6. Southern Blotting

Southern blotting is a technique for detecting specific **DNA** fragments in a complex mixture. The technique was invented in mid-1970s by Edward Southern. It has been applied to detect Restriction Fragment Length Polymorphism (**RFLP**) and Variable Number of Tandem Repeat (**VNTR**) Polymorphism. The latter is the basis of DNA fingerprinting.

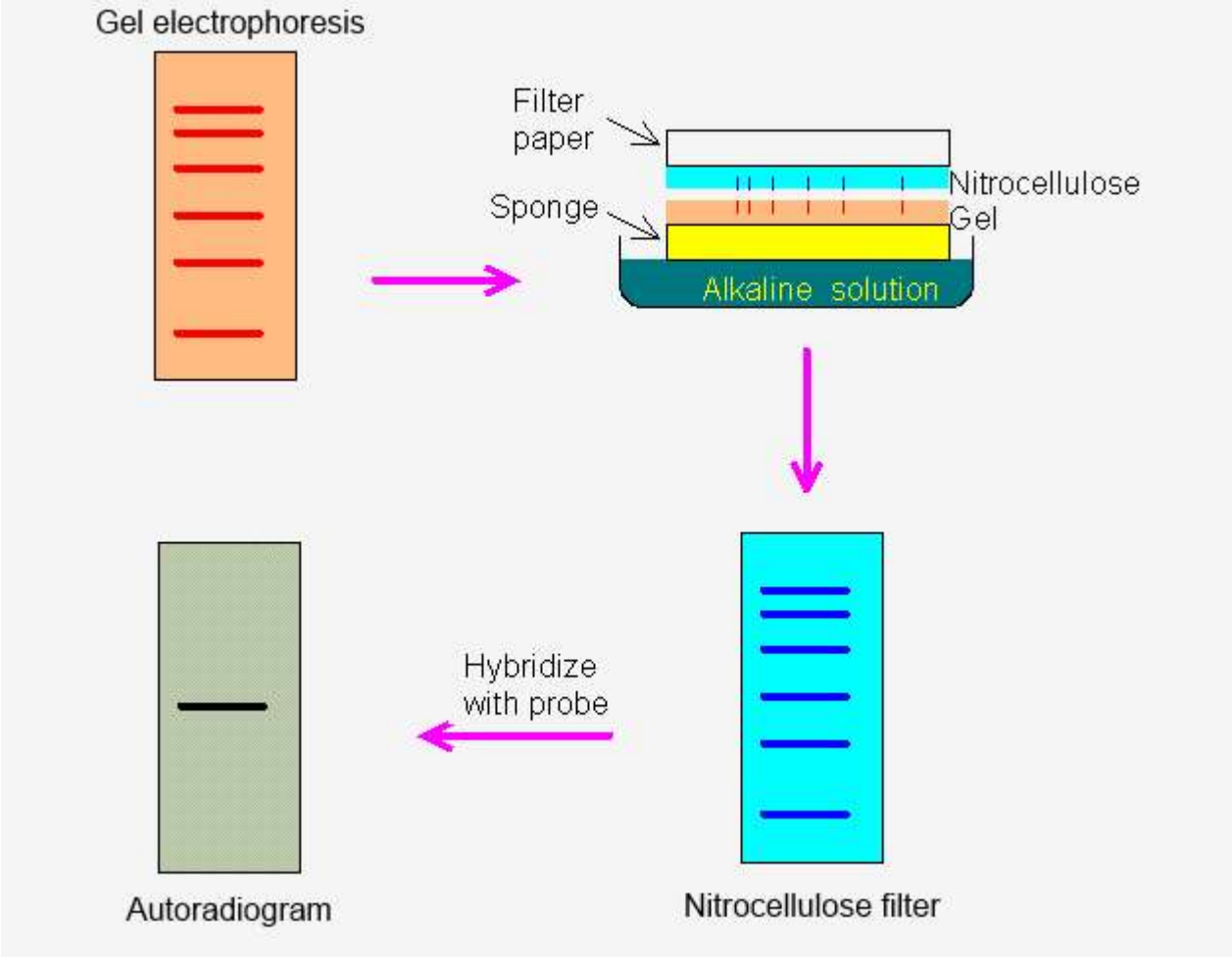

Figure 11**.** Southern blotting. **(a)** The DNA to be analyzed is digested with restriction enzymes and then separated by agarose gel electrophoresis. **(b)** The DNA fragments in the gel are denatured with alkaline solution and transferred onto a nitrocellulose filter or nylon membrane by blotting, preserving the distribution of the DNA fragments in the gel. **(c)** The nitrocellulose filter is incubated with a specific probe.

The location of the DNA fragment that hybridizes with the probe can be displayed by autoradiography.

6.1 Principle of Southern Blotting

The process involves the transfer of electrophoresis-separated DNA fragments to a carrier membrane which is usually nitrocellulose and the subsequent detection of the target DNA fragment by probe hybridization. Hybridization refers to the process of forming a double-stranded DNA molecule between a single-stranded DNA probe and a single-stranded target DNA. Since the probe and target DNA are complementary to each other, the reaction is specific which aids in the detection of the specific DNA fragment (Fig 11).

6.2 Steps involved in southern blotting

a. DNA extraction and purification

DNA is first separated from target cells following standard methods of genomic DNA extraction and then purified.

b. Restriction Digestion

Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments. One or more restriction enzymes can be used to achieve such fragments.

c. Electrophoresis

The separation may be done by agarose gel electrophoresis in which the negatively charged DNA fragments move towards the positively charged anode, the distance moved depending upon its size.

d. Depurination

Partial depurination is done by the use of dilute HCl which promotes higher efficiency transfer of DNA fragments by it breaking down into smaller pieces.

e. Denaturation

DNA is then denatured with a mild alkali such as an alkaline solution of NaOH. This causes the double stranded DNA to become single-stranded, making them suitable for hybridization. DNA is then neutralized with NaCl to prevent re-hybridization before addition of the probe.

f. Blotting

The denatured fragments are then transferred onto a nylon or nitrocellulose filter membrane which is done by placing the gel on top of a buffer saturated filter paper, then laying nitrocellulose filter membrane on the top of gel. Finally some dry filter papers are placed on top of the membrane. Fragments are pulled towards the nitrocellulose filter membrane by capillary action and result in the contact print of the gel.

g. Baking

The nitrocellulose membrane is removed from the blotting stack, and the membrane with single stranded DNA bands attached on to it is baked in a vacuum or regular oven at 80 °C for 2-3 hours or exposed to ultraviolet radiation to permanently attach the transferred DNA onto the membrane.

h. Hybridization

The membrane is then exposed to a hybridization probe which is a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.

i.Washing

After hybridization, the membrane is thoroughly washed with a buffer to remove the probe that is bound nonspecifically or any unbound probes present.

j. Autoradiograph

The hybridized regions are detected autoradiographically by placing the nitrocellulose membrane in contact with a photographic film which shows the hybridized DNA molecules. The pattern of hybridization is visualized on X-ray film by autoradiography in case of a radioactive or fluorescent probe is used or by the development of color on the membrane if a chromogenic detection method is used.

6.3 Application

- Identifying specific DNA in a DNA sample.
- Preparation of RFLP (Restriction Fragment Length Polymorphism) maps
- Detection of mutations, deletions or gene rearrangements in DNA
- For criminal identification and DNA fingerprinting (VNTR)
- Detection and identification of trans gene in transgenic individual
- Mapping of restriction sites
- For diagnosis of infectious diseases
- Prognosis of cancer and prenatal diagnosis of genetic diseases
- Determination of the molecular weight of a restriction fragment and to measure relative amounts in different samples.

7. Restriction Fragment Length Polymorphism (RFLP)

Polymorphism refers to the DNA sequence variation between individuals of a species. If the sequence variation occurs at the restriction sites, it could result in RFLP. The most well known example is the RFLP due to β globin gene mutation (Fig 12).

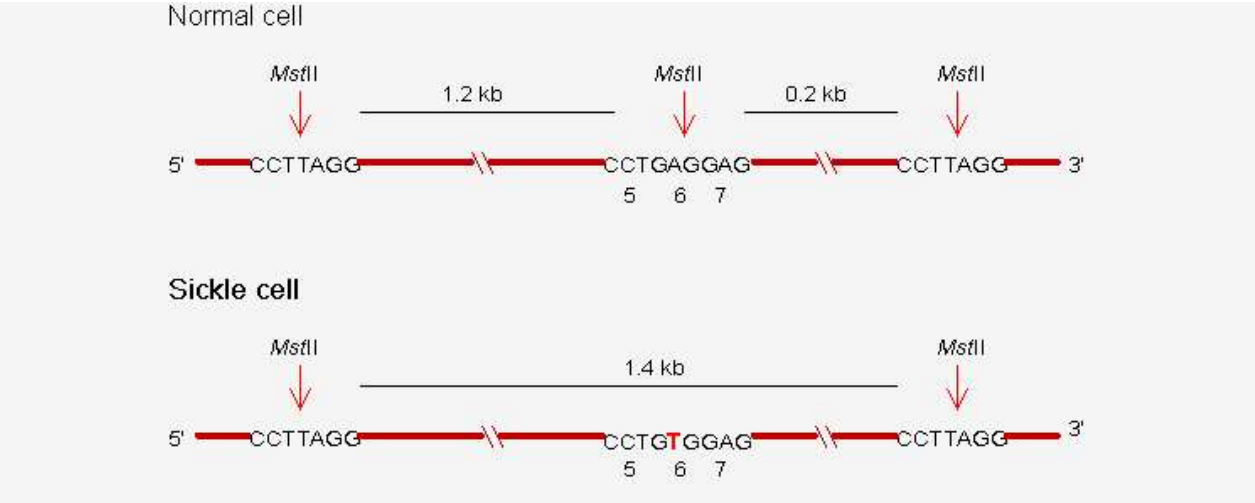

Figure 12 RFLP resulting from β-globin gene mutation. In the normal cell, the sequence corresponding to 5th to 7th amino acids of the β-globin peptide is CCTGAGGAG, which can be recognized by the restriction enzyme MstII. In the sickle cell, one base is mutated from A to T, making the site unrecognizable by MstII. Thus, MstII will generate 0.2 kb and 1.2 kb fragments in the normal cell, but generate 1.4 kb fragment in the sickle cell. These different fragments can be detected by the southern blotting.

8. Northern Blotting

Northern Blotting is a technique used for the study of gene expression. It is done by detection of particular RNA (or isolated mRNA). mRNA is generally represented as 5% of the overall RNA sequence. This method reveals the identity, number, activity, and size of the particular gene. This blotting technique can also be used for the growth of a tissue or organism. In different stages of differentiation and morphogenesis the abundance of an RNA changes and this can be identified using this technique. It also aids in the identification of abnormal, diseased or infected condition at the molecular level. The northern blot technique was developed in 1977 by James Alwine, David Kemp and George Stank at Stanford University. The technique got its name due to the similarity of the process with Southern blotting. The primary difference between these two techniques is that northern blotting concerns only about RNA.

8.1 Principle

As all normal blotting technique, northern blotting starts with the electrophoresis to separate RNA samples by size. Electrophoresis separates the RNA molecules based on the charge of the nucleic acids. The charge in the nucleic acids is proportional to the size of the nucleic acid sequence. Thus the electrophoresis membrane separates the Nucleic acid sequence according to the size of the RNA sequence. In cases where our target sequence is an mRNA, the sample can be isolated through oligo cellulose chromatographic techniques, as mRNA are characterized by the poly(A)-tail. Since gel molecules are fragile in nature, the separated sequences are transferred to the nylon membranes. The selection of nylon membrane is contributed to the factor that nucleic acids are negatively charged in nature. Once the RNA molecules are transferred it is immobilized by covalent linkage. The probe is then added, the probe can be complementary an ss DNA sequence. Formamide is generally used as a blotting buffer as it reduces the annealing temperature (Fig 13).

8.2 Procedure

1. The tissue or culture sample collected is first homogenized. The samples may be representative of different types of culture for comparison or it can be for the study of different stages of growth inside the culture.
2. The RNA sequence is separated in the electrophoresis unit an agarose gel is used for the purpose of the nucleic acid separation.
3. Now the separated RNA sequence is transferred to the nylon membrane. This is done by two mechanisms capillary action and the ionic interaction.

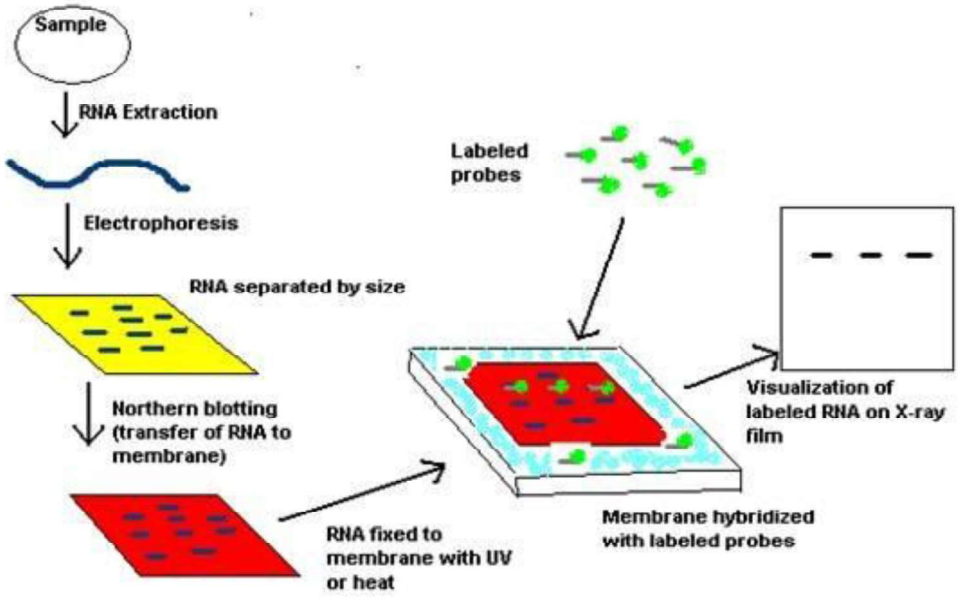

Figure 13 Northern Blotting

4. The transfer operation is done by keeping the gel in the following order. First, the agarose gel is placed on the bottom of the stack, followed by the blotting membrane. On top of these paper towels a mild weight (glass plate) is placed. The entire setup is kept in a beaker containing transfer buffer.
5. RNA transferred to the nylon membrane is then fixed using UV radiation.
6. The fixed nylon membrane is then mixed with probes. The probes are specifically designed for the gene of interest, so that they will hybridize with RNA sequences on the blot corresponding to the sequence of interest.
7. The blot membrane is washed to remove unwanted probe
8. Labeled probe is detected by chemiluminescence or autoradiography. The result will be dark bands in x ray film.

9. Western blotting

Western blotting (or immunoblotting) is a widely used method to detect proteins as well as posttranslational modifications on proteins, using antibody based probes to obtain specific information about target proteins from complex samples. It is a routine method in a molecular

biology, biochemistry and cell biology field with the multitude of applications. It can provide semi-quantitative or quantitative data about the target protein in simple or complex biological samples.

Since western blotting is a multistep protocol, variations and errors can occur at any step reducing the reliability and reproducibility of this technique. Recent reports suggest that a few key steps, such as the sample preparation method, the amount and source of primary antibody used, as well as the normalization method utilized, are critical for reproducible western blot results. This method relies on the fact that most epitopes (sites recognized by antibodies, generally comprising several amino acids) inspite of denaturation of proteins can still be recognized. Due to high affinities of antibody toward their epitopes, it is a very sensitive method and even picogram quantities of a target protein can be detected. The two primary advantages of western blotting are sensitivity and specificity. Western blotting has advantages over other protein detection techniques. Silver staining, another technique of protein detection detects 10 ng of protein and all proteins in a given sample. Whereas, western blotting can detect as little as 0.1ng of protein, and it selectively detects only the protein of interest. Thus a complex mixture containing only traces of the desired protein may be analyzed accurately with this technique. Western blotting was first described by Harry Towbin in 1979. It was in 1981 when W. Neal Burnette developed an improved version of the method and gave the name “western blotting” simply because of the location of laboratory. Towbin’s landmark paper has been cited more than 54,000 times (Fig 14).

Below is a general procedure for blotting, and every step is critical for obtaining high-quality, reliable and analyzable data.

1. Homogenize the sample.
2. Separation of the molecule of interest by an electrophoresis membrane.
3. Transferring the molecules to a nitro cellulosic membrane/ nylon membrane.
4. Hybridization or identification of the molecule

Western blotting is the technique used for separation or identification of protein molecules. This technique can be used for both the active 3D protein and denatured long peptide chains. The 3-D protein in its active structure has sulfur-hydroxyl bonds in the structure. This methodology classifies protein based on the molecular weight and charge. These techniques have application in the identification of a wide variety of infectious diseases like HIV, Hepatitis B, Herpes type 2,

and feline immunodeficiency disease. The identification of these diseases is done by using the antibody of the particular disease as the probe and these probes are produced in vitro condition. Western blotting is also used for research purpose. This technique can be used in the study of the properties and activity of a protein molecule of interest.

9.1 PROCEDURE

9.1.1 Sample Preparation

1. Wash cells in the tissue culture flask or dish by adding cold Phosphate Buffered Saline (PBS) and rocking gently. Flask or dish should be kept on ice throughput the process. Discard PBS.
2. Add PBS and use a cell scraper to dislodge the cells. Pipette the mixture into microcentrifuge tubes.
3. Centrifuge at 1500 RPM for 5 minutes. Discard the supernatant.
4. Add 180 ul of ice cold lysis buffer solution to 20 ul of fresh protease inhibitor. This prevents the protease enzyme. Incubate for 30 minutes.
5. Incubate for 30 min on ice, and centrifuge this solution for 10 minutes at 12000 RPM at 4^0c and the sample solution is ready.
6. Transfer supernatant (or protein mix) to a fresh tube and store on ice or frozen at -20°C or -80°C.
7. Measure the concentration of protein using a spectrophotometer and determine the volume of protein extract to ensure 50 μg in each well.
8. Add 5 μL sample buffer to the sample, and make the volume in each lane equalized using double distilled H_2O (dd H_2O). Mix well.
9. Heat the samples with a dry plate for 5 minutes at 100°C.

9.1.2 Gel Preparation

The gel has two parts stacking gel and separation gel. 10% stacking gel and 6% separating gel are generally used. Add the stacking gel solution into the assembly carefully and then add H_2O to the top. Wait for 15–30 minutes until the gel solidifies. Overlay the stacking gel with the separating gel, after removing the water. Insert the comb, ensuring that there are no air bubbles. Wait until the gel is solidified.

9.1.3 Electrophoresis

Pour the running buffer into the electrophorator. Place gel inside the electrophorator and connect to a power supply. (Tip: When connecting to the power source always connect red to red, and black to black). Make sure buffer covers the gel completely, and remove the comb carefully. Load marker (6 µL) followed by samples (15 µL) in to each well. Run the gel at 40 volts until the sample reaches the stacking gel and changed into 80 volts from the separation gel. Run the gel for approximately an hour, or until the dye front runs off the bottom of the gel

9.1.4 Fixing and blotting

Fixing is done 5% of bovine serum albumin solution. Cut 6 filter sheets to fit the measurement of the gel and one polyvinylidene fluoride (PDVF) membrane with the same dimensions. Wet the sponge and filter paper in transfer buffer, and wet the PDVF membrane in methanol. Separate glass plates and retrieve the gel. Create a transfer sandwich as follows – Sponge, 3 Filter Papers, Gel PVDF, 3 Filter Papers (Ensure there are no air bubbles between the gel and PVDF membrane, and squeeze out extra liquid). Relocate the sandwich to the transfer apparatus, which should be placed on ice to maintain 4°C. Add transfer buffer to the apparatus, and ensure that the sandwich is covered with the buffer. Place electrodes on top of the sandwich, ensuring that the PVDF membrane is between the gel and a positive electrode. Blotting is carried in two ways, capillary blotting or through electroblotting. Usually, electroblotting is carried out at 40 volts. For capillary blotting, the gel is stacked in the following order: electrophoretic gel followed by blotting membrane followed by wet tissue and lid glass plate.

9.1.5 Blocking and Incubation

Block the membrane with 5% skims milk in TBST for 1 hour. Add primary antibody in 5% bovine serum albumin (BSA) and incubate overnight in 4°C on a shaker. Wash the membrane with TBST for 5 minutes. Do this 3 times. (Tip: All washing and antibody incubation steps should be done on a shaker at room temperature to ensure even agitation). Add secondary antibody in 5% skim milk in TBST, and incubate for 1 hour. Wash the membrane with TBST for 5 minutes. Do this 3 times.

9.1.6 Detection

Detection and identification can be carried out by a number of methods like radiography, chemiluminescence, colorimetric and x-ray methods.

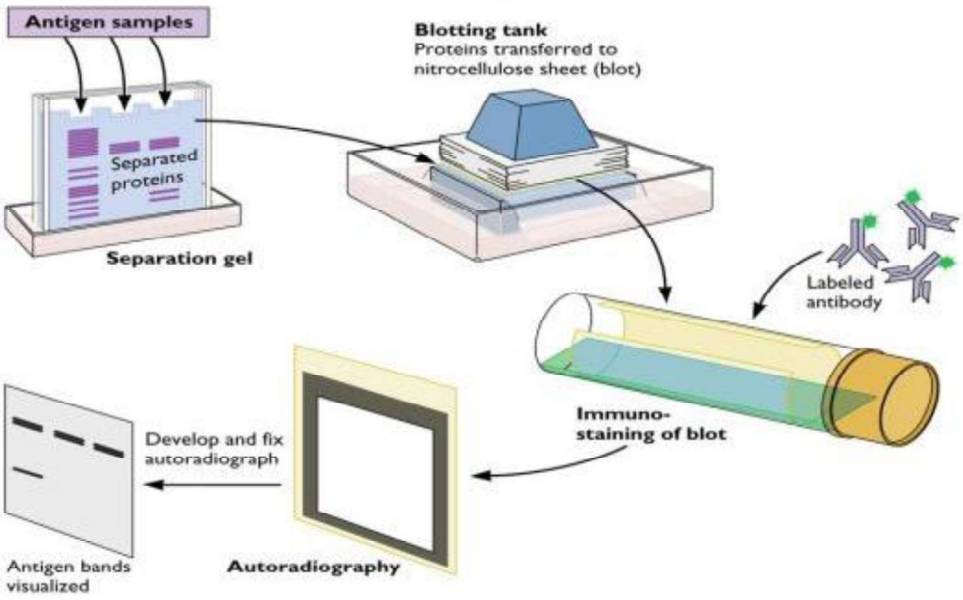

Figure 14 Western Blotting