

## Lecture 13

## Electrophoresis (Part-I)

**Introduction :** “Electrophoresis” literally means running in the electric field. The charged molecule moves to their counter charge electrodes but electric field is removed before it reaches the electrode. Movement of charged species in an electric field gives differential mobility to the sample based on the charge and consequently resolve them. Movement of the charged particle is retarded with the addition a polymeric gel so that a sufficient time is available for resolving the sample. The polymeric gel is inert, uncharged and does not cause retardation by binding the molecule. Instead it, forms pores of different size (depending on the concentration of polymer) and sample pass through these pore and as a result their electrophoretic mobility is reduced (Figure 13.1).

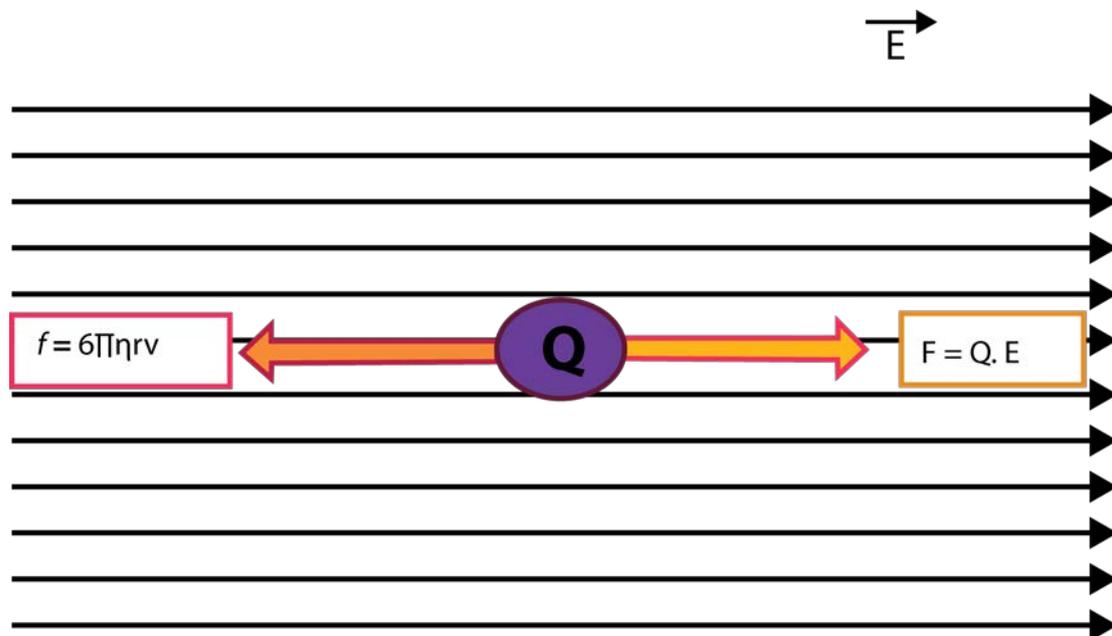


Figure 13.1: Movement of the charged particle in an external field.

Suppose a charged particle has net charge  $Q$  and the external electric field is  $E$ , then the force  $F$  responsible for giving electrophoretic mobility,

$$F = Q.E \dots \dots \dots Eq (13.1)$$

The friction forces  $F$  which is opposing the movement of the charged particle is as follows

$$F = f.v \dots \dots \dots Eq (13.2),$$

here  $f$  is the friction coefficient and the  $v$  is the velocity of the electrophoretic mobility. The movement of a spherical through a liquid medium (gel) of the viscosity  $\eta$ , the friction coefficient  $f$  is given by :

$$f = 6\pi\eta rv \dots \dots \dots \text{Eq (13.3)}$$

The force where,  $F = F$  or  $QE = 6\pi\eta rv$

The electrophoretic mobility  $v$  is given by:  $v = \frac{Q}{6\pi\eta r}$

As  $Q = ze$ , where  $z$  is the valency and  $e$  is the electronic charge, the electrophoretic mobility can be expressed as:

$$v = \frac{ze}{6\pi\eta r}$$

Hence, electrophoretic mobility  $v$  is directly proportional to the charge and inversely proportional to the viscosity of the medium, size and shape of the molecule. In the case of relative mobility, it is directly related to the charge/radius of the molecule. For a globular protein, the radius ( $r$ ) of the molecule is related to the molecular mass of the macromolecule. The relative mobility,  $v'$  is as follows

$$v' = \frac{\text{Charge}}{\text{mass}} \dots \dots \dots \text{Eq (13.4)}$$

**Electrophoretic techniques:** Different types of electrophoresis techniques are designed depending upon whether it carried out in the presence or absence of a supporting media.

**Moving boundary electrophoresis-**In this method, the electrophoresis is carried in solution, without a supporting media. The sample is dissolved the buffer and molecules move to their respective counter charge electrodes. Moving boundary electrophoresis is carried out in a U shape tube with platinum electrodes attached to the end of both arms (Figure 13.2). At the respective ends, tube has refractometer to measure the change in refractive index of the buffer during electrophoresis due to presence of molecule. Sample is loaded in the middle of the U tube and then the apparatus is connected to the external power supply. Charged molecule moves to the opposite electrode as they passes through the refractometer, a change can be measured. As the desirable molecule passes, sample can be taken out from the apparatus along with the buffer.

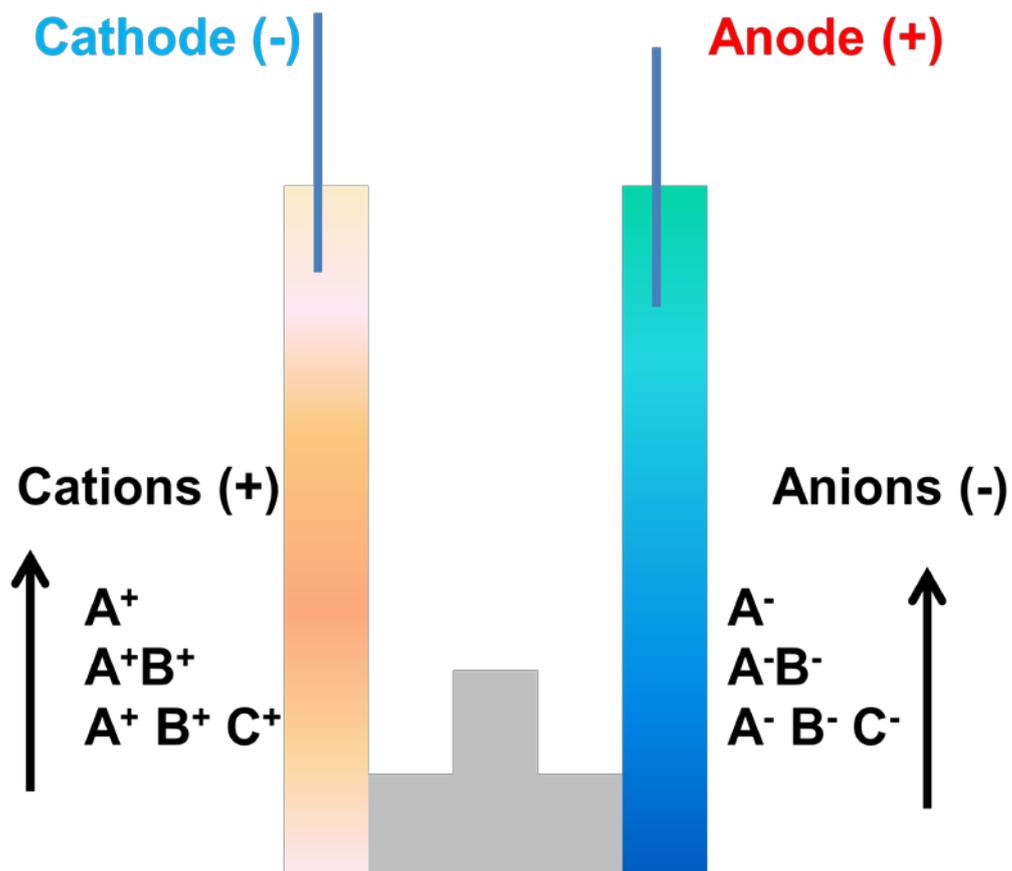


Figure 13.2: Movement of the charged particle in a moving boundary electrophoresis.

**Disadvantages of Moving Boundary electrophoresis-**The resolution of the technique is very low due to the mixing of the sample as well as over-lapping of the sample components. The electrophoresis technique is not good to separate and analyze the complex biological sample instead it can be used to study the behavior of the molecule in an electric field.

**Zone electrophoresis-**In this method, an inert polymeric supporting media is used between the electrodes to separate and analyze the sample. The supporting media used in zone electrophoresis are absorbent paper, gel of starch, agar and polyacrylamide. The major advantage of presence of supporting media is that it minimizes mixing of the sample and immobilization of the molecule after electrophoresis. It makes the analysis and purification of the molecule from the gel much easier than the moving boundary electrophoresis. The gel electrophoresis is the best example of zone electrophoresis.

**Experiment 13.1 : Analysis of crude cell lysate in SDS-PAGE and determination of molecular weight of unknown protein.**

**Principle of the technique:** In the discontinuous system, negatively charged detergent, sodium dodecyl sulphate (SDS), is used to denature protein and impart a constant negative charge/mass ratio, as a result separation of the protein largely depends on size only. This technique uses 3 different buffers (1) Running Buffer (2) Stacking gel buffer of pH 6.8 (3) Resolving gel Buffer of pH 8.8. Discontinuous gel system concentrate the diluted protein sample into a narrow band, allows application of diluted protein sample. **What is the mechanism of stacking protein sample in the discontinuous gel system?** Stacking gel buffer is composed of TrisHCl pH 6.8, SDS where as resolving gel buffer contains Tris pH 8.8, SDS and the pore size is large compared to the resolving gel. The mobility of chloride ion present in the buffer is more than the protein in the sample. The glycine moves slower than the protein sample and as a result protein sample get sandwiched between fast moving chloride ion and slow moving glycine. Due to high electrophoretic mobility, the protein molecules run fast and stack between the leading and trailing ions. Once protein sample enters into the resolving gel (resolving buffer pH 8.8), glycine ions doesn't stop the migration of protein and protein molecules separate as per their size or molecular weight.

**Vertical Gel Instrument-** The schematic diagram of a vertical gel electrophoresis apparatus is given in Figure 13.3. It has two buffer chamber, upper chamber and a lower chamber. Both chamber are fitted with the platinum electrodes connected to the external power supply from a power pack which supplies a direct current or DC voltage. The upper and lower tank filled with the running buffer is connected by the electrophoresis gel casted in between two glass plates (rectangular and notched). There are additional accessories needed for casting the polyacrylamide gel such as comb (to prepare different well), spacer, gel caster etc.

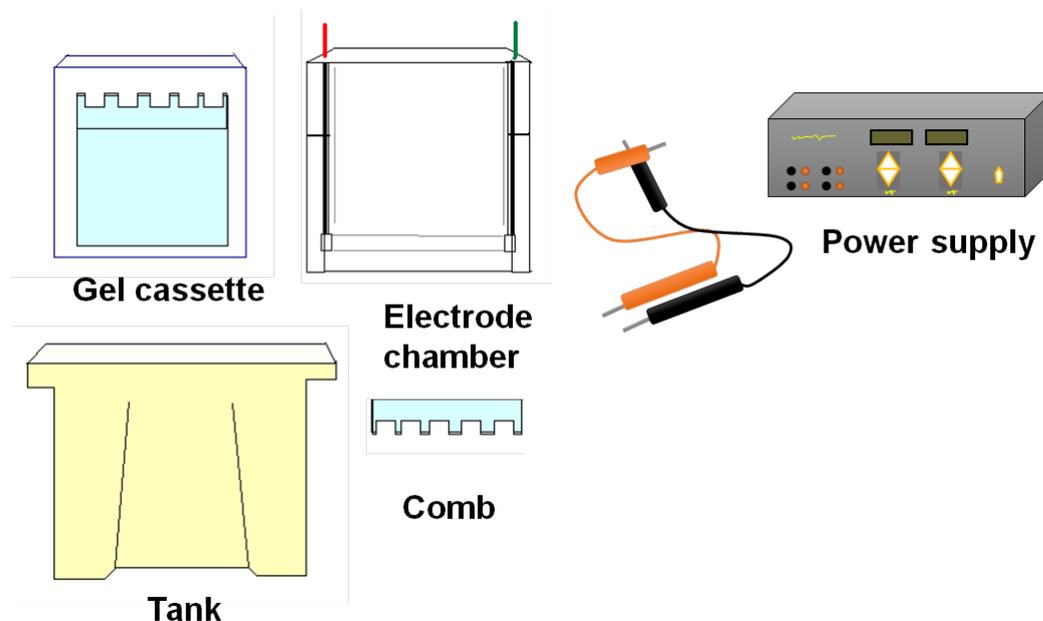
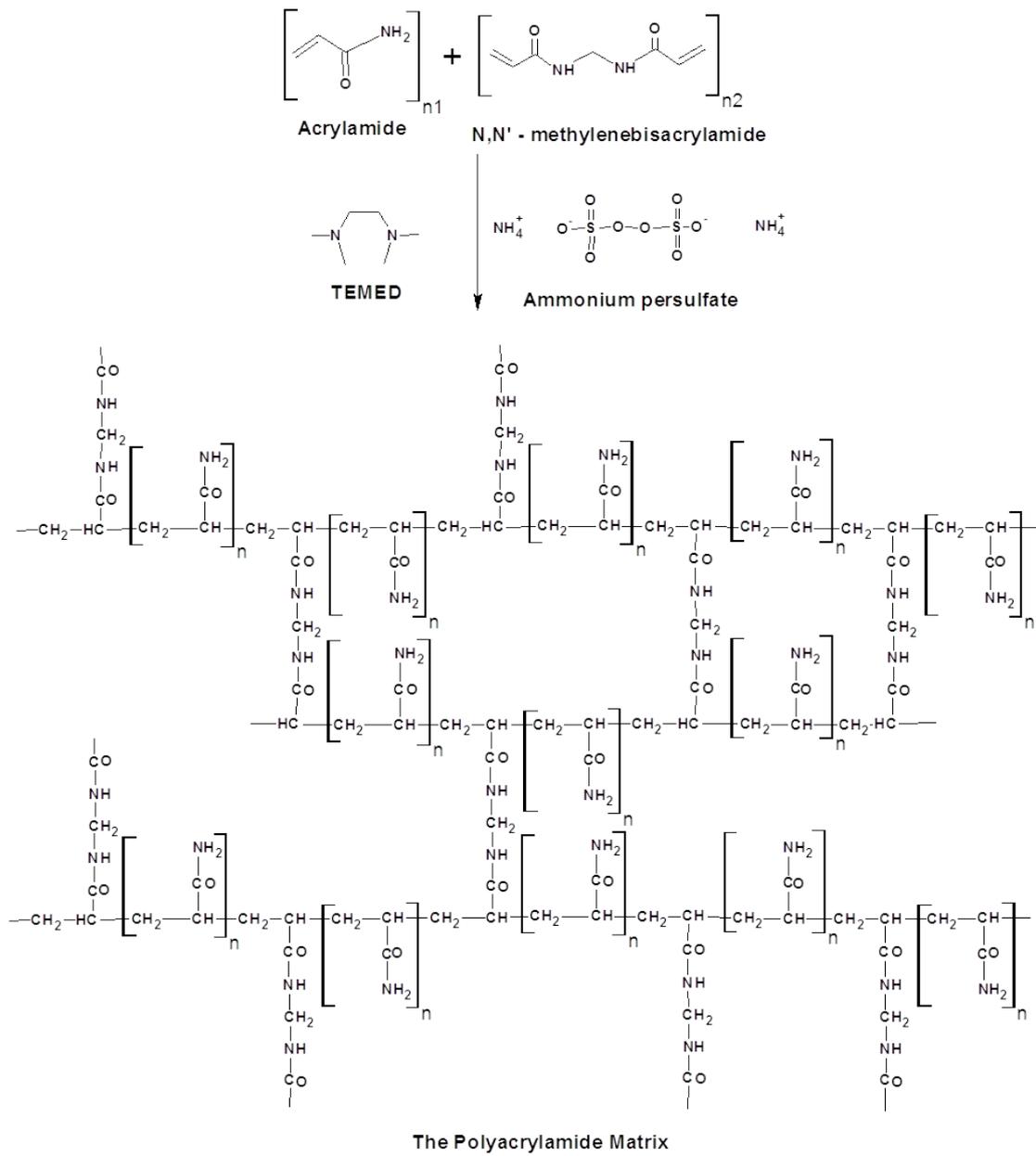


Figure 13.3: Different components of vertical gel electrophoresis apparatus.

**Buffer and reagent for electrophoresis-** The different buffer and reagents with their purpose for vertical gel electrophoresis is as follows-

1. **N, N, N', N'-tetramethylethylenediamine (TEMED)**-it catalyzes the acrylamide polymerization.
2. **Ammonium persulfate (APS)**-it is an initiator for the acrylamide polymerization.
3. **Tris-HCl**- it is the component of running and gel casting buffer.
4. **Glycine**- it is the component of running buffer.
5. **Bromophenol blue**- it is the tracking dye to monitor the progress of gel electrophoresis.
6. **Coomassie brilliant blue R250**-it is used to stain the polyacrylamide gel.
7. **Sodium dodecyl sulphate**-it is used to denature and provide negative charge to the protein.
8. **Acrylamide**- monomeric unit used to prepare the gel.
9. **Bis-acrylamide**- cross linker for polymerization of acrylamide monomer to form gel.

**Casting of the gel:** The acrylamide solution (a mixture of monomeric acrylamide and a bifunctional crosslinker bisacrylamide ) is mixed with the TEMED and APS and poured in between the glass plate fitted into the gel caster. **What is the mechanism of acrylamide polymerization?** Ammonium persulfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acrylamide monomer to form a linear polymer (Figure 13.4). These linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of acrylamide and amount of bis-acrylamide in the gel. In a vertical gel electrophoresis system, we cast two types of gels, stacking gel and resolving gel. First the resolving gel solution is prepared and poured into the gel cassette for polymerization. A thin layer of organic solvent (such as butanol or isopropanol) is layered to stop the entry of oxygen (oxygen neutralizes the free radical and slow down the polymerization) and make the top layer smooth. After polymerization of the resolving gel, a stacking gel is poured and comb is fitted into the gel for construction of different lanes for the samples (Figure 13.5).



**Figure 13.4: Mechanism of acrylamide polymerization.**

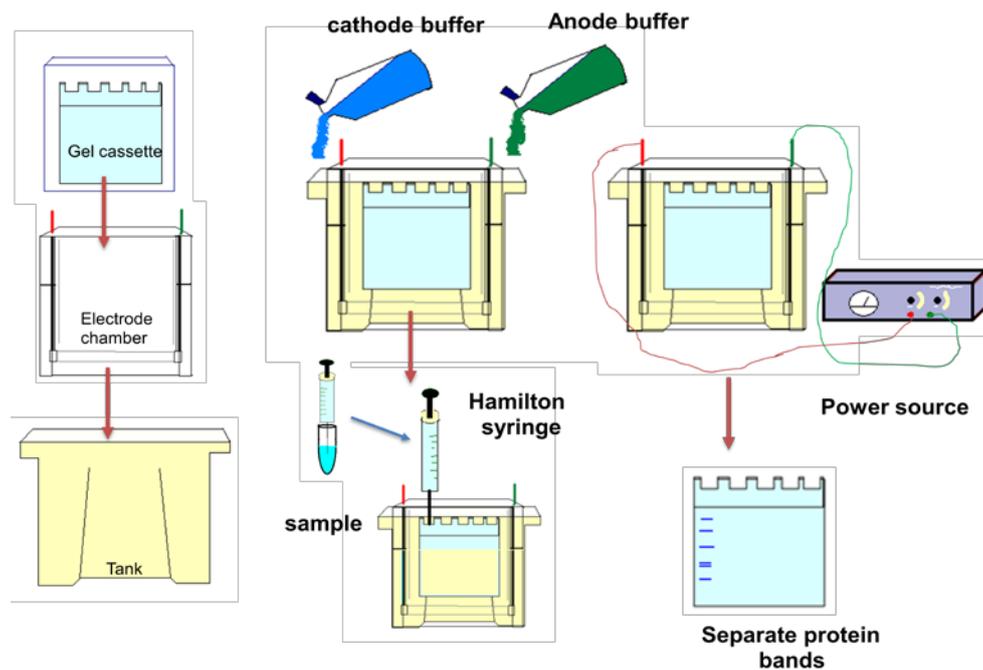


Figure 13.5: Different steps in performance of vertical gel electrophoresis to resolve sample.

**Running of the gel:** The sample is prepared in the loading dye containing SDS,  $\beta$ -mercaptoethanol in glycerol to denature the sample and presence of glycerol facilitates the loading of sample in the well. As the samples are filled vertically there is a distance drift between the molecules at the top Vs at the bottom in a lane. This problem is taken care once the sample run through the stacking gel. The pH of the stacking gel is 6.8 and at this pH, glycine is moving slowly in the front where as Tris-HCl is moving fast. As a result, the sample gets sandwiched between glycine-Tris and get stacked in the form of thin band. As the sample enters into the resolving gel with a pH 8.8, the glycine is now charged, it moves fast and now sample runs as per their molecular weight (due to SDS they have equal negative charge). After tracking dye reaches to the bottom of the gel, gel is taken out from the glass plate with the help of a spatula. Gel is stained with coomassie brilliant blue R250 dye. The dye stains protein present on the gel. A typical SDS-PAGE pattern is given in the Figure 13.6.

**Potentials of discontinuous PAGE :**

**1. Number of disulfide bonds:** Comparison of reducing and non-reducing denaturing gels can be used to provide information related to the number of disulfide bonds present in the protein.

**2. Separating Proteins based on size alone:** In the presence of SDS and reducing environment, PAGE gel resolve two proteins of on the basis of molecular masses and the concentration of gel concentration.

In SDS-PAGE, the relative mobility and the log molecular weight as given by

$$v' = V_0 \frac{A - \log M}{A} \dots\dots\dots \text{Eq (13.5)}$$

**Molecular weight of a protein can be determined by plotting relative migration Rf with the log molecular weight of standard protein.**

$$Rf = \frac{\text{migration of protein from the lane}}{\text{migration of tracking dye}} \dots\dots\dots \text{Eq (13.6)}$$

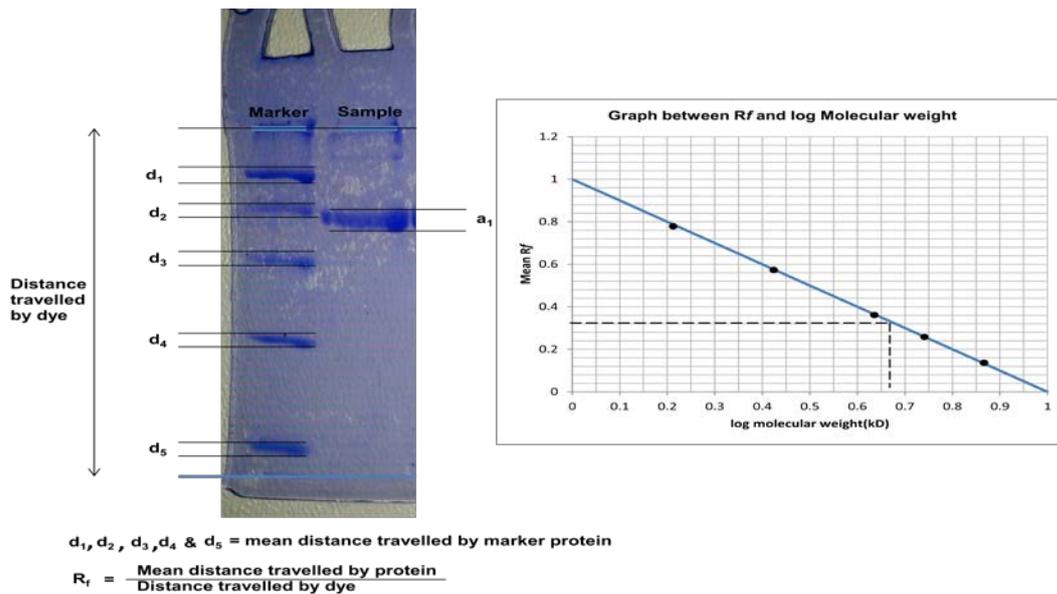


Figure 13.6: Determination of molecular weight using SDS-PAGE. (A) SDS-PAGE (B) Determinaion of Rf.

**Calculation of molecular weight of the unknown protein sample is a 5 step process:**

1. Resolve the protein sample on the SDS-PAGE along with the molecular weight markers.
2. Calculate the relative mobility (Rf) using the following formula:

$$Rf = \frac{\text{migration of protein from the lane}}{\text{migration of tracking dye}} \dots\dots\dots \text{Eq (13.6)}$$

3. Plot log molecular mass (y-axis) versus relative mobility (x-axis) of the standards.

4. Perform a linear regression using a calculator or using regression software such as Microsoft Excel.

5. Use the linear regression equation ( $y=mx+c$ ) to estimate the mass of the unknown protein.

$$\text{Log Molecular Weight}=(\text{slope})(\text{mobility of the unknown})+\text{Yintercept}$$

**Troubleshooting : The pattern of the protein bands on polyacrylamide gel depend on several factors. As a result several types of defects are observed. Now we will discuss few of these defects and responsible factors.**

**1. Smiling :** Uneven heating of the gel causes differential migration of proteins, with outer lanes moving slower than the middle lanes. Rapid heat transfer eliminates this defect and be achieved by filling the lower tank with the buffer until sample height.

**2. Diffuse Protein Bands:** Diffused pattern of the protein band appears on the PAGE. Diffused protein bands pattern can be corrected by increasing running current by 25-50%, higher concentration of acrylamide.

**3. Vertical Streaking:** Vertical streaking of the protein bands appears due to overloading of the protein sample. It can be corrected by either reducing amount of the protein sample or running the gel at lower current.

**4. Protein Runs faster than expected:** In few cases migration of protein is not proportional to the molecular weight, it is either more or less on gel than the expected place. It is due to the unusual very high proportion of basic or acidic amino acids.

**5. Double Bands:** Appearance of double bands is due to partial oxidation or degradation of the protein sample. Addition of more amount of reducing reagent or preparing a fresh sample will reduce these artifacts.

**6. Distorted Protein Bands:** Appearance of distorted or uneven protein band is due to stacking gel polymerization. It can be corrected by increasing amount of ammonium persulfate and TEMED or deaerating the stacking gel.

**7. Lateral Spreading:** if protein bands appears laterally spreading, it can be avoided by reducing the time between loading of the sample and running.

## Lecture 14

## Staining of PAGE Gels

### Other Variants of PAGE:

**Native PAGE:** SDS-PAGE discussed in the previous lecture is using anionic detergent sodium dodecyl sulfate and  $\beta$ -mercaptoethanol to give equal charge to all protein and breaks the disulphide linkage. As a result, the 3-D structure of the protein is destroyed and it migrate as per their subunit molecular weight. In the native PAGE, sample is prepared in the loading dye does not contains detergent or denaturing agent and as a result, sample runs on the basis of charge/mass. In native PAGE, the 3-D conformation as well as activity of the protein remains unaffected.

**Urea PAGE:** In this method, insoluble protein is dissolved in Urea and samples separate based on their charge/subunit mass. A gradient Urea PAGE is used to monitor the folding states of a protein.

**Staining methods in Polyacrylamide Gel:** There are different staining protocols are developed to stain the protein bands in the gel. Few of the selected staining protocol are given in the Table 14.1 with brief over-view of their advantage and disadvantages.

Table 14.1: Different Stains available for detection of protein in polyacrylamide gels

S.No	Type of Stain	Features
1	Coomassie Brilliant Blue R250 (CBB)	Non-Specific, One step simple staining procedure, detection limit 0.3-1 $\mu$ g/protein band
2	Colloidal Solution	CBB 5-10 fold more sensitive than CBB, No destaining step
3	Silver Stain	100 fold more sensitive than CBB, No simple staining procedure, Mass-spectrometry incompatible
4	SYPRO Ruby	Mass-spectrometry compatible, sensitivity upto 1-2ng/protein band.
5	SYPRO Orange	Sensitivity upto 4-8ng/band.
6.	SYPRO Tangerine	Sensitivity upto 4-8ng/band, can be used to detect blotted protein bands.

**Laboratory Experiment 14.1 : Analyze the crude mixture of protein sample and stain the gel with Coomassie Brilliant Blue R250 dye and perform image analysis.**

**Background Information :** Coomassie Brilliant Blue R250 non-specifically stains almost all proteins and the staining procedure is rapid, simple and inexpensive. The detection limit of the stain is 0.3 to 1 mg/protein band.

**Materials:**

1. Polyacrylamide gel containing protein bands.
2. Coomassie Brilliant Blue R250 staining solution:
3. Destaining Solution: 15% (v/v) Methanol and Acetic Acid (10% v/v) in Triple Distilled water.
4. plastic or glass container with lid.
5. platform shaker

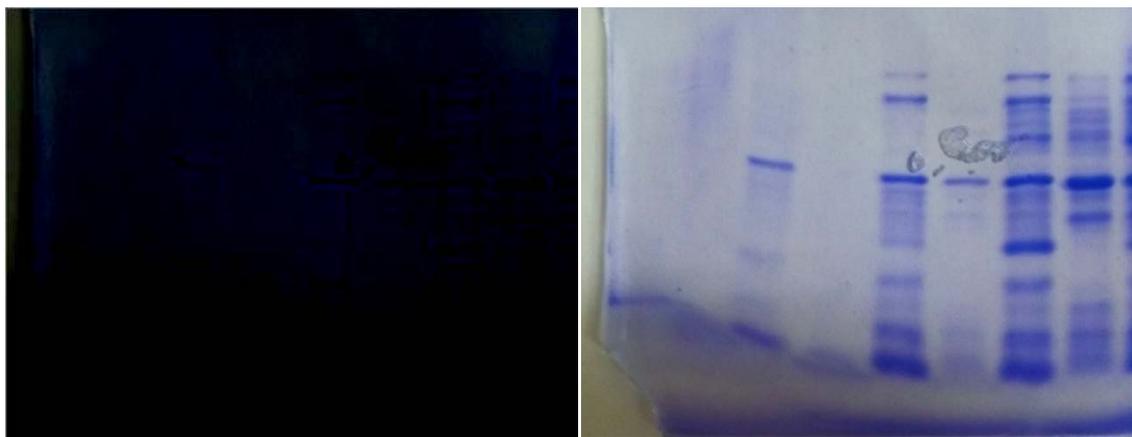
**Procedure:**

1. Remove polyacrylamide gel from the electrophoresis unit and place in plastic container with ~10 volumes Coomassie Brilliant Blue R250 staining solution.
2. Agitate slowly on a platform shaker for 30-60mins.
3. Discard staining solution and wash the gel with triple distilled water.
4. Add 5 to 10 gel volumes of destaining solution.
5. Agitate slowly on a platform shaker for 30-60mins.
6. If the color of the destaining solution is intense blue, replace it with the new destaining solution.

**Results:** A typical gel picture during coomassie brilliant blue R250 staining and destaining is given in the Figure 14.1. In the beginning, whole gel will appear blue/black but the dye will be removed from the background and give discrete appearance of protein bands.

**AFTER STAINING WITH CBB**

**AFTER DE-STAINING**



**Figure 14.1: Coomassie Brilliant Blue R250 Staining of Polyacrylamide gel.**

**Image acquisition:**

The image acquisition in typical gel documentation has multiple steps as given below:

1. Electrophoresis separation and a suitable staining of the protein bands within the gel.
2. Preview the gel bands and optimize parameters such as camera focus, brightness and capture time, magnification, gain, spatial resolution, contrast, gamma etc.
3. Once all parameters are optimized and shows good quality image, image can be captured.
4. Save the image in a suitable digital image format.

Before discussing image analysis, we will discuss few features of the camera and their benefits.

- 1. Number of Pixels :** Number of pixels in a given area increase the spatial resolution but it comprises a lot on sensitivity and accuracy.
- 2. Size of Pixels:** Smaller pixel size increases the resolution whereas larger pixel size is more accurate, sensitive and takes lesser time to collect signal.
- 3. Size of detector:** Size of detector decides the field of viewing but it increases the cost of the optics.

**4. Cooling Temperature:** Cooling reduces the noise but it is irrelevant for fast exposures.

**5. Readout Noise Specification:** lower values can allow to acquire low light intensity but a slower acquisition may be required to achieve lower readout noise values.

**6. Quantum Efficiency Value:** Higher Quantum efficiency is better but it should be considered along with camera noise.

**Analysis of Digital Image:** Table 14.2 lists popular imaging and analysis softwares. Image analysis typically involved several key steps.

**STEP 1: Detection of Lane Position:** The first step in image analysis is to identify and mark the lane position. There are different ways to do this task. For gels with well defined bands, automatic lane detection algorithm can be able to detect lane. If the bands are smiling or not straight, then a manual positioning and identifying lane is the best. There is necessarily consideration while doing manual positioning, the bracket used should be large enough to cover whole lane but it should not wide to include bands of other lane.

**STEP 2: Detection of Band:** Once the lane is defined, protein bands in the lane can be defined by systematically scanning lane profile and identify the region of local maxima as band.

**STEP 3: Background subtraction:** Background plays important role in identifying the protein band as well as measuring the band intensity. Background of a gel picture is non-uniformly distributed and made the measurement less accurate. Many methods of background subtraction are possible. In one of the method, a replica image can be generated and then digitally subtracted from the original image to correct the background.

**Table 14.2: Different Softwares for 1-D Polyacrylamide Gel Image Analysis**

<b>S.No</b>	<b>Software</b>	<b>Company</b>
1	VisionWorkLS, DocItLS	UVP Incorporation
2	TotalLab Series	Non-Linear Dynamics
3	Gel-Pro, ImagePro	MediaCybernetics
4	NIH Image	NIH
5	Melanie, Image Master	Expasy
6.	ImageMaster	GE Healthcare

**STEP 4: Measurement:** Once lane and bands are defined, it is possible to perform quantification and characterization step. The amount in each band is quantified in comparison to the background information and the total intensity present in all pixels present in the band. If the known amount of the protein sample is loaded then a calibration curve can be drawn and use to more accurately quantitate the band intensity.

**Laboratory Experiment 14.1 : Analyze the crude mixture of protein sample and stain the gel with Colloidal Silver Stain.**

**Background Information :** Silver Staining is 50-100 more sensitive than Coomassie Brilliant Blue R250 stain. But this method is not compatible with the down-stream mass spectrometry applications.

**Materials:**

1. Polyacrylamide gel containing resolved protein sample.
2. Fixing Solution A: 40% (v/v) ethanol and 10% (v/v) acetic acid in water.
3. Fixing Solution B: 5% (v/v) ethanol and 5% (v/v) acetic acid in water.
4. 0.5M sodium acetate/1% (v/v) glutaraldehyde
5. 0.05% (w/v) 2,7-naphthalene disulphonic acid.
6. Ammonical Silver Nitrate Solution
7. Citric Acid developing Solution
8. Stop Solution: 5% (w/v)Tris base containing 2% (v/v) acetic acid.
9. plastic or glass container with lid.
10. platform shaker

**Procedure:**

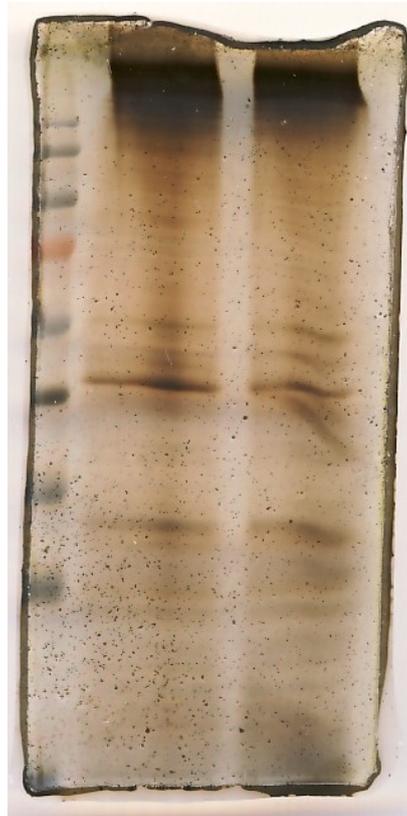
1. Remove PAGE gel from the glass plate and wash with deionized water.
2. remove water and fix the gel in the following manner:
  - A. 1 hr in fixing solution A (10-20 gel volume).
  - B. 2 hr to over-night in fixing solution B (10-20 gel volume).
  - C. wash with deionized water.
  - D. 30mins in 0.5M sodium acetate/1% (v/v) glutaraldehyde (5-10 gel volume)
3. Wash with deionized water.
4. Incubate gel in 5-10 gel volume 0.05% (w/v) 2,7-naphthalene disulphonic acid solution for 30mins.
5. Wash with deionized water.
6. Stain gel with 5-10 volume of freshly prepared Ammonical Silver Nitrate Solution.
7. Wash with deionized water.
8. Develop gel with Citric Acid developing Solution. Development will continue until appearance of bands.
9. Remove developing solution and stop the reaction with 5% (w/v) Tris base containing 2% (v/v) acetic acid.
10. Wash the gel with deionized water.

**Results:** A typical SDS-PAGE stained with colloidal silver stain is given in Figure 14.2. Silver staining gives brown colored bands and the number of bands appeared will be much more than CBB stained gel.

**STAINED WITH CBB**



**STAINED WITH SILVER STAIN**



**Figure 14.2: Comparison of PAGE Gel stained with CBB and colloidal silver stain.**

## Lecture 15

## Western Blotting

**Lab Experiment 15.1 : Analyze the E.Coli Over-expressing cells for the expression of GFP inside the cells.**

**Background Information:** The western blot is a popular technique to detect the specific protein present in the crude lysate or homogenate. It uses the separation of different proteins in the gel electrophoresis (preferably SDS-PAGE) and then the transfer of protein onto a solid support such as nitrocellulose membrane. A primary antibody direct against protein of interest and then a secondary antibody is used to detect the primary antibody and gives either colored or chemiluminescent signal.

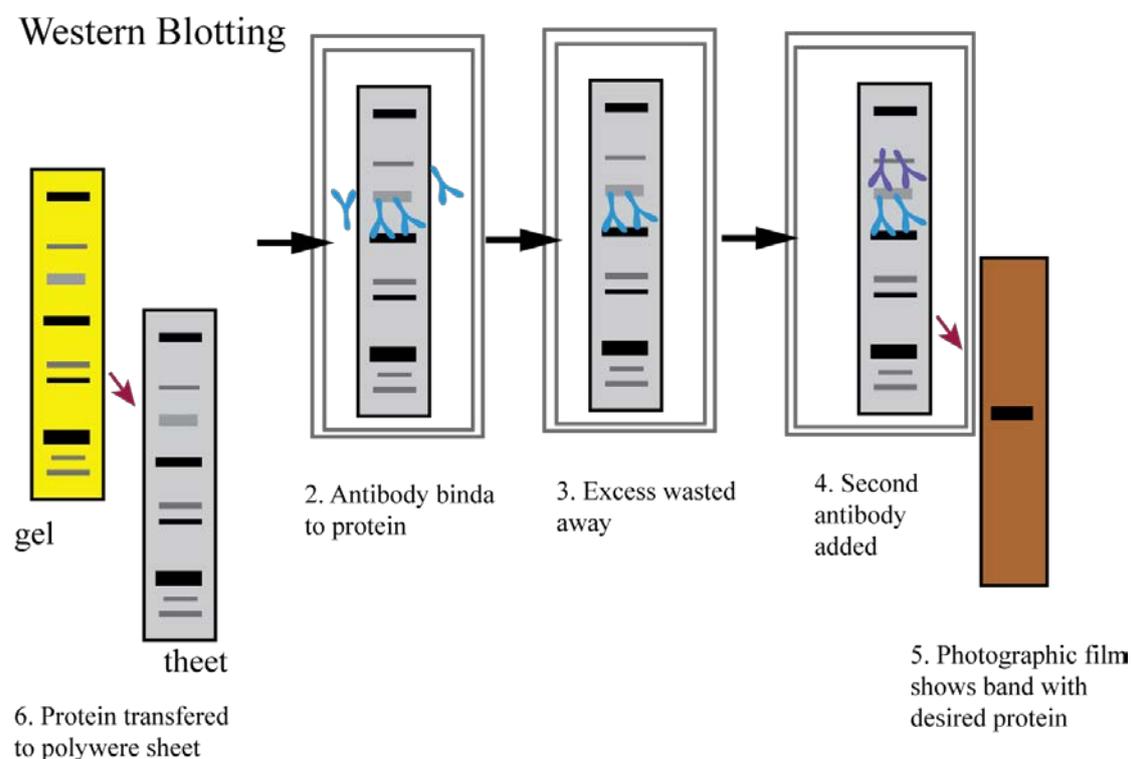


Figure 15.1: Salient Steps in Western Blotting.

### Materials:

1. E.coli cells over-expressing GFP.
2. Protein Standard Markers
3. Transfer Buffer
4. Transfer Membrane: 0.45 $\mu$ m nitrocellulose membrane or PVDF membrane.
5. Plastic Tray
6. Spatula
7. Blotting sheets: 3mm thick cellulose blotting sheets.
8. Semi-Dry Electroblotting Unit.
9. Reagents for performing electrophoresis.

10. anti-GFP antibodies
11. anti rabbit IgG-HRP.
12. Developing reagents

**Methods :** Western Blotting is the multi step process to detect the protein present in the complex mixture. These Steps are as follows:

**STEP 1: Preparation of Sample: Preparation of sample depends on the sample type.**

**For Tissue:** For solid tissue such as tumor or whole liver, brain, it is first mechanically broken down into individual cells using a blender, homogenizer or by sonication. Once individual cells are obtained it will be processed as described.

**For Cells:** Individual cells are incubated with the lysis buffer containing detergent along with protease and phosphatase inhibitor cocktail to protect the sample from degradation.

**STEP 2: Electrophoresis of Sample:** Sample was resolved on SDS-PAGE as described previously.

**STEP 3: Transfer of protein gel on blotting membrane:**

**A. Preparation of Transfer membrane:** Cut the membrane of the same size as gel.

**A1: For nitrocellulose membrane:** Place the membrane in the transfer buffer and observe that the liquid has wicked the membrane. Areas appeared as white spot needs special consideration.

**A2: For PVDF membrane:** Immerse the membrane into the 100% methanol for 15-30mins. Decant the methanol and submerge the membrane into the transfer buffer for additional 10-30mins.

**B. Assembly of transfer cassette:**

1. Remove the stacking gel from the PAGE and equilibrate the gl in transfer buffer for 10-30mins.
2. Place a pair of blotting sheet (already saturated with transfer buffer) on the anode plate (usually this plate is black colored).

3. Place the transfer membrane on the top of blotting sheets and remove trapped air bubble by rolling test tube or glass rod.
4. Place the PAGE gel on the top of membrane and gently remove trapped air bubble by rolling test tube or glass rod.
5. Place another blotting sheet (already saturated with transfer buffer) on the top and remove trapped air bubble by rolling test tube or glass rod.
6. Finally keep cathode plate (usually red colored) and tight the transfer cassette by four screws. The whole transfer assembly is given in the Figure 15.2.

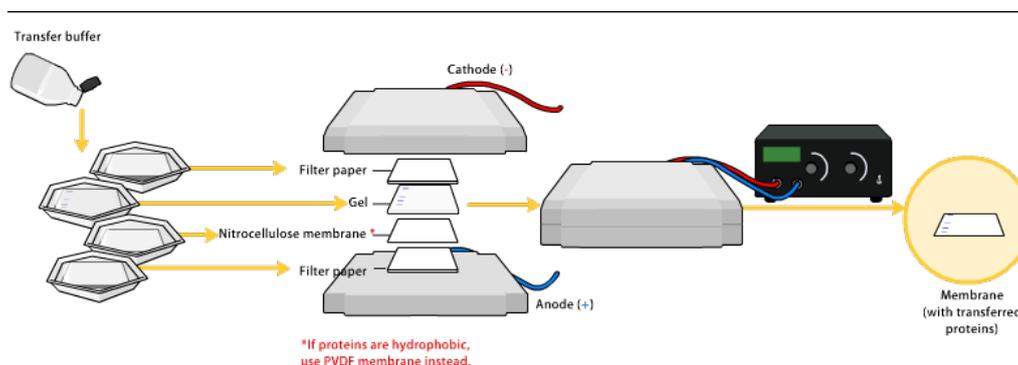


Figure 15.2: Steps in electroblotting of protein on the membrane.

**C. Transfer protein from gel to the membrane:** Place the transfer cassette in the tank filled with the transfer buffer. Connect the transfer apparatus to the power supply unit and apply constant voltage (100 volts for 1hrs). After transfer, disassembly the whole cassette and carefully remove transfer membrane and check the protein transfer by ponceau S stain. Use a pencil and label different lanes.

**STEP 4: Blocking:** Wash the membrane with distilled water to remove any remaining ponceau S stain. Put the membrane in blocking buffer containing 5% skimmed milk (for detection of cytosolic protein) or 5% Fat free BSA (for detection of phosphor protein) for 30 mins.

**STEP 5: Probing:** In western blotting, probing can be done in two different ways.

**A. Two Step Probing:** In two step probing scheme, membrane is first probed with the primary antibody to recognize the protein of interest. (Generation of monoclonal or polyclonal antibody is discussed in the later lectures). Membrane is probed with the primary antibody with an appropriate dilution for 1hrs at room temp (RT). Membrane is washed with buffer containing non-anionic detergent Triton X-100 and reprob

with another antibody directed against the primary antibody. The secondary antibody is coupled with an enzyme (either HRP or alkaline phosphatase) or a fluorescent dye. Washed membrane is incubated with the secondary antibody with an appropriate dilution for 1hrs at room temp (RT). Membrane is washed with buffer containing non-anionic detergent Triton X-100 and developed. Use of two different antibodies increases sensitivity as well as giving flexibility to plan multiple probing.

**B. One Step Probing:** In one step probing, primary antibody contains enzyme or fluorescent label for detection. One step probing is not very common.

**STEP 6: Blot Development :** There are multiple ways to develop the blot and detect the protein present on the membrane. A list of reagents to develop blot are given in the Table 15.1.

**A. Colorimetric Detection:** The different colorimetric reagents are given in the Table 15.1. Wash the membrane with TBS to remove detergent. Place the membrane into the colorimetric reagent and protein bands appeared in 10-30mins. Stop the reaction by washing in distilled water. Air dry the membrane and photograph for permanent record.

**B. Chemiluminescent Detection:** The different chemiluminescent reagents are given in the Table 15.1. Transfer the membrane into the chemiluminescent reagent. Soak the membrane for 30sec to 5mins. Drain off the reagent and wrap the membrane into the plastic wrap. Place it in film cassette and expose the membrane to film for a few seconds to several hours.

**C. Fluorescent detection:** Secondary antibodies labeled with fluorescent dye and captured in the scanner.

**Table 15.1: Different Reagents Available to develop western blot.**

<b>System</b>	<b>Reagent</b>	<b>Reactions</b>
<b>Chromogenic</b>		
HRP Based	4-chloro-naphthol	Oxidized Product to form purple precipitate
	DAB/NiCl <sub>2</sub>	Forms dark brown precipitate
	TMB	Forms dark purple stain
Alkaline phosphatase	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT.
<b>Luminescent</b>		
HRP Based	Luminol/H <sub>2</sub> O <sub>2</sub>	Oxidized luminal gives off blue light.
Alkaline Phosphatase	Substituted 1,2 dioxetane phosphate	Dephosphorylated substrate gives off light

## Lecture 16 II)

## Electrophoresis (Part-

**Background Information:** The complex biological samples are efficiently resolved in 2-D gel electrophoresis. It involves the combination of charge and molecular weight to provide much greater separation in comparison to use of the individual property. The 2-D electrophoresis is a combination of isoelectrofocusing (IEF) followed by SDS-PAGE in a perpendicular direction (Figure 16.1). The isoelectric point (pI) separates the sample based on their isoelectric pH (it is indirectly related to the charge present on the protein) whereas SDS-PAGE separates the molecules based on size (it is indirectly related to the molecular weight). In general analysis of complex bacterial lysate or tissue extract produces 1000-2500 well separated spots. With a sensitive detection tool and image analysis software, individual can be identified and compared under different conditions.

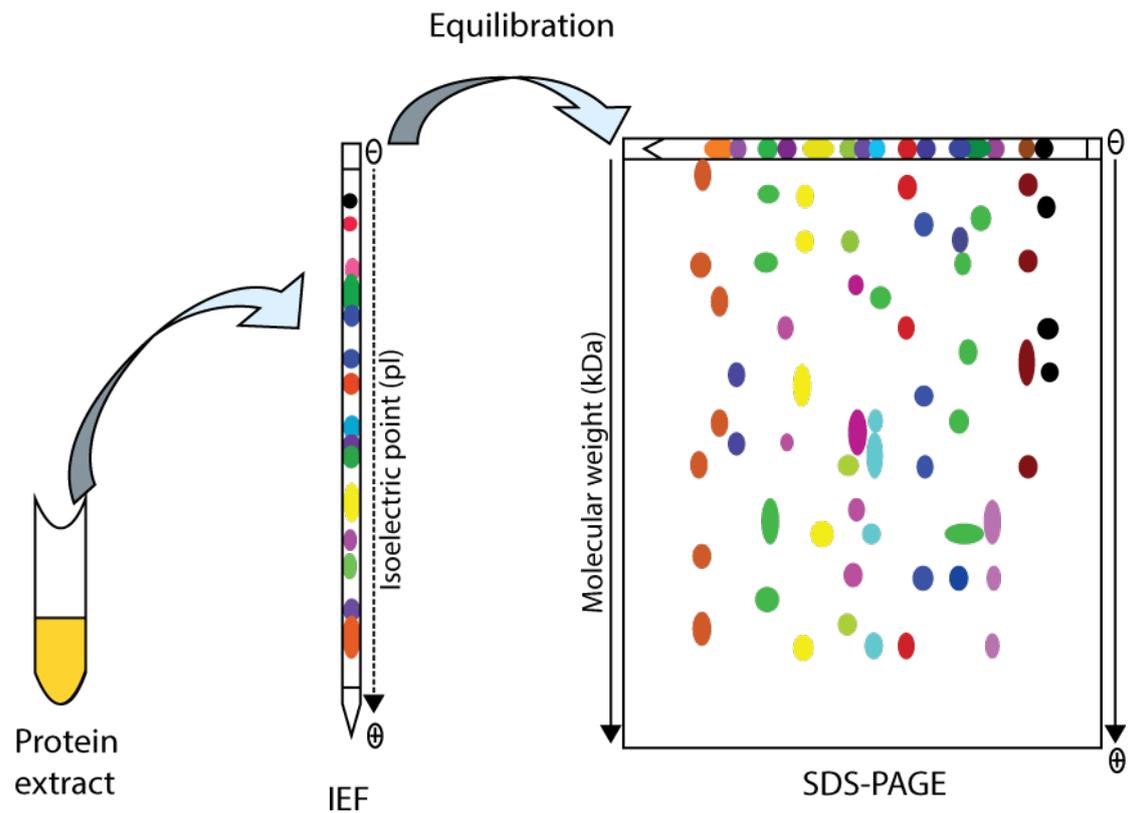


Figure 16.1: An Over-view of 2-Dimensional Gel Electrophoresis. needs redraw from

## **Lab Experiment 16.1 : Analyze the complex E.coli lysate in 2-D Gel electrophoresis.**

### **Material and methods:**

IPG

Reagents for SDS-PAGE

Reagents for silver stain

Trypsin

MALDI-Tof

**Protocol :** The complete process of 2-D gel electrophoresis is given in the Figure 16.2 and it has following steps:

### **STEP 1: Protein Extraction**

1. Tissue or cells were frozen in liquid and ground to fine powder. The powder was mixed with chilled (200C) 10% Trichloacetic acid in acetone with 1% DTT.
2. The tissue suspension was incubated for 1hr at 20<sup>0</sup>C and the mixture is centrifuged at 35000g for 15mins at 4<sup>0</sup>C.
3. Discard the supernatant and carefully dissolve the pellet into the ice cold acetone containing 1% DTT. Incubate the suspension for 1hr at -20<sup>0</sup>C and subsequently centrifuge the the mixture at 35000g for 15mins at 4<sup>0</sup>C.
4. Discard the supernatant and pellet was freeze dried multiple times.
5. 25mg of pellet were suspended in 750µl of lysis buffer (9M Urea, 4% CHAPS, 0.8% ampholyte pH 3-10 and 1% DTT) and incubate for 1hr at 37<sup>0</sup>C.
6. Centrifuge the suspension at 12000g at room temperature and collect the supernatant in fresh tube.
7. Determine the protein concentration by lowry or Bradford method.

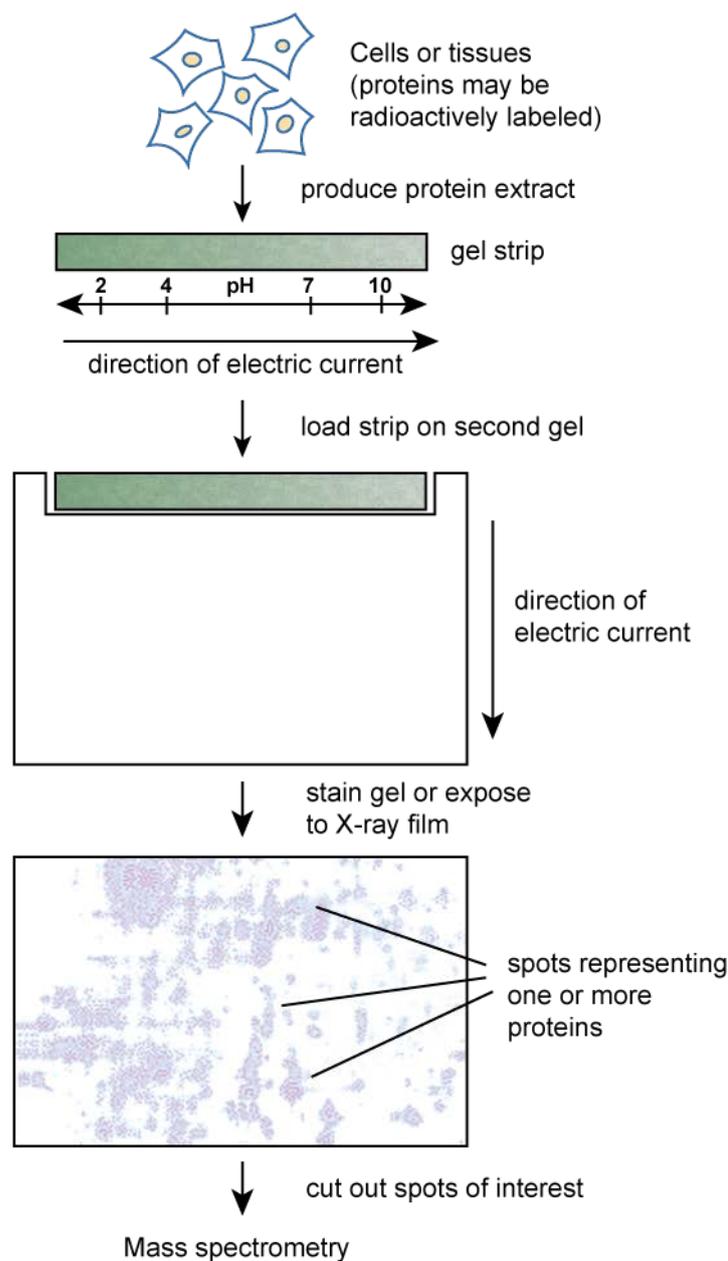


Figure 16.2: Different Steps in 2-Dimensional Gel Electrophoresis. needs redraw from

## STEP 2: First Dimension Gel Electrophoresis

1. IPG strips (pH 3-10) were rehydrated overnight with 350  $\mu$ l of rehydration buffer (8M Urea, 2% CHAPS, DTT (7 mg per 2.5 ml of rehydration buffer) and 0.5% (v/v) IPG buffer pH 3-10).
2. IPG rehydrated strip loaded with 100 $\mu$ g proteins in a reswelling tray at room temperature.
3. The focused strips were equilibrated twice (15min each time), 1st equilibration in 50mM Tris-HCl buffer containing 30% (w/v) glycerol, 2% (w/v) SDS, 6M urea, 1%

(w/v) DTT and and 2<sup>nd</sup> equilibration was performed in a solution containing 4% (w/v) iodoacetamide instead of DTT.

4. Isoelectric focusing (IEF) was conducted at 20°C with running conditions as follows: 1st hr at 500V , followed by 1000V for 2<sup>nd</sup> hr and finally 16 hrs at 3000V.

The IPG strips will be taken out from the apparatus and 2<sup>nd</sup> dimensional separation will be perform by SDS-PAGE in a vertical slab of acrylamide.

### **STEP 3: Second Dimension SDS-PAGE Electrophoresis**

The protocol to perform SDSPAGE is discussed in a earlier lecture.

### **STEP 4: Gel Silver Staining**

The protocol to perform silver staining is discussed in a earlier lecture.

## Lecture 17

### Electrophoresis

## Horizontal Gel

**Horizontal gel electrophoresis-** The electrophoresis in this gel system is performed in a continuous fashion with both electrodes and gel cassette submerged within the buffer. The schematic diagram of a vertical gel electrophoresis apparatus is given in Figure 17.1. The electrophoresis chamber has two platinum electrodes placed on the both ends are connected to the external power supply from a power pack which supplies a direct current or DC voltage. The tank filled with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories needed for casting the agarose gel such as comb (to prepare different well), spacer, gel caster etc.

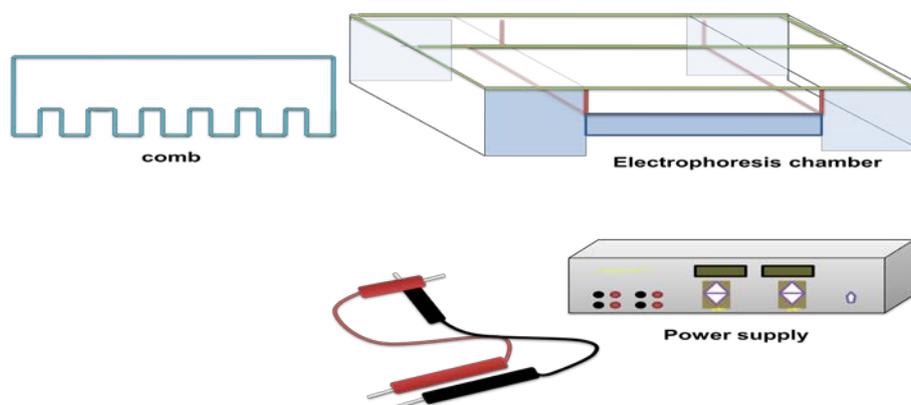


Figure 17.1: Different components of horizontal gel electrophoresis apparatus.

**Buffer and reagent for electrophoresis-** The purpose of each reagents used in horizontal gel electrophoresis are as follows-

1. **Agarose-** polymeric sugar used to prepare horizontal gel for DNA analysis.
2. **Ethidium bromide-** for staining of the agarose gel to visualize the DNA.
3. **Sucrose-** For preparation of loading dye for horizontal gel.
4. **Tris-HCl-** The component of the running buffer.
5. **Bromophenol blue-** Tracking dye to monitor the progress of the electrophoresis.

**Casting of the agarose gel-** Different steps to cast the agarose gel for horizontal gel electrophoresis are given in Figure 17.2. The agarose powder is dissolved in a buffer (TAE or TBE) and heated to melt the agarose. Hot agarose is poured into the gel cassette and allowed it to set. A comb can be inserted into the hot agarose to cast the

well for loading the sample. In few cases, we can add ethidium bromide within the gel so that it stains the DNA while electrophoresis.

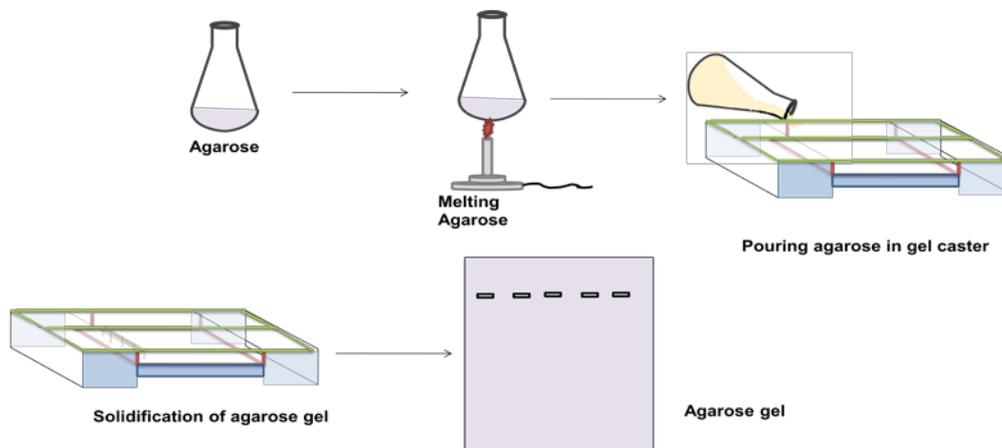


Figure 17.2: Different steps in casting of the agarose gel for horizontal gel electrophoresis apparatus.

**Running and staining-**The gel cassette is placed in the electrophoresis tank submerged completely and DNA loaded into the well with the help of pipetman and run with a constant voltage. DNA runs from negative to positive end and ethidium bromide (EtBr) present in the gel stain the DNA. Observing the agarose gel in a UV-chamber shows the DNA stained with EtBr as orange colored fluorescence (Figure 17.3).

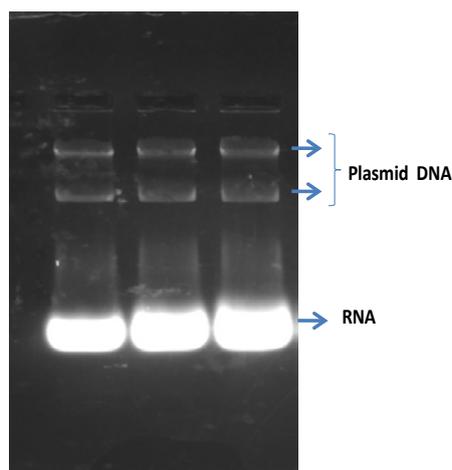


Figure 17.3: Observation of DNA stained with EtBr in a UV chamber.

**Laboratory Experiments related to the Horizontal Agarose Gel Electrophoresis:**

Horizontal gel electrophoresis is used to answer several biological, molecular biology and cell biology question. Here, in today’s lecture we will discuss few selected example of experimental approaches of horizontal gel electrophoresis-

**Lab Experiment 17.1 : Analyze the given DNA sample and determine the molecular weight using horizontal gel electrohpresis.**

**Background Information:** The size of a DNA can be determined by comparing the size of the known DNA molecules. The DNA of known sizes are resolved on 0.8% agarose along with the unknown sample. **Calculation of molecular weight of the unknown DNA sample is a 5 step process:**

1. Resolve the DNA on the Agarose Gel along with the DNA molercular weight markers.
2. Calculate the relative mobility (Rf) using the following formula:  
Rf=.....
3. Plot log molecular mass (y-axis) versus relative mobility (x-axis) of the standards.
4. Perform a linear regression using a calculator or using regression software such as Microsoft Excel.
5. Use the linear regression equation ( $y=mx+c$ ) to estimate the mass of the unknown DNA.

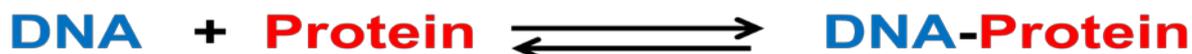
$$\text{Log Molecular Weight}=(\text{slope})(\text{mobility of the unknown})+\text{Yintercept}$$

**Materials Required:** Agarose, Buffers and reagents for horizontal gel as discussed before.

**Procedure:** The value of the relative migration (Rf) of each DNA band is calculated from the agarose gel. The values of relative migration (Rf) and size of the DNA is used to draw the calibration curve to calculate the size of the unknown DNA samples.

**Lab Experiment 17.2 : Analyze the interaction between single stranded binding protein and different DNA species.**

**Background Information:** DNA is a negatively charged molecule and it interact with positively charged protein to form DNA-protein complex. The size and the hydrodynamic volume changes when DNA is interacting with protein to form DNA-protein complex.



Hence, at the end of the experiment, we can be able to understand several aspects of DNA-protein interaction:

1. Whether protein-X has a affinity for DNA and the interaction is specific or non-specific in nature.
2. What will be affinity parameters of the interaction of DNA to protein in making DNA-protein complex?

**Materials Required:** Agarose, TAE Buffer, EtBr, Pure SSB, DNA

**Procedure:** To study the DNA-protein interaction, a fix amount of DNA is incubated with the increasing concentration of protein (Figure 17.4). Due to the formation of DNA-protein complex, the hydrodynamic volume of the complex increases and a shift in band is observed. The DNA has a extended structure and it provides docking site for several protein molecules such as single stranded binding protein (SSB).

**Observation:** As a result, a gradual shift in DNA band will be observed until the DNA binding site is not saturated with the protein molecules.

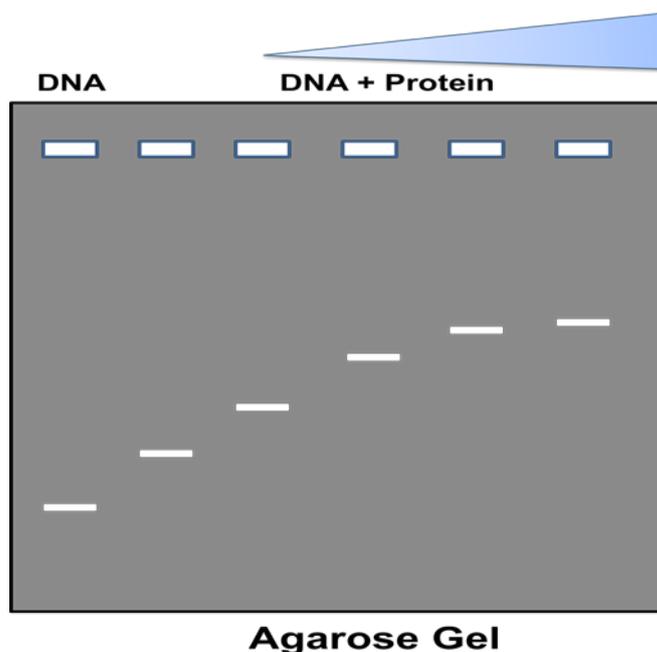


Figure 17.4: DNA-Protein interaction analysis by agarose gel electrophoresis.

### Lab 17.3: Electroelute the given protein sample and determine its concentration.

**Background Information-** protein band present within the polyacrylamide gel block is removed by electroelution for further usage (Figure 17.5). During electrophoresis the protein band migrate and ultimately comes out from the gel block. Due to dialysis bag, salt and other small molecule contaminant moves out of the dialysis bag but protein remain trapped within the dialysis bag. Protein can be recovered from the dialysis bag for further use in downstream processing.

**Materials Required:** Agarose, Dialysis, Horizontal Gel Tank, Horizontal Tank Buffer, Dialysis Clips.

**Procedure:** In this procedure, a gel band is cut from the SDS-PAGE and placed in a dialysis bag and sealed from both ends. The dialysis bag is chosen so that the molecular weight cut off of dialysis membrane should be lower than the protein of interest. The dialysis bag is placed in the horizontal gel apparatus with buffer and electrophoresis is performed with a constant voltage.

**Results and Conclusions:** After running the protein isolated from the excised slice gives protein band in the SDS-PAGE.

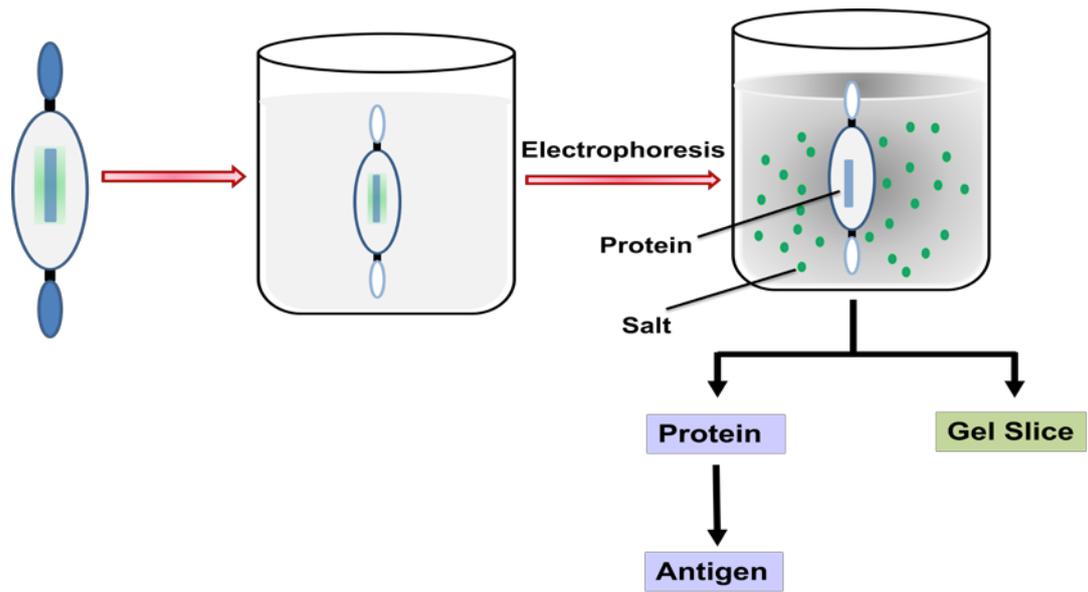


Figure 17.5: Electroelution using horizontal gel electrophoresis apparatus.

## Lecture 18

## RNA gel

**Lab Experiment 18.1 : Resolve the mRNA isolated from the liver on the denaturing Agarose gel electrophoresis.**

**Background Information:** The protocol for RNA gel is similar to the DNA gel discussed in the previous lecture except that the RNA gels are performed under denaturing conditions. RNA sample and the agarose gel contains formaldehyde to denature the secondary structure present in the RNA and prevent the re-formation of double stranded region in the RNA structure (formation of secondary structure affects the migration and resolution of RNA on agarose gel). Presence of secondary structure in RNA allows the faster migration of RNA on agarose gel. As a result, it gives less time for the molecule to interact with the agarose gel and consequently less resolution within the different RNA species. Destruction of secondary structures in the RNA structure minimizes these effects and allows better separation on agarose gel.

### Materials:

1. Agarose, Molecular Biology Grade.

2. 10x MOPS Buffer:

Composition:

0.4M 3-(N-morpholino)-propanesulphonic acid (MOPS), pH 7.0

0.1M sodium acetate

10mM EDTA, pH 8.0

MOPS buffer can be stored upto 3 months at 4<sup>0</sup>C. For preparing 1xMOPS buffer, it can be diluted with RNase free water.

3. 37% formaldehyde, pH > 4.0

4. RNA molecular weight ladder

5. 0.5M ammonium acetate

6. 0.5µg/ml ethidium bromide in 0.5M ammonium acetate

7. RNase Free H<sub>2</sub>O

8. Formamide

9. Formaldehyde loading buffer:

Composition:

1mM EDTA, pH 8.0

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

50% Glycerol.

Loading buffer can be filter sterilized by passing through 0.22µm filter. It can be aliquot into the small volume and store at -20<sup>0</sup>C.

10. Autoclave gloves

11. 55<sup>0</sup>C water bath

12. Horizontal Gel electrophoresis system
13. Power supply
14. RNase free container for staining and destaining
15. Shaker
16. UV chamber
17. Gel Doc
18. Flask for preparing agarose gel

### Methods:

**1. Isolation of mRNA-** The structure of a typical mRNA is given in Figure 18.1. it has a n-terminal CAP structure, coding sequence and a c-terminal poly A tail. The Nucleotide A forms 2 hydrogen bonding with nucleotide T and this pairing is very specific. Exploiting this feature, m-RNA population can be isolated from RNA pool using a poly-T affinity column. The Steps in m-RNA isolation from cell is given in Figure 18.2. it has following steps:

1. Release of total RNA either by a lysis buffer containing detergent or by homogenization in the case of hard tissue.



Figure 18.1: Structure of a typical mRNA.

2. Mixing of poly-T containing beads with the total RNA preparation. Due to mutual exclusive affinity, mRNA binds to the poly-T beads.
3. Wash the beads with washing buffer to remove non-specific cross contaminating species.
4. Elute the mRNA from beads and its purity can be checked on polyacrylamide gel (discussed later in future lectures).

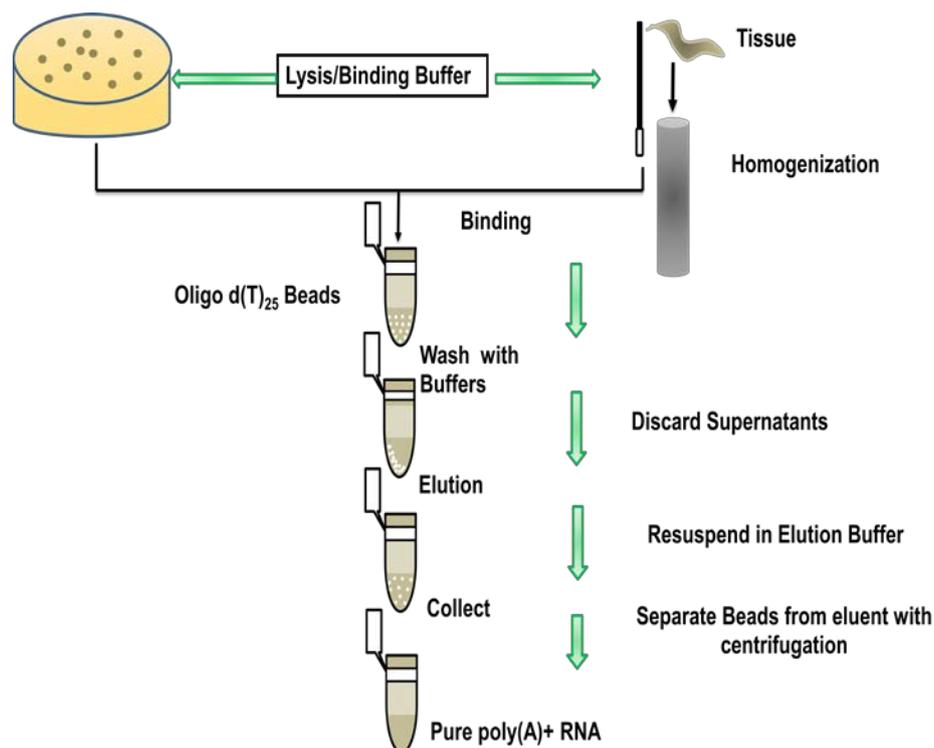


Figure 18.2: Isolation of mRNA from total cell lysate.

## 2. Preparation and casting of denaturing agarose gel:

### (A) Preparation of RNase Free Water:

1. Dissolve diethylpyrocarbonate (DEPC) in deionized distilled water to a final concentration of 0.1%.
2. Stir this solution for 12hrs at RT and then autoclave to disintegrate DEPC. Store at RT.

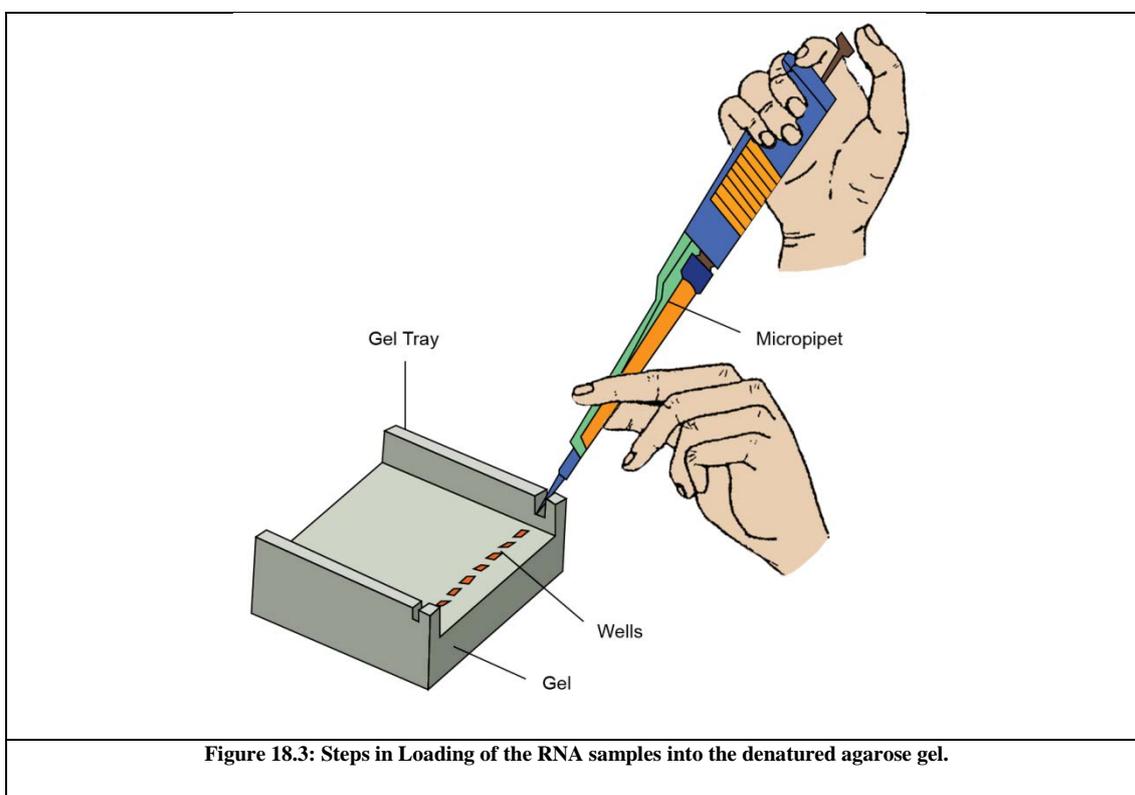
### (B) Casting of agarose gel:

1. In a flask, add 1g agarose to the 75ml of RNase free water.
2. Heat the solution to melt the agarose and observe the disappearance of agarose flecks.
3. Allow the solution to cool down upto 55°C. Inside a fuming hood, add 10ml 10x MOPS buffer and 18ml 37% formaldehyde.
4. Setup the casting tray with comb and pour the gel in fuming hood. [Caution: formaldehyde is very toxic and can be easily absorbed through skin, wear gloves and mask].

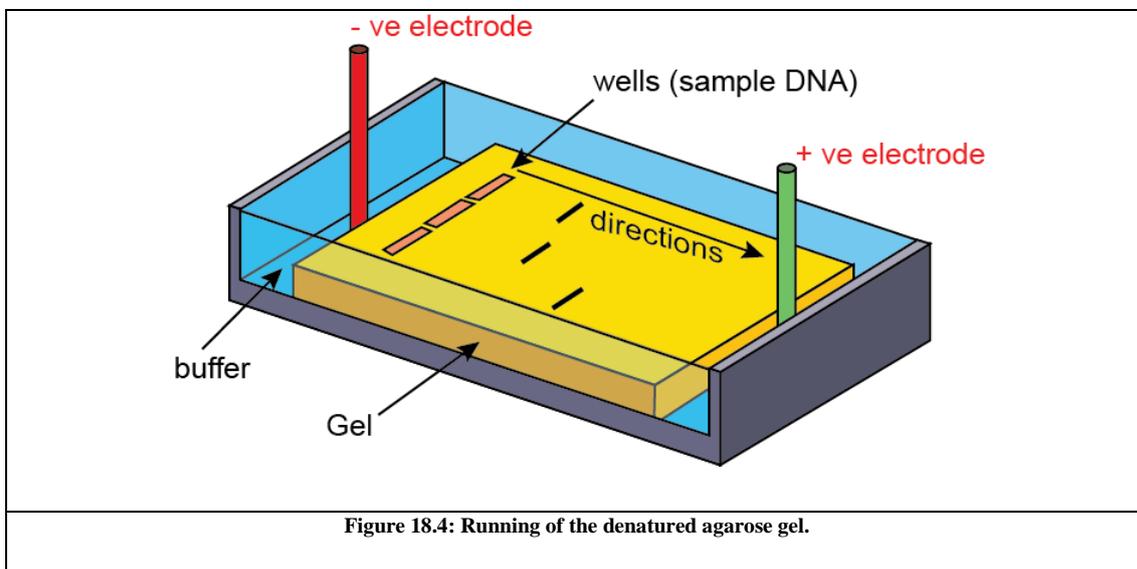
### 3. Prepration of RNA sample:

1. Take the RNA sample and make up-to 6.5 $\mu$ l with appropriate quantity.
2. To each sample, add 2.5 $\mu$ l 10x MOPS running buffer, 4.5 $\mu$ l 37% formaldehyde, 11.5 $\mu$ l formamide.
3. Mix by vortexing and briefly spin to collect the sample at the bottom.
4. Inside the hood, add 5 $\mu$ l RNA loading buffer, mix by vortexing and briefly spin to collect the sample at the bottom.

**4. Loading of the RNA sample:** Fill the agarose denatured gel prepared with the 1x MOPS running buffer. Load the RNA sample into the lane (Figure 18.3).



**5. Running the denaturing agarose gel:** Place the lid onto the buffer chamber and perform electrophoresis at 5v/cm until the dye front reaches the 2/3 length of the gel (Figure 18.4).



### 6. Staining of agarose gel:

1. In a RNase-free container, agarose gel is dipped into the 0.5M ammonium acetate for 40mins at RT.
2. Drain off the solution and dip the block in the 0.5M ammonium acetate containing 0.5 $\mu$ g/ml ethidium bromide.
3. Incubate on RT for 30-40mins.
4. If required and the stain is too intense, it can be destain by 0.5M ammonium acetate for another 60mins.
5. Transfer the gel to a UV chamber and capture the image with the gel documentation unit.

## Lecture 19

## Nucleic acid Detection

### Lab Experiment 19.1 : Detect the LDH gene in the human genome using southern blotting.

**Introduction:** In southern blotting, the genomic DNA is digested with the EcoRI or BamHI and the DNA fragments are resolved on the agarose gel. The gel is incubated in an alkaline solution to denature the double stranded DNA to single stranded form. DNA is transferred on the nitrocellulose membrane by capillary action by applying a uniform pressure either by suction pressure or by placing wet paper towels. The membrane is incubated with a non-specific DNA such as sonicated calf thymus genomic DNA to block the binding sites on the membrane. A single stranded radioactive probe is added to the membrane and allowed to bind. Membrane is washed and the blot is developed by autoradiography. The DNA fragment complementary to the probe sequence binds the radioactive probe and give positive signal (Figure 19.1).

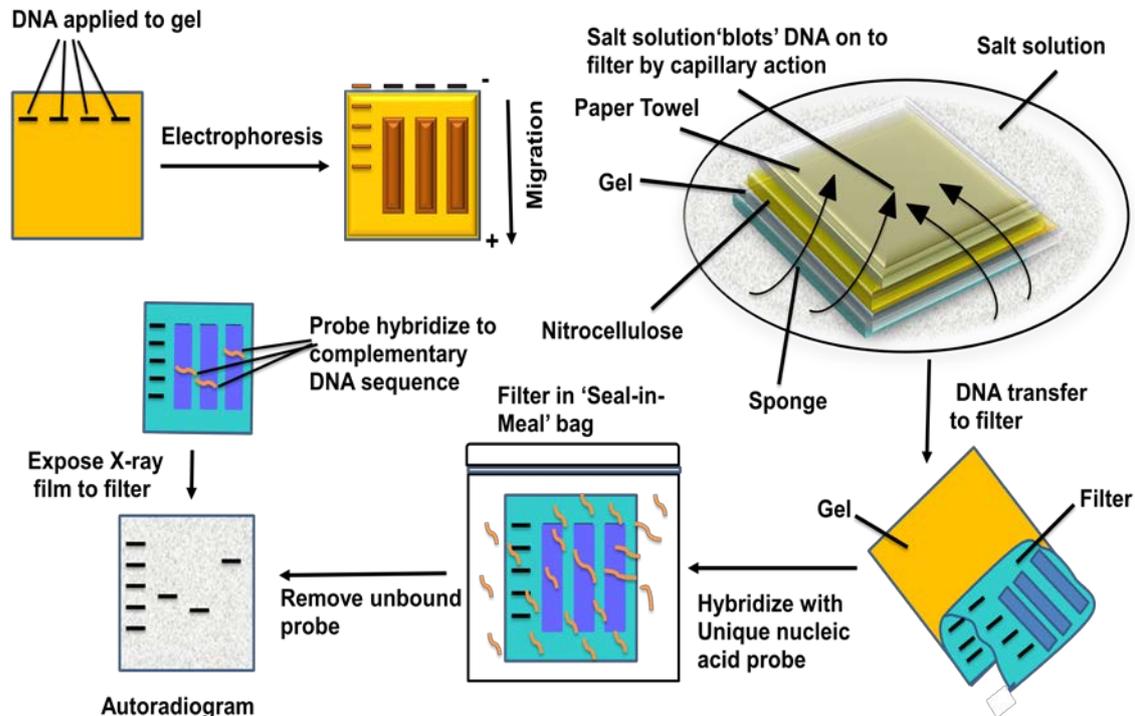


Figure 19.1: General Over-view of different steps in Southern Blotting.

Materials and Instruments.

### **Methodology:**

#### **A. Restriction Digestion of Genomic DNA:**

1. Isolate the high weight molecular weight genomic DNA from the cells.
2. Digest 10-20 $\mu$ g of the genomic DNA with EcoRI (2-3 unit for each  $\mu$ g genomic DNA) over-night in the presence of appropriate buffer and BSA (if required).
3. Take out small aliquot from the restriction digestion reaction and check on 0.8% agarose gel. Appearance of smear with visible bands indicate complete digestion of genomic DNA and the suitability of the sample for southern blotting.

**B. Separation of DNA on agarose gel:** Agarose gel electrophoresis is a standard gel to resolve the DNA and it is discussed in detail in the lecture Lecture 18. As a standard practice, agarose used for southern blotting is of ultra-pure quality to avoid contaminants affecting migration of DNA. Observe the gel in UV-transilluminator and record the pattern in a gel documentation system.

**C. Blotting of DNA on the nylon membrane:** Here I am discussing two most popular methods to transfer the DNA onto the nitrocellulose membrane.

**i. Capillary Transfer method:** The setup to perform capillary transfer of DNA from the gel to the nitrocellulose membrane is given in the Figure 19.2.

1. Soak the gel in 0.25N HCl for 10mins with shaking at RT. This step depurinate the DNA and facilitates the transfer of larger DNA fragments. This step can be omitted if the fragmented DNA is of smaller size.
2. Remove the depurination solution. Wash the gel with double distilled water. Submerge the gel into the alkaline solution for 30mins at RT with gentle shaking. Alkaline solution will denature the DNA.
3. Remove the denaturation solution. Wash the gel with double distilled water. Submerge the gel into the neutralization solution for 30mins at RT with gentle shaking. This step will neutralize gel without denaturing DNA.

4. Pour off the neutralization solution and incubate the gel in the 10xSSC buffer for 30mins with gentle shaking.

**5. Preparation of nitrocellulose membrane for transfer:** cut a piece of nylon membrane to the size of agarose gel, mark the nylon membrane by cutting on one corner. Wash the nylon membrane with the double distilled water and then soak the membrane in transfer buffer.

6. Cut 2 pieces of 3mm whatman filter paper slightly bigger size to the gel. Wet the filter paper with the transfer buffer.

7. Setup the blotting tray as given in Figure 20.2. Add 500-800ml transfer buffer (10xSSC) in the transfer tray. On a glass solid support, place a piece of non-woven wiper with the size of the solid support to serve as wick. Dip both ends of the wick in the transfer buffer. Wet the wick and remove the trapped air bubble by rolling glass rod.

8. Place the gel on the top of wick and remove bubbles by rolling glass rod.

9. Over-lay nylon membrane on the gel and carefully remove the trapped air bubble by rolling glass rod. (gel and nylon membrane should be handled with wearing gloves).

10. Carefully lay the filter paper on the membrane, remove the trapped air bubble by rolling glass rod. Cover the whole setup with saran wrap to avoid loss of untargeted loss of water.

11. Place a stack of paper towel over the filter paper. Place the glass plate and a weight (0.5-1kg) on the top of glass plate.

12. Allow the capillary transfer for a period of 4-16 hrs. During this period, if filter paper gets wet, change the filter to maintain the capillary action. Do not allow the setup to dry out.

13. Remove the paper towel stack, remove the membrane and rinse with 2xSSC buffer.

14. Check the DNA transfer blotting on the membrane by visualizing the membrane in UV light. Under the UV light, mark the positions of DNA and different lanes.

15. Immobilize the DNA on the membrane with a UV cross linker with an optimal setting.

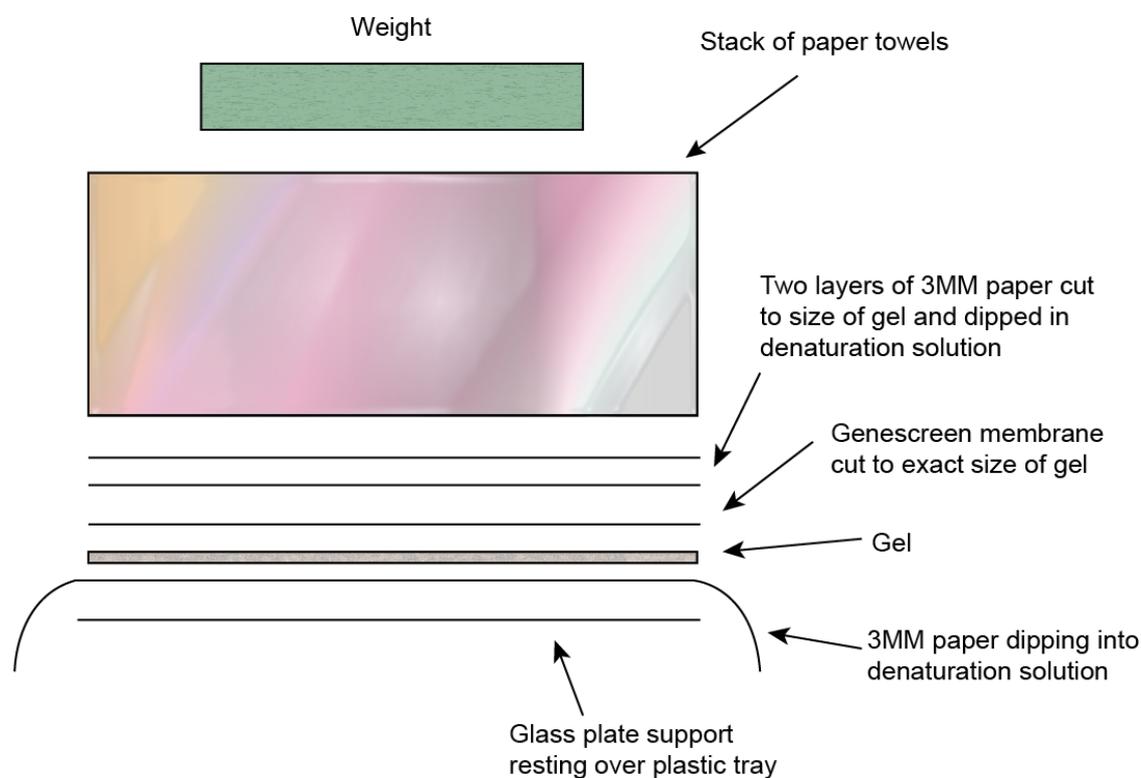


Figure 19.2: Step up for Capillary Transfer of DNA from gel to the nitrocellulose membrane [Redraw

**ii. Vacuum Transfer Methods:** Compare to the capillary transfer method, vacuum transfer method is more efficient. Complete transfer of DNA from gel to the nylon membrane takes place in couple of hours compared to 16-20hrs in capillary transfer. Setup of the vacuum blot to transfer the DNA from gel to the membrane is given in the Figure 19.3.

1. Treat the gel as described in the step 1-5 of the capillary methods.
2. Cut the nylon membrane and the silicon mask with an opening smaller than the size of the gel.
3. Place the wet 3mm whatman filter paper on the metal grid.
4. Place the nylon membrane on the 3mm whatman filter paper and subsequently put mask.
5. Position the gel on the mask and place the cover of the vacublot to hold the mask.

6. Apply the vacuum (~75mm Hg) and initiate the transfer process.
7. Pour 5-10ml of depurination solution to cover the gel and let the solution permeate the gel.
8. Remove the depurination solution and replace it with 20ml denaturation solution and let the solution permeate through the gel.
9. Replace the denaturation solution with neutralization solution and let the solution permeate through gel.
10. Replace the neutralization solution with 10x SSC buffer and let the solution permeate through gel for 30-40mins.
11. Remove excess buffer and turn off the vacuum.
12. Remove the gel and check the transfer under UV.
13. Store the wrapped membrane at room temperature.

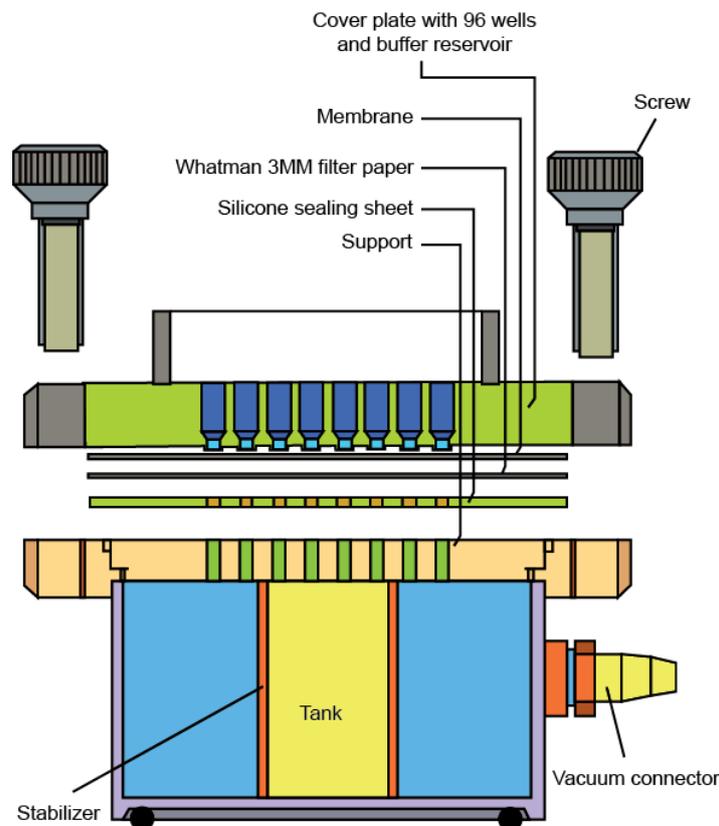


Figure 19.3: Step up for vacuum Transfer of DNA from gel to the nitrocellulose membrane

#### D. Preparation of labeled probe:

**1. Preparation of radioactive probe.** There are two different methods used to label a single stranded DNA probe either at terminal or throughout the sequence.

**A. Random primer method-** In this method, a random primer is used to anneal to the template and then a PCR reaction is performed in the presence of radiolabeled nucleotide. After PCR, newly synthesized DNA strand is labeled with radioactive nucleotide. The whole process is given in Figure 19.4, and it has following steps-

1. The source double stranded DNA is denatured to generate the single stranded DNA template.
2. A random primer is added and allowed it to anneal to the template strand. It will anneal to the random position throughout the sequence at multiple places.
3. Primer will anneal to the template strand and now Klenow will start the synthesis of new DNA strand.
4. Newly synthesized DNA will give short stretches of multiple labeled DNA probes.

**B. Terminal transferase-** In this method, a terminal transferase enzyme will label the probe at the ends to the last nucleotide of the probe. Probe is incubated with the labeled nucleotide and terminal transferase enzyme will add the labeled nucleotide at the end. A partial purification with gel filtration column will give labeled primer.

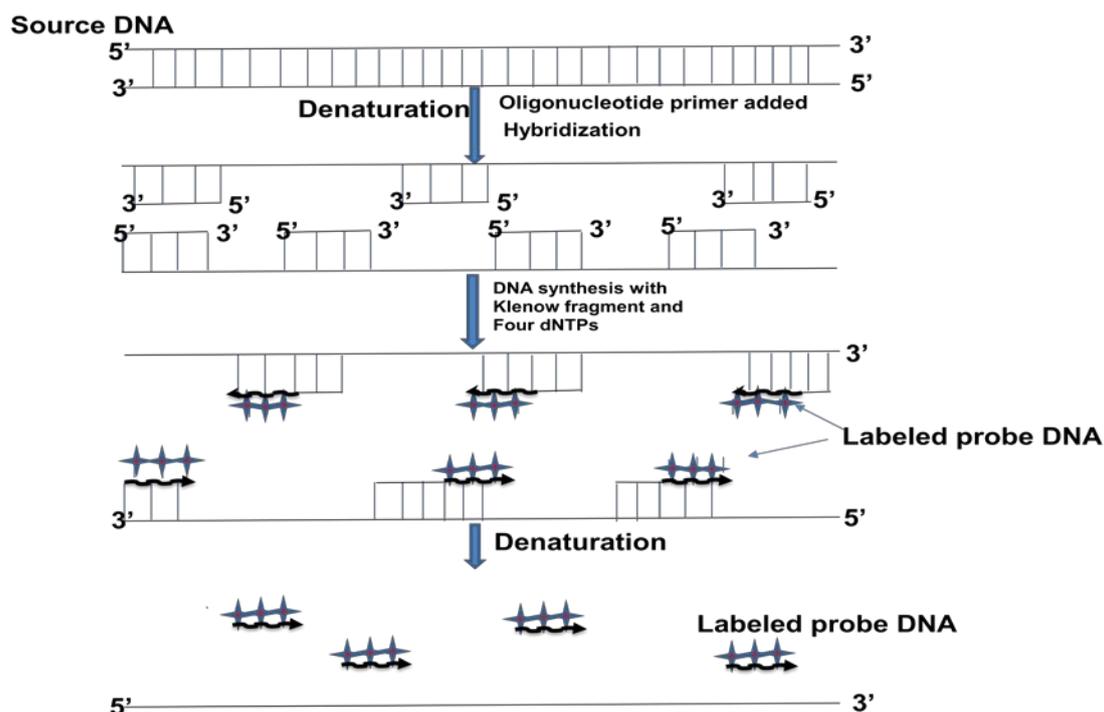


Figure 19.4: Preparation of radioactive probe by random primer method.

#### E. Hybridization and development of Blot:

1. Place the membrane in a hybridization tube and pre-hybridize in 10-20 ml hybridization solution at 65<sup>0</sup>C for atleast 30mins.
2. **Preparation of probe for hybridization:** Denature the probe by boiling for 10mins and quench on ice.
3. Add the probe to the hybridization solution and incubate over-night. Use high temperature (~65<sup>0</sup>C) for high stringency or moderate temperature (50-55<sup>0</sup>C) for low stringency conditions.
4. Rinse the membrane thoroughly with 10ml of washing solution 2xSSC containing 0.1% SDS at room temperature.
5. Expose the membrane to an x-ray film with an intensifying screen.