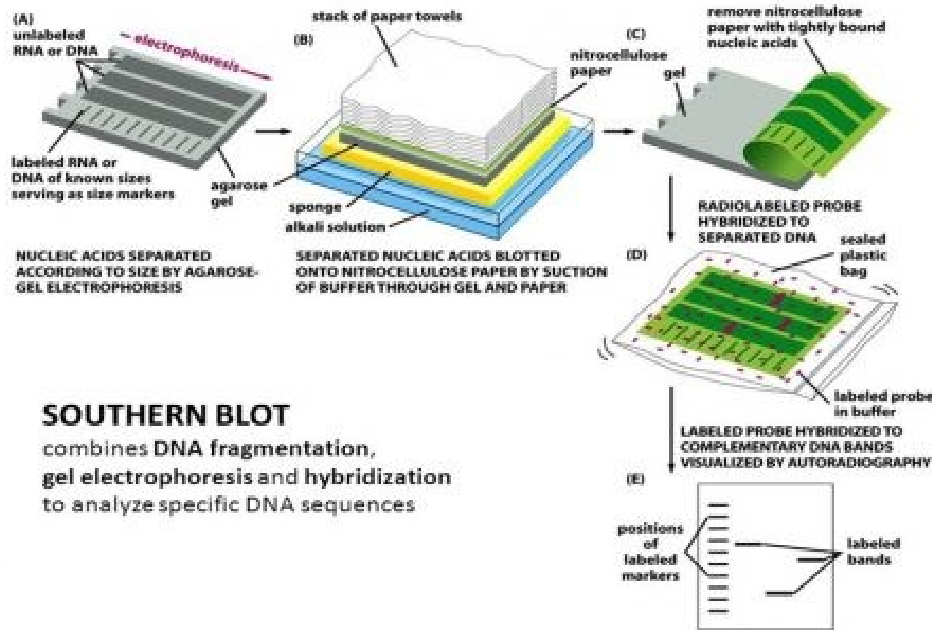


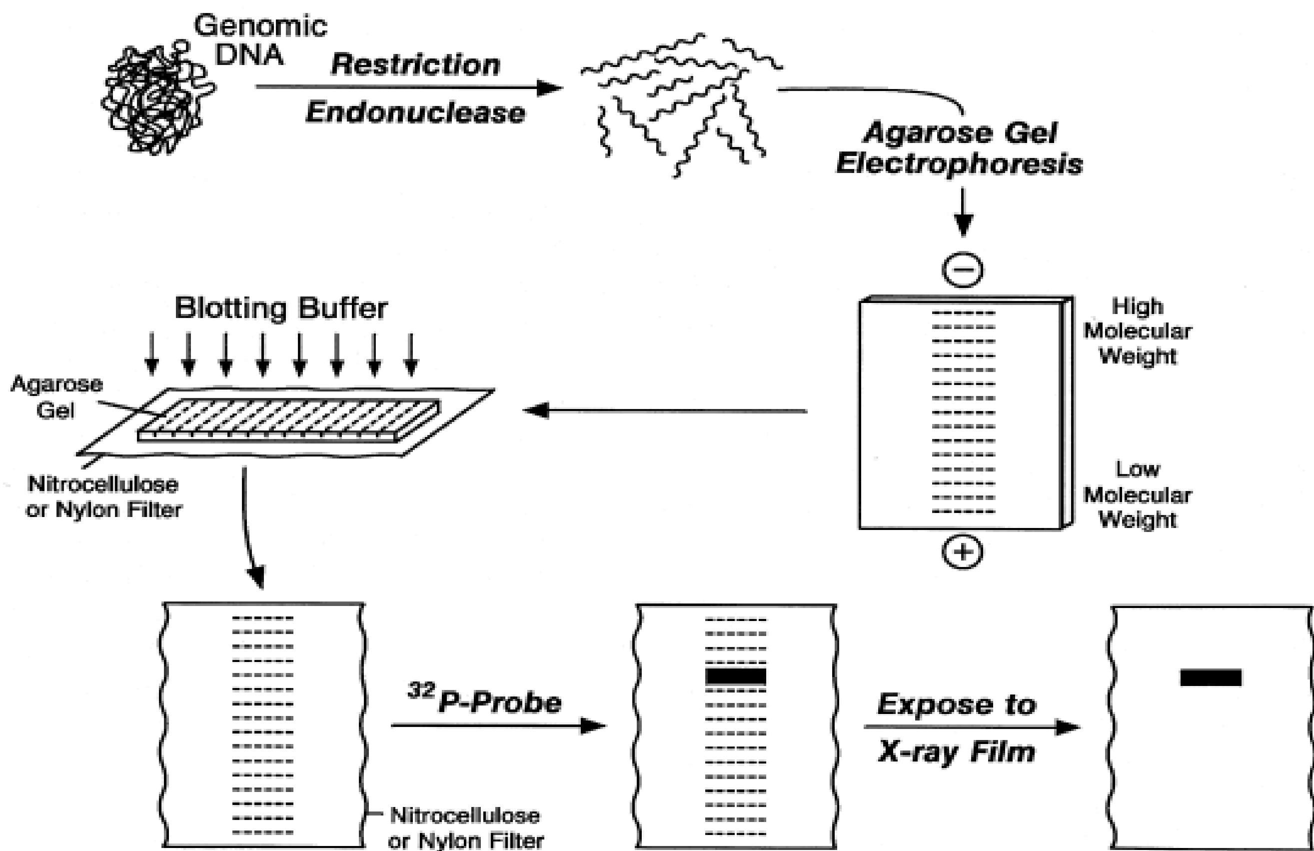
Southern Blotting

Principle:

- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/ Steps

1. Restriction digest: by RE enzyme and amplification by PCR
2. Gel electrophoresis: SDS gel electrophoresis
3. Denaturation: Treating with HCl and NaOH
4. Blotting
5. Baking and Blocking with casein in BSA
6. Hybridization using labelled probes
7. Visualization by autoradiogram



Step I: Restriction digest

- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments
- The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

- The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.
- DNA strands get separated

Step IV: Blotting

- The separated strands of DNA is then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

- The DNA bound to membrane is then treated with labelled probe
- The labelled probe contains the complementary sequences to the gene of interest
- The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Application of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting
3. Used for paternity testing, criminal identification, victim identification
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism
6. To identify mutation or gene rearrangement in the sequence of DNA
7. Used in diagnosis of disease caused by genetic defects
8. Used to identify infectious agents

Western Blot Definition

Western blot, also known as immunoblotting, is the process of separating proteins and identifying them in a complex biological sample.

- The use of polyacrylamide gel electrophoresis is a prerequisite for western blotting in order to separate proteins prior to their identification.
- The process of western blotting involves the transfer of proteins separated by SDS PAGE into an absorbent membrane. The proteins can then be identified on the membrane by different means.
- Western blotting has revolutionized the field of immunology with the use of antibody probes against membrane-bound proteins.
- The immunodetection of proteins has a wide application in biochemistry and other sciences as it can detect and characterize a multitude of proteins.
- The sensitivity of the process depends on the efficiency of transfer retention of proteins during processing and the final detection.
- Western blotting or protein blotting depends on the specificity of interaction between the protein of interest and the probe used for the detection of the protein.
- Unlike Southern blotting that utilizes radio-labeled nucleic acid probes, western blotting usually uses a second antibody tagged with an enzyme.
- Western blotting has a number of advantages over other similar techniques as the process only requires the use of a small amount of reagents, and the same protein transfer can be used for multiple analyses.

Principle of Western Blot

- The principle of western blotting is the interaction between the proteins and the probes used for the detection of the proteins.
- The proteins used for western blotting are separated by gel electrophoresis to obtain them on a gel matrix.
- The proteins are then transferred to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane, where they are immobilized. The transfer of the protein is known as blotting.
- The protein on the membrane can either be detected by the use of a reporter-labeled primary antibody directed against the protein or a reporter-labeled secondary antibody directed at the primary antibody.
- The reporter or probe present on the antibody can be an enzyme that produces a color reaction or a luminescent signal at the antigen-antibody binding site that produces a fluorescent signal in the presence of a particular substrate.
- The signal or color generated by the probe requires a detection system that is appropriate for the signal or intensity generated.

Procedure of Western Blot

The process of western blotting consists of the following steps;

1. Sample Preparation

- The most commonly used samples for western blot are cell lysates which are collected by the process of extraction.
- The extraction can be achieved by different means like mechanical destruction, chemical extraction, or the use of enzymes.
- The extraction of often performed at cold temperature in the presence of protease inhibitors in order to prevent the denaturation of the proteins.

2. Gel Electrophoresis

- The protein sample is diluted with the sample buffer and is heated and shaken for 10 minutes at 70°C.
- The sample is then centrifuged at 5000g.
- The gel case is removed from the pouch and is placed in the buffer tank against the rubber seal with the gel walls facing the inside of the tank reservoir.
- The running buffer is poured onto the upper reservoir while ensuring that no buffer leakage occurs on the lower tank.
- Each of the wells is then loaded with an equal volume of heat-denatured sample, and one of the lanes is reserved for the protein ladder.
- The lid is placed on the tank, and it is connected to the power supply.
- The run is allowed to run at 200 V constant for 50 minutes.

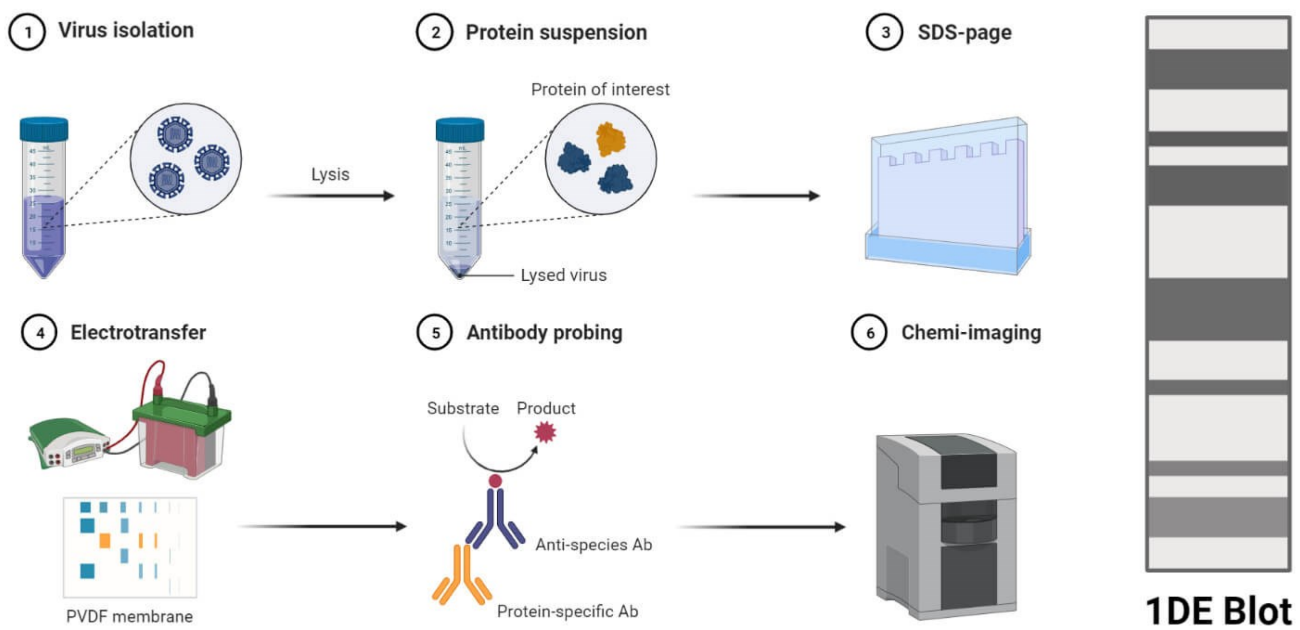
3. Protein Transfer

- The transfer buffer is prepared by adding 10% methanol to the buffer.
- The transfer case is taken and laid out. It is then covered with a transfer buffer.
- A foam sponge is taken and laid on the backside, over which goes the filter paper. These should be placed to ensure that both of them are wet and slightly submerged.
- The gel is taken out from the tank and placed on the wet filter paper.
- The nitrocellulose membrane is wet with the transfer buffer and is placed on top of the gel in a way that there are no bubbles between the gel and the membrane.
- The transfer case is placed into the transfer tank, which is further filled with transfer buffer.
- The tank is then connected to power at 100V for 1 hour.
- Once the transfer is complete, the transfer case is removed, and the nitrocellulose membrane is removed from the gel.

4. Immunodetection

- The membrane is washed with Tris-buffered saline for 5 minutes in a Petri dish.
- The 10% nonfat dry milk is mixed with the Tris buffer, and the membrane is covered with the mixture for 30 minutes at room temperature.
- The membrane is washed with the Tris buffer to remove any excess mixture remaining on the membrane.

- With the help of the forceps, the membrane is transferred to a new Petri dish onto which the primary antibody is added.
- The membrane with the antibody is incubated for 3 hours at room temperature. The membrane is washed after incubation with the Tris buffer.
- The membrane is transferred again to a new Petri dish, where a secondary HRP-conjugated antibody is added. The membrane is incubated for 1 hour. The concentration of secondary antibodies often remains at 1 μg/ml, but this also depends on the dilution.
- The membrane is washed again with Tris buffer to remove excess antibodies from the surface.
- The membrane is incubated with the substrate for 5 minutes, and the observation is made.



Applications of Western Blot

1. Western blotting is an excellent method with high sensitivity in order to detect a particular protein even in low quantity.
2. Western blotting has been used in the clinical diagnosis of different diseases. The confirmatory test for HIV involves a western blot by detecting anti-HIV antibodies in the serum.
3. The technique has been used to quantify proteins and other gene products in gene expression studies.
4. Since western blotting detects the proteins by their size and ability to bind to the antibody, it is appropriate for evaluating the protein expressions in cells and further analysis of protein fractions during protein purification.
5. Western blotting is also used for the analysis of different biomarkers like growth factors, cytokines, and hormones.

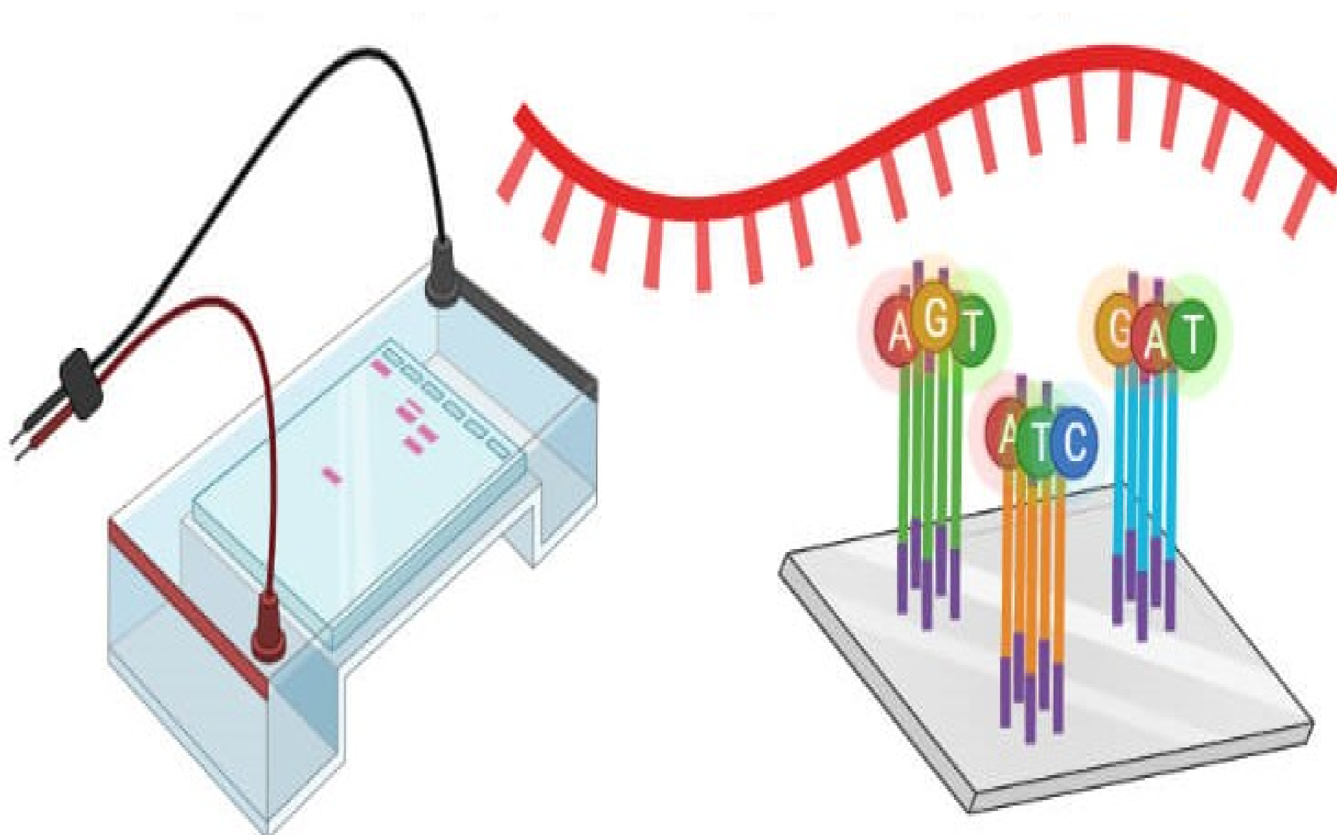
Limitations of Western Blot

1. Since it is a very sensitive process, any imbalance in the process can affect the results of the entire process.
2. In some cases, no bands or erroneous bands might be observed due to the insufficient transfer of the proteins.
3. The test can only be used as a semi-quantitative test as the estimation is not always precise.
4. The process is time-consuming and complex, thus can only be performed by well-trained personnel.
5. Western blotting can only be performed for proteins if the primary antibodies for the proteins are available.
6. Some antibodies might exhibit off-target effects by interacting with more than one protein in the sample.
7. The technique is a costly process with the cost of antibodies and expensive detection methods.
8. Small proteins might not be retained by the membrane, whereas larger proteins are difficult to transfer to the membrane.

Northern Blot Definition

Northern blot is a technique based on the principle of blotting for the analysis of specific RNA in a complex mixture.

- The technique is a modified version of the Southern Blotting, which was discovered for the analysis of DNA sequences.
- The detection of certain sequences of nucleic acids extracted from different types of biological samples is essential in molecular biology, which makes blotting techniques imperative in the field.
- The principle is identical to southern blotting except for the probes used for the detection as northern blotting detects RNA sequences.
- This technique provides information about the length of the RNA sequences and the presence of variations in the sequence.
- Even though the technique is primarily focused on the identification of RNA sequences, it has also been used for the quantification of RNA sequences.
- Since the discovery of the technique, several modifications have been made in the technique for the analysis of mRNAs, pre-mRNAs, and short RNAs.
- Northern blotting was employed as the primary technique for the analysis of RNA fragments for a long time; however, new, more convenient, and cost-effective techniques like RT-PCR have slowly replaced the technique.



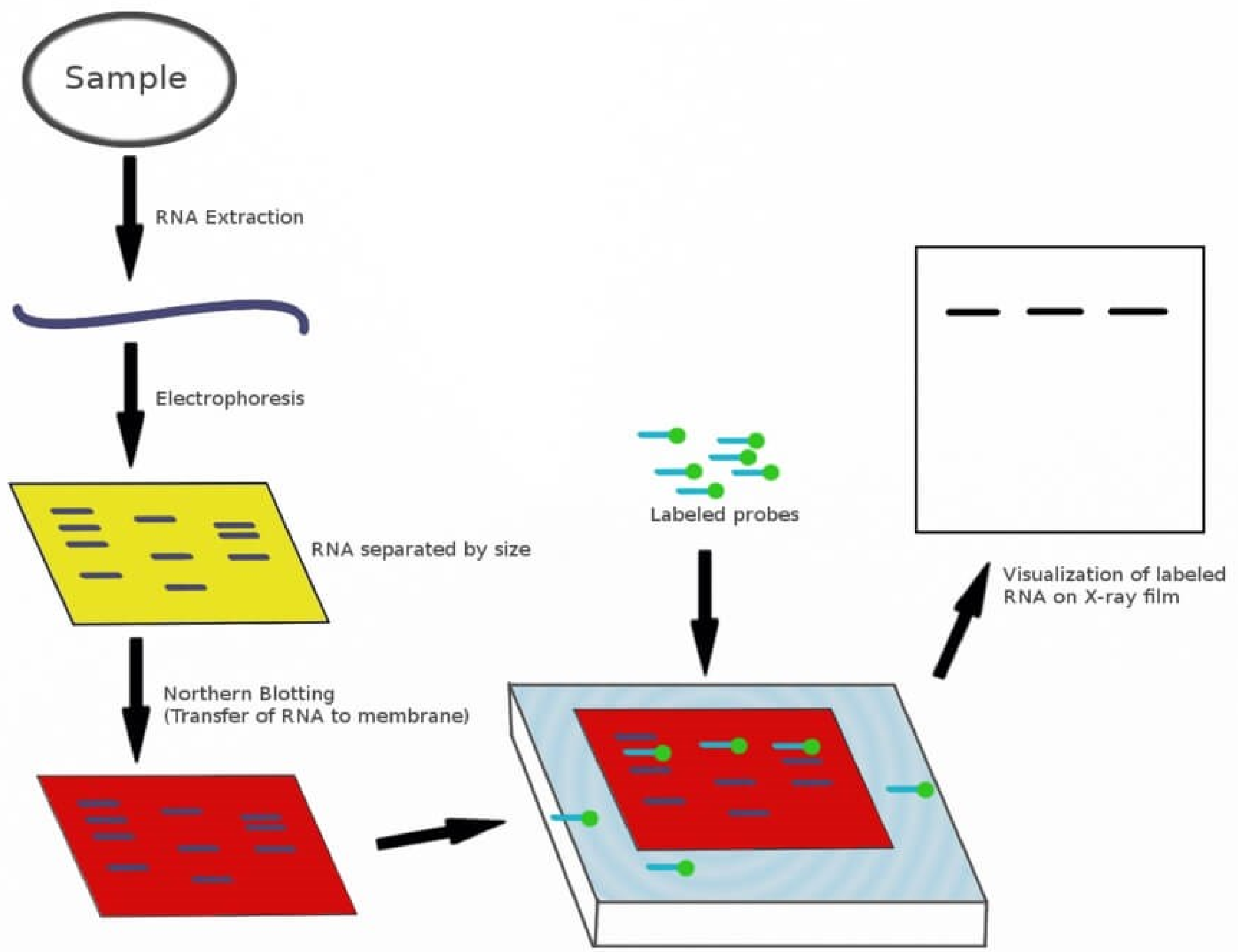
Principle of Northern Blot

- The principle of the northern blot is the same as all other blotting technique that is based on the transfer of biomolecules from one membrane to another.
- The RNA samples are separated on gels according to their size by gel electrophoresis. Since RNAs are single-stranded, these can form secondary structures by intermolecular base pairing. The electrophoretic separation of the RNA segments is thus performed under denaturing conditions.
- The separated RNA fragments are then transferred to a nylon membrane. Nitrocellulose membrane is not used as RNA doesn't bind effectively to the membrane.
- The transferred segments are immobilized onto the membrane by fixing agents. The RNA fragments on the membrane are detected by the addition of a labeled probe complementary to the RNA sequences present on the membrane.
- The hybridization forms the basis of the detection of RNA as the specificity of hybridization between the probe, and the RNA allows the accurate identification of the segments.
- Northern blot utilizes size-dependent separation of RNA segments and thus can be used to determine the sizes of the transcripts.

Procedure/Steps of Northern Blot

a. Separation of RNA on a denaturing gel

- The RNA gel solution is prepared by adding formaldehyde to the agarose solution.
- The cast is assembled, and the prepared denaturing gel is poured into the cast. As the gel begins to set, a comb with appropriate teeth is added to form wells.
- Once the gel is set, the comb is removed, and the gel is equilibrated with a running buffer for 30 minutes before running.
- 15 µg RNA sample is mixed with an equal volume of RNA loading buffer. Three µg of RNA markers are added in the same volume of RNA loading buffer.
- The samples are incubated at 65°C on a heating block for about 12-15 minutes.
- The samples are loaded to the equilibrated gel, and the first row of wells is filled with RNA markers.
- The gel is then run at 125V for about 3 hours.



b. Transfer of RNA from gel to the nylon membrane

- A nylon membrane is cut that is larger than the size of the denaturing gel, and a filter paper with the same size as the nylon membrane is also prepared.
- Once the electrophoresis process is complete, the RNA gel is removed from the tank and rinsed with water.
- An oblong sponge that is slightly larger than the gel is placed on a glass dish, and the dish is filled with SSC to a point so as to leave the soaked sponge about half-submerged in the buffer.
- A few pieces of Whatman 3mm papers are placed on top of the sponge and are wetted with SSC buffer.
- The gel is then placed on top of the filter paper and squeezed out to remove air bubbles by rolling a glass pipette over the surface.
- The nylon membrane prepared is wetted with distilled water on an RNase-free dish for about 5 minutes.
- The wetted membrane is placed on the surface of the gel while avoiding any air bubbles formation.
- The surface is further flooded with SSC, and a few more filter papers are placed on top of the membrane.
- A glass plate is placed on top of the structure in order to hold everything in place. The structure is left overnight to obtain an effective transfer.

c. Immobilization

- Once the transfer is complete, the gel is removed and rinsed with SSC, and allowed to dry.
- The membrane is placed between two pieces of filter paper and baked in a vacuum oven at 80°C for 2 hours.
- In some cases, the membrane can be wrapped in a UV transparent plastic wrap and irradiates for an appropriate time on a UV transilluminator.

d. Hybridization

- The DNA or RNA probes to be used are to be labeled to a specific activity of >108 dpm/μg, and unincorporated nucleotides are to be removed.
- The membrane carrying the immobilized RNA is wetted with SSC.
- The membrane is placed in a hybridization tube with the RNA-side-up, and 1 ml of formaldehyde solution is added.
- The tube is placed in the hybridization oven and incubated at 42°C for 3 hours.
- If the probe used is double-stranded, it is denatured by heating in a water bath or incubator for 10 minutes at 100°C.
- The desired volume of the probe is pipette into the hybridization tube and further incubated at 42°C.
- The solution is poured off, and the membrane is washed with a wash solution. The membrane is then observed under autoradiography.

Applications of Northern Blot

1. The technique can be used for the identification and separation of RNA fragments collected from different biological sources.
2. Northern blotting is used as a sensitive test for the detection of transcription of DNA fragments that are to be used as a probe in Southern Blotting.
3. It also allows the detection and quantification of specific mRNAs from different tissues and different living organisms.
4. Northern blotting is used as a tool for gene expression studies related to overexpression of cancer-causing genes, and gene expression during transplant rejects.
5. Northern blotting has been used as a molecular tool for the diagnosis of diseases like Crohn’s disease.
6. The process is used as a method for the detection of viral microRNAs that play important roles in viral infection.