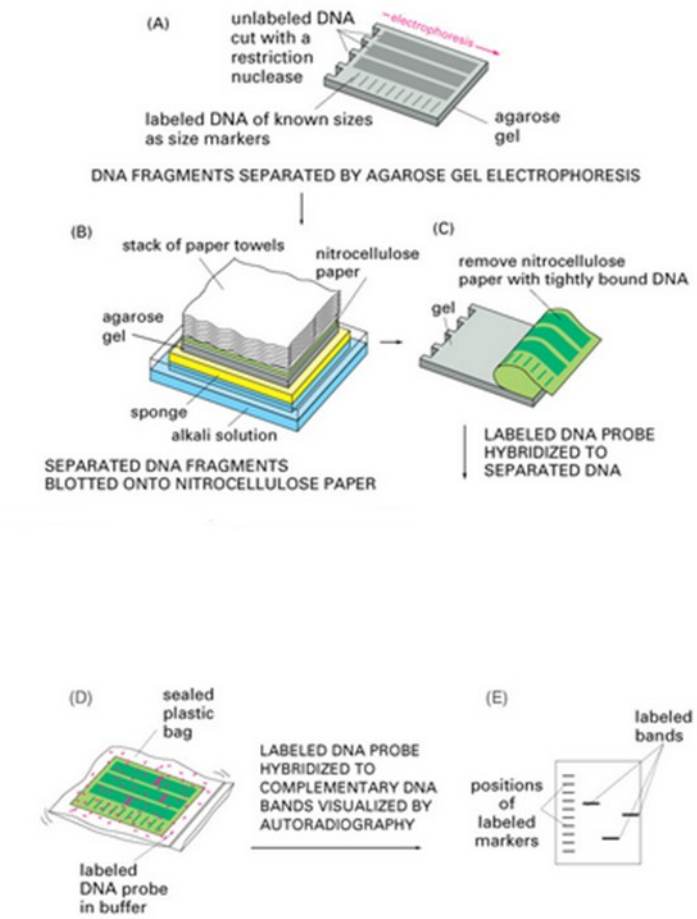


Fig 4: DNA Denaturation by NaOH

Methods in Southern Blotting (contd…)

- Membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.
- The probe is added to the matrix to bind to the molecules.
- Any unbound probes are then removed.
- The place where the probe is connected corresponds to the location of the immobilized target molecule.



Southern Blotting Applications

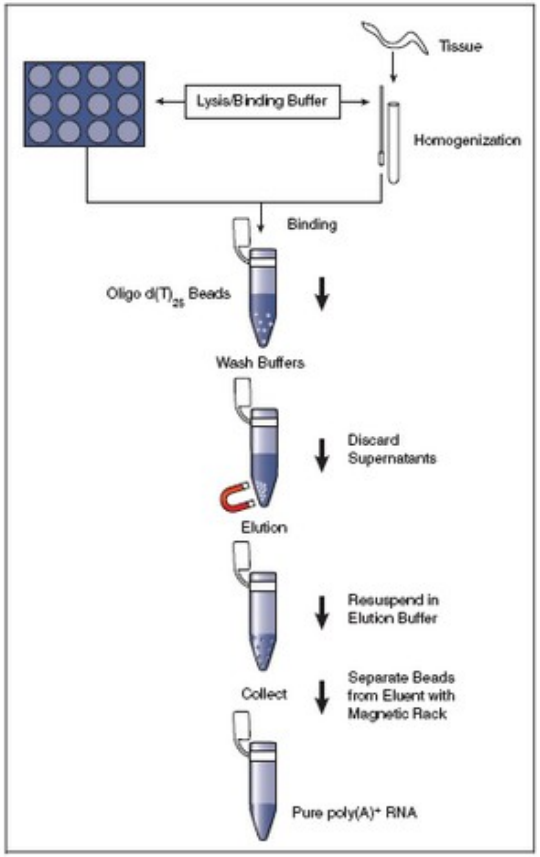
- Identify specific DNA sequences in DNA samples
- Isolate desired DNA for construction of rDNA.
- Identify mutations, deletions and gene rearrangements.
- In GMOs, used for testing to ensure that a particular section of DNA of known genetic sequence has been successfully incorporated into the genome of the host organism.
- Phylogenetic analysis
- To determine the number of copies of a particular DNA sequence
- In DNA Fingerprinting for:
  - Paternity & Maternity tests
  - Forensics
  - Personal/ Bio identification

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 in 1979 and was named such by analogy to Southern blotting.
- Northern blot analysis reveals information about RNA identity, size, and abundance, allowing a deeper understanding of gene expression levels.
- Northern blotting involves the use of electrophoresis to separate RNA samples by size and detection with a hybridization probe complementary to part of or the entire target sequence.

Northern Blotting Method

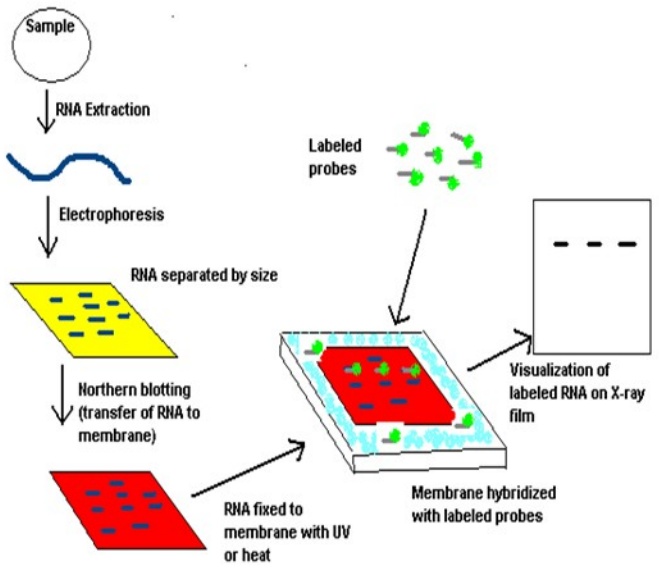
- Extraction of total RNA from a homogenized tissue sample or from cells.
- mRNA isolated through the use of oligo (dT) cellulose chromatography to isolate only those RNAs with a poly(A) tail.
- RNA samples are then separated by gel electrophoresis.
- Transferred to a nylon membrane through a capillary or vacuum blotting system.



Facilitate the isolation of poly(A)+ RNA using the Magnetic mRNA Isolation kit

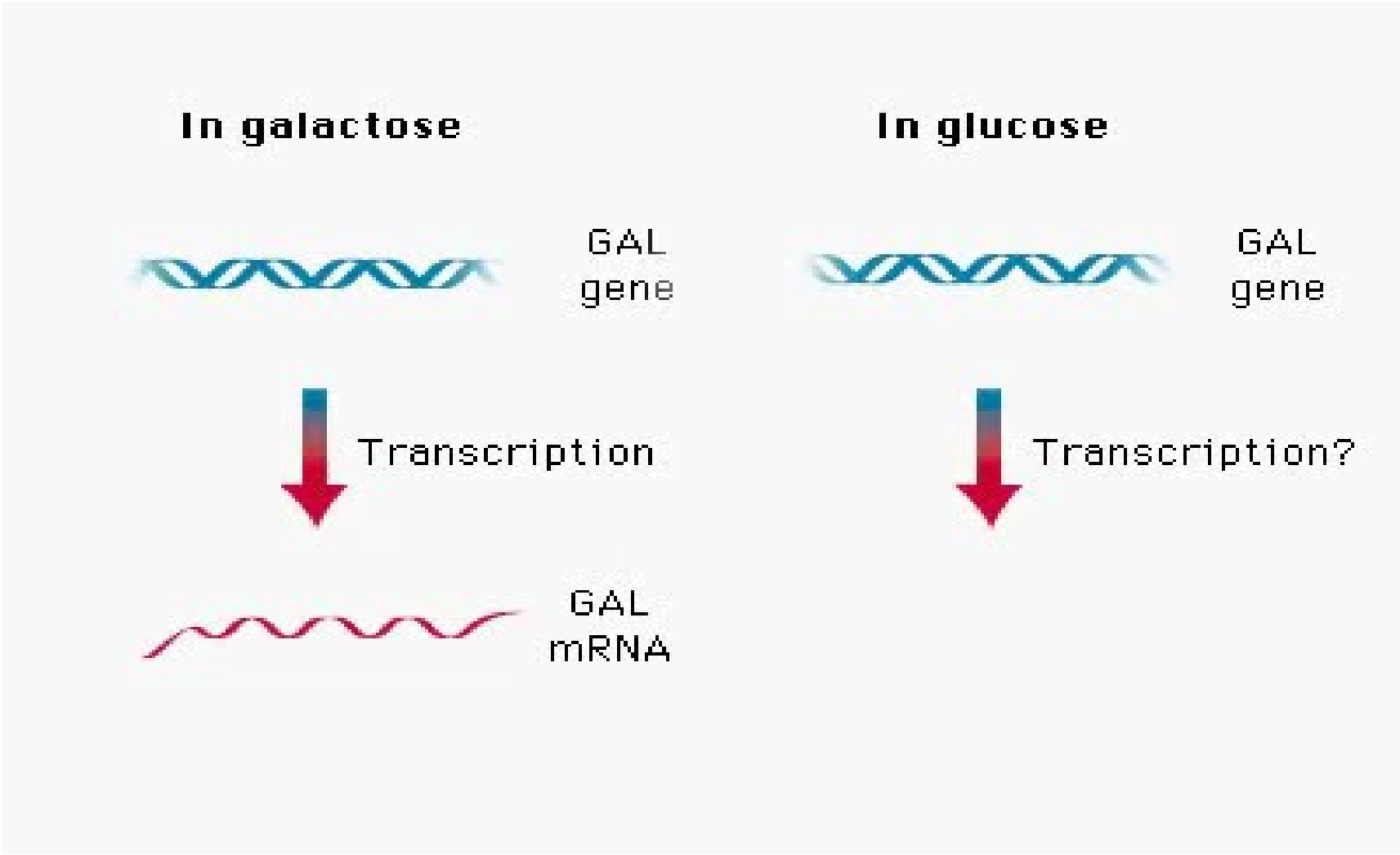
Northern Blotting Method

- A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them.
- Once RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane.



Flow diagram outlining the general procedure for RNA detection by northern blotting.

Northern Blotting Video



Northern Blotting Application

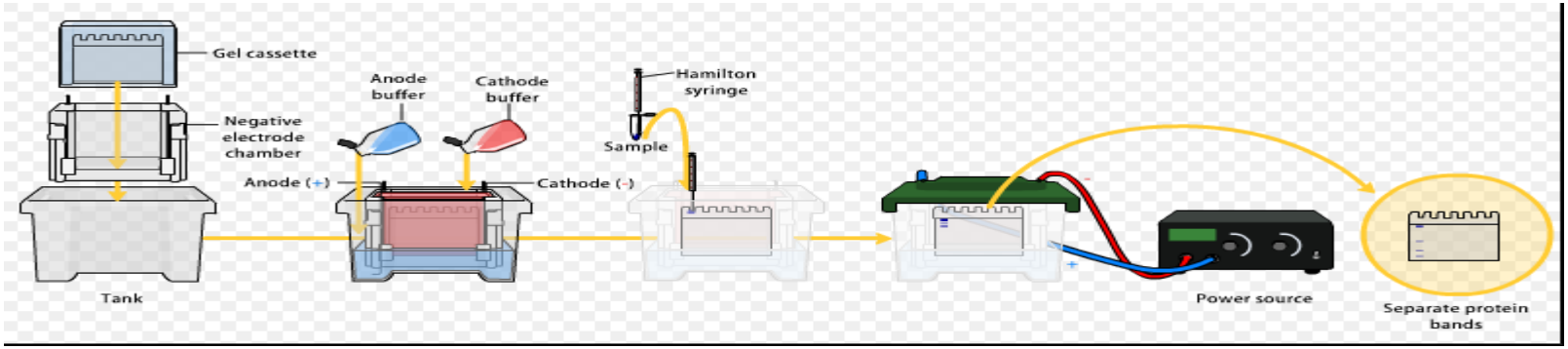
- Observe a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment.
- Used to show overexpression of oncogenes and down regulation of tumor-suppressor genes in cancerous cells.
- Detecting a specific mRNA in sample, used for screening recombinants which are successfully transformed with transgene.
- mRNA splicing studies

Western Blotting

- Detect specific proteins in a sample of tissue homogenate or extract using labelled antibodies.
- The method originated in the laboratory of Harry Towbin at the Friedrich Miescher Institute, Switzerland in 1979.
- The name western blot was given to the technique by W. Neal Burnette and is a play on the name Southern blot.

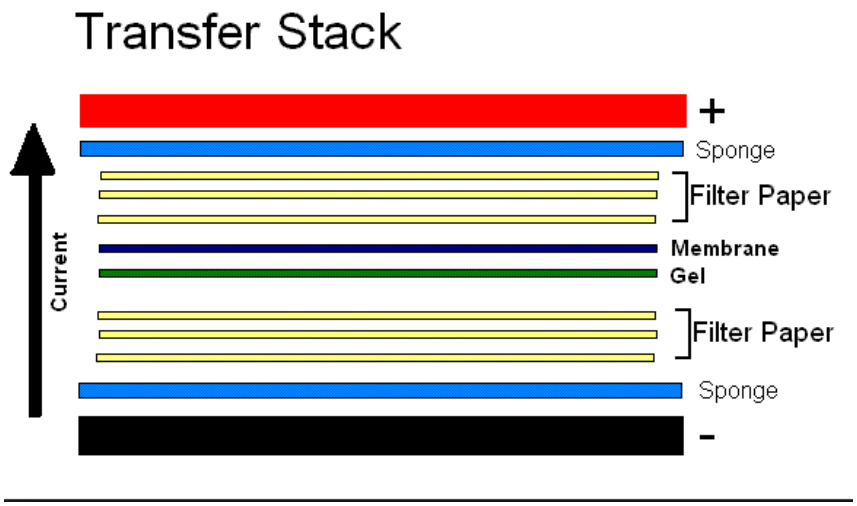
Western Blotting Method

- Proteins of the sample are separated by gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors.
- Most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight.



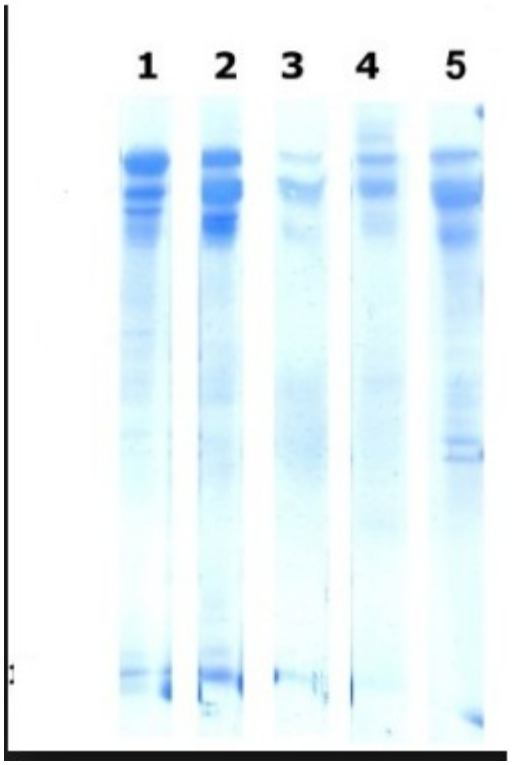
Western Blotting Method (contd.)

- Proteins are transferred from the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF) for accessibility to antibodies.
- Transferring by electroblotting uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel.



Western Blotting Method (contd.)

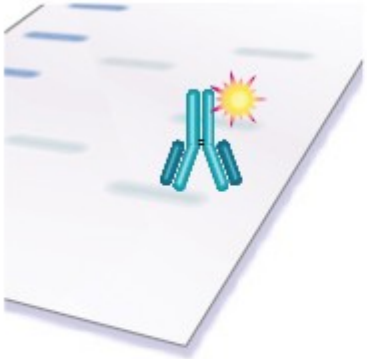
- Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.
- The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie Brilliant Blue.
- Blocking of non-specific binding to the membrane by dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk Tris-Buffered Saline (TBS) with a minute percentage (0.1%) of detergent such as Tween 20 or Triton X-100.



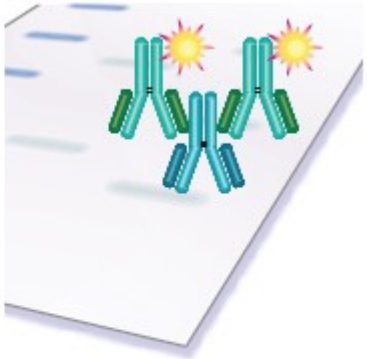
Coomassie stained PVDF membrane

Western Blotting Method (contd.)

- The membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate, this enzyme drives a colourimetric reaction and produces a color.



Direct detection uses a labeled primary antibody to identify the target protein.



Indirect detection uses an unlabeled primary followed by labeled secondary antibodies.

References:

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Western Blotting Applications

- The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample (ELISA).
- Technique for the detection and analysis of proteins.
- Diagnostics of various diseases (viral and autoimmune disease included)