

**20BTC502J MOLECULAR BIOLOGY AND GENE MANIPULATION
LABORATORY MANUAL**



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIOENGINEERING
SRM INSTITUTE OF SCIENCE AND TECHNOLOGY
KATTANKUALTHUR -603202
2020-2021**

BONAFIDE CERTIFICATE

Register no:

Certified to be the bonafied record of work done by

----- of M Tech Biotechnology
Degree course in the 20BTC502J Molecular Biology and Gene manipulation
laboratory at SRM IST Kattankulathur, during academic year 20 -20

Lab In charge

Head of Department

Submitted for End Semester Examination held in **Molecular Biology Laboratory**
at SRM IST, Kattankulathur

Date:

Examiner I

Examinaer II

DATE:

EXPERIMENT NO. 1

ISOLATION OF PLASMID FROM *E. coli* CELLS

AIM:

To isolate the plasmid DNA from *E. coli* cells.

PRINCIPLE:

Bacterial plasmid DNAs are widely used as cloning vehicles in recombinant DNA research. After new plasmids are constructed, they may be isolated and characterized with respect to their size and restriction enzyme pattern by gel electrophoresis. Plasmid-containing cells are suspended in Solution I which provides alkaline pH. Divalent cations (Mg^{2+} , Ca^{2+}) are essential for DNase activity and the integrity of the bacterial cell wall. EDTA chelates divalent cations in the solution preventing DNases from damaging the plasmid and also helps by destabilizing the cell wall. Plasmid containing cells are lysed completely solution II. SDS is there to solubilize the cell membrane. NaOH helps to break down the cell wall, but more importantly it disrupts the hydrogen bonding between the DNA bases, converting the double-stranded DNA in the cell, including the genomic DNA and also plasmid DNA to single stranded DNA. At an alkaline pH, OH-groups are predominant. They remove the hydrogen- bonds-contributing protons from guanine and thymine, thus breaking the hydrogen bonds between the two oligonucleotides. Addition solution III decrease alkalinity. Under these conditions the hydrogen bonding between the bases of the single stranded DNA can be re-established, so the ssDNA can re-nature to dsDNA. This is the selective part. While it is easy for the small circular plasmid DNA to re-nature it is impossible to properly anneal those huge genomic DNA stretches. While the double-stranded plasmid can dissolve easily in solution, the single stranded genomic DNA, aggregates to form an insoluble network. Simultaneously, the high concentration of potassium acetate causes precipitation of protein-SDS complexes and of high molecular weight RNA. On phenol chloroform extraction, phenol keeps the protein

unfolded and chloroform increases the efficiency of phenol for denaturation of the protein. Here, chloroform allows proper separation of the organic phase and aqueous phase which keeps DNA protected into the aqueous phase. The Plasmid DNA present in the aqueous phase is ethanol precipitated.

MATERIALS REQUIRED

1. Table top cooling centrifuge
2. Micropipettes and Tips
3. 2 mL Microcentrifuge tubes
4. Isopropanol

BUFFERS AND REAGENTS

Alkaline Lysis Solution I

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Prepare Solution I from standard stocks in batches of approx. 100 mL, autoclave for 15 min at 15 psi (1.05 kg/cm²) on liquid cycle, and store at 4°C.

Alkaline Lysis Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1% (w/v) SDS

Prepare Solution II fresh and use at room temperature.

Alkaline Lysis Solution III

5 M potassium acetate, 60.0 mL

Glacial acetic acid, 11.5 mL

H₂O, 28.5 mL

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

TE Buffer, 10×

100 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. Store the buffer at room temperature.

Phenol-chloroform mixture

Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE (pH 8.0) buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it on ice.

70% Ethanol

With 70 ml of 99.9% ethanol add 30 ml of H₂O. Store the solution at 4°C and transfer it to an ice bucket just before use

PROCEDURE:

1. Take 2 ml overnight culture and harvest cells by centrifugation for 5 minutes. Discard the supernatant carefully.
2. Add 100 µl of solution I to the cell pellet and resuspend the cells by gentle mixing.
3. Incubate the above mixture at room temperature for 5 minutes.
4. Add 200 µl of solution II to the mixture and mix by inverting the tubes for 5 minutes.
5. Incubate for 5-10 minutes at room temperature.
6. Add 500µl of ice cold solution III to the mixture and mix by inverting the tube.
7. Incubate on ice for 10 minutes.
8. Centrifuge at 10,000 rpm for 5 minutes.
9. Transfer the supernatant into fresh tube.
10. Add 400 µl of phenol-chloroform mixture to the contents, mix well by inverting and incubate them at room temperature for 5 minutes.
11. Centrifuge at 10000 rpm for 5 minutes.
12. Collect the supernatant using microtips and transfer to a fresh tube.
13. Add 0.8 ml of isopropanol and mix gently by inversion.

14. Centrifuge the contents at 10,000 rpm for 10 minutes.
15. Discard the supernatant after centrifugation.
16. Add 0.5 ml of 70% ethanol. Centrifuge the contents at 5,000 rpm for 5 minutes
17. Discard the supernatant
18. After air-drying for 5 minutes, add 30 μ l of TE buffer or autoclaved distilled water to the pellet to resuspend the plasmid DNA.

RESULT:

DATE:

EXPERIMENT NO. 2

QUALITATIVE ANALYSIS OF PLASMID DNA

AIM:

Analysis of Plasmid DNA by agarose gel electrophoresis

PRINCIPLE:

Electrophoresis through agarose gels is used to separate, analyze, identify, and purify DNA fragments. DNA contain negative charges because of the back bone PO_4 . This property is exploited to separate DNA based on their size in an electric field with an agarose gel as a stationary phase. The location of bands of DNA within the gel can be determined directly by staining with low concentrations of fluorescent intercalating dyes, such as ethidium bromide; bands containing as little as 20pg of double-stranded DNA can then be detected by direct examination of the gel in ultraviolet (UV) light.

The agarose, for agarose gel electrophoresis is purified from agar. Agarose is a linear polymer made up of repeating units of 1,3 –linked β D galactopyranose and 1, 4 linked 3,6 anhydro a L galactopyranose [P-D –gal (1-4)-3,6 anhydro – a L Gal (1-3) -]n

Agarose has an average MW of 12,000 and contains about 35-40 agarobiose units. Agarose in solution exist as a left handed double helices. About 7 to 11 such helices form bundles which extend as long rods and appear to intertwine with one another, further strengthening the frame work of the gel. The cross links are held together by hydrogen and hydrophobic bonds. By changing the gel concentration, the pore size can be altered. Higher the concentration of agarose smaller the pore size and vice versa.

Effect of agarose concentration on separation ranges

The following table describes the relationship between agarose concentration and separation range of nucleic acid

<i>Agarose Concentration (%)</i>	<i>Separation range (Kb)</i>
0.3	5 to 60
0.6	1-20
0.8	0.8 to 10
1.0	0.4 to 8
1.2	0.3 to 6
1.5	0.2 to 4

Running Buffer

TRIS Acetate EDTA (TAE) buffer, TRIS Borate EDTA (TBE) buffer both slightly alkaline (pH 8.00 to 8.5) buffer are used in electrophoresis. Electrophoresis that separates on the basis of charge, ions in the buffer transmit the charge necessary for separation. The buffer, also keeps the pH within a narrow range. This is important because the structure and charge nucleic acid will change if subjected to significant pH changes, thus preventing proper separation. The alkaline pH of the buffer keeps the DNA stable and EDTA chelates di valent cations like Mg²⁺ which is required for DNAase activity.

Gel Loading Dye

Gel-loading buffers are mixed with the samples before loading into the slots of the gel. These buffers serve three purposes: They increase the density of the sample, ensuring that the DNA sinks evenly into the well; they add color to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates and used for tracking the sample. Bromophenol blue migrates through agarose gels 2.2-fold faster than xylene cyanol FF, independent of the agarose concentration

Staining the Gel

A convenient and commonly used method to visualize DNA in agarose gels is staining with the fluorescent dye ethidium bromide which contains a tricyclic planar group that intercalates between the stacked bases of DNA. Ethidium bromide intercalates into double-stranded DNA in a sequence-independent fashion

at a maximum stoichiometry of about two dye molecules per turn of the helix. Ethidium bromide can be used to detect both single-stranded and double-stranded nucleic acids (both DNA and RNA). However, the affinity of the dye for single-stranded nucleic acid is relatively low, and the fluorescent yield is comparatively poor

Materials Required

Electrophoretic apparatus – Tank, Tray and Comb.

Power supply device

Micropipettes and Microtips

Buffers and Reagents

TBE buffer (5X)

54 g of Tris base

27.5 g of boric acid

20 mL of 0.5 M EDTA (pH 8.0)

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•2H₂O to 800 mL of H₂O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

The 0.5X working solution is 45 mM Tris-borate/1 mM EDTA.

Gel Loading Buffer (6X)

3.9 mL glycerol

500 µL 10% (w/v) SDS

Dissolve 10g in 100 ml of water

200 µL 0.5 M EDTA

0.025 g bromophenol blue

0.025 g xylene cyanol

Bring to 10 mL total volume with H₂O

PROCEDURE

1. Weigh 0.5 g agarose and dissolve it in 50 mL of 0.5x TBE Buffer. (Note: Use 250 ml conical flask for preparing 50 ml solution to avoid overflow of gel solution while heating and to avoid its loss.)
2. Heat the solution over a hot plate to boiling constituency marked with a clear solution
3. Leave the solution to cool and add 2 μ l of EtBr solution mix it well by gentle swirling.
4. Pour it in the gel tray-comb set up. Also be sure the gel plates have been taped securely and contain the well combs prior to pouring.
5. Allow the gel to set completely (30–45 min at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer, and carefully remove the tape. Mount the gel in the electrophoresis tank.
6. Add just enough electrophoresis buffer to cover the gel to a depth of approximately 1 mm.
7. Mix the samples of DNA with 0.20 volume of the desired 6x gel-loading buffer
8. Slowly load the sample mixture into the slots of the submerged gel using a micropipette,
9. Load size standards into slots on both the right and left sides of the gel.
10. Close the lid of the gel tank, and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1–5 V/cm (measured as the distance between the positive and negative electrodes
11. When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electric current and remove the leads and lid

from the gel tank.

12. Place the gel on the working surface of a UV illuminator. Capture an image of the gel under UV transillumination on a CCD camera equipped with the appropriate screens and emission filters.

RESULTS

DATE:

EXPERIMENT NO. 2

QUANTITATIVE ANALYSIS OF PLASMID DNA

AIM:

Quantitative analysis of Plasmid DNA by spectrometry

PRINCIPLE:

Quantification of nucleic acids is commonly used in molecular Biology to determine the concentration of DNA. DNA exhibit strong absorbance of UV due to the presence of conjugated double bonds of the constant purine and pyrimidine bases and these have characteristics of OD (optical density) of absorbance maximum at 260nm which is linearly related with the concentration of the DNA in the solution up to the OD value of 2. The spectroscopic method is used to check the purity of DNA. Proteins are the major contaminants in the nucleic acid extracts and these have the maximum absorbance at 280nm. Value less than 1.8 signifies the presence of proteins as impurities.

MATERIALS REQUIRED

DNA samples

UV Spectrophotometer

Reagents and Buffer

TE Buffer, 10×

100 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. Store the buffer at room temperature.

PROCEDURE:

1. Take the DNA sample (10 μ l) in TE buffer.
2. Now dilute the above sample by the factor of 100 i. e, by taking 10 μ l of the sample in 990 μ l of TE buffer.
3. After doing this take the optical density value at A260 & A280 and calculate the amount of DNA recovered.
4. Use the following formula to determine the concentration of DNA:

$$\text{Total DNA } (\mu\text{g}) = 50 \text{ mg/ml} \times \text{Absorbance at A260} \times \text{dilution factor} \times 0.1$$

ml

Where 0.1 ml is the total volume of the DNA.

Quality: DNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm. Clean DNA has a OD₂₆₀/OD₂₈₀ between 1.8 and 2.0

RESULTS

DATE:

EXPERIMENT NO. 3
RESTRICTION DIGESTION OF DNA

AIM:

To digest the λ DNA with *EcoRI* and pUC18 with *EcoRI* and *Hind III* restriction enzymes.

PRINCIPLE:

Restriction enzymes are also called 'molecular scissors' as they cleave DNA at or near specific recognition sequences known as restriction sites. These enzymes make one incision on each of the two strands of DNA and are also called restriction endonucleases. Viruses infect the host cells by injecting their DNA into the cells. This viral DNA uses the host cell's machinery for reproduction of viral progeny, resulting in the host cell's death. To overcome the viral infection, many bacteria and archaea have evolved several mechanisms. A major protective mechanism involves the use of restriction enzymes to degrade the invading viral DNA by cleaving it at specific restriction sites. At the same time, the host cell protects its own DNA from being cleaved by employing other enzymes called methylases, which methylate adenine or cytosine bases within host recognition sequences. For each of the restriction enzyme, the host cell produces a corresponding methylase that methylates and protects the host DNA from degradation. These enzymes make up the restriction-modification (R-M) systems. The restriction enzymes catalyse the hydrolysis of the bond between the 3'-oxygen atom and the phosphorus atom in the phosphodiester backbone of DNA. The enzymes require Mg^{2+} or other divalent ions for their activity. Restriction digestion is accomplished by incubation of the target DNA molecule with restriction enzymes - enzymes that recognize and bind specific DNA sequences and cleave at specific nucleotides either within the recognition sequence or outside of the recognition sequence. Restriction digestion can result in the production of blunt ends (ends of a DNA molecule that end with a base pair) or sticky ends (ends of a DNA molecule that end with a nucleotide overhang).

By definition, 1 unit of restriction enzyme will completely digest 1 μ g of substrate DNA in a 50 μ l reaction in 60 minutes. However 5–10 fold over digestion is recommended to overcome variability in DNA source, quantity and purity.

EcoRI,

E-genus Escherichia, co –Species coli, R –strain RY13, I- first endonuclease isolated. ↓

EcoRI 5'GAATTC.....3'

3'CTTAAG.....5'
↑

HindIII

H-genus Haemophilus, in –Species influenza, d –strain Rd, III- third endonuclease isolated.

HindIII 5'AAGCTT....3'

3'TTCGAA....5'

MATERIALS REQUIRED

λ DNA

pUC18

Restriction enzymes (*HindIII* and *EcoRI*)

10X buffer (D)

1Kb or 0.5Kb DNA Ladder

Sterile water

Agarose

6X loading dye

BSA

Ethidium Bromide

1X TAE buffer

Electrophoresis Apparatus

Power supply

Micropipettes

Microtips

PROCEDURE:

1. Prepare the samples according to the following table

Single Digestion

λ DNA (1 μ g)	
Sterile water	
Buffer (D) (10X)	
<i>EcoRI</i> (1U/ μ l)	
Total	10 μl

Double Digestion

pUC18 DNA (1 μ g)	
Sterile water	
Buffer (D) (10X)	
<i>EcoRI</i> (1U/ μ l)	
<i>HinD III</i> (1U/ μ l)	
Total	10 μl

3. Incubate the tubes at 37°C for 1 hr.
4. Add 2 μ l of 6X DNA loading dye
5. Load 10 μ l of digested DNA onto 0.8% agarose gel
6. Load the undigested λ DNA and puC18 DNA as control

RESULTS

DATE:

**EXPERIMENT NO. 4
LIGATION OF DNA**

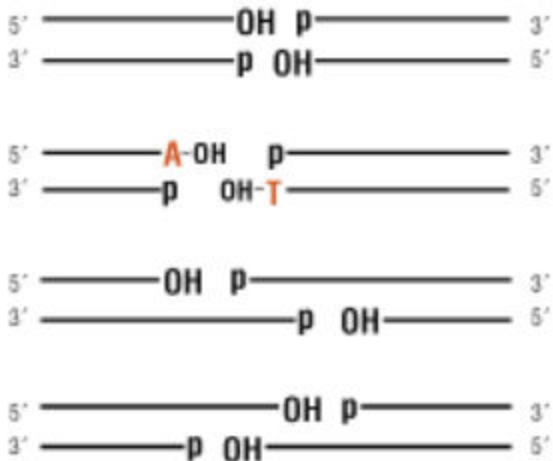
AIM:

To ligate λ DNA/EcoR1 with T4 DNA ligase

PRINCIPLE:

DNA ligase (EC 6.5.1.1) is the covalently joins the phosphate backbone of DNA with blunt or compatible cohesive ends and it's natural role is in repairing double strand breaks in DNA molecules. In molecular biology it is commonly used for the insertion of restriction enzyme-generated DNA fragments into vector backbones. Commercial ligases are supplied with a reaction buffer containing ATP and Mg^{2+} , which are both essential for ligase activity.

Ligase can establish phosphor diester bond in 5'P and 3'OH both in DNA and RNA



MATERIALS REQUIRED

λ DNA/EcoR1

T4 DNA Ligase

Ligase Buffer

1Kb or 0.5Kb DNA Ladder

Sterile water

Agarose

6X loading dye

Ethidium Bromide

1X TAE buffer

Electrophoresis Apparatus

Power supply

Micropipettes

Microtips

PROCEDURE

1. Prepare the sample according to the table

λ DNA/EcoR1 (1ug)	
T4 DNA ligase	
Ligase Buffer(10X)	
Water	
Total	10

2. Mix the samples and incubate them at 16°C for 1 hr

3. Add 2ul of 6X gel loading buffer

4. Load the sample in 1% agarose gel

5. Load λ DNA/EcoR1 as control

RESULTS:

DATE:

EXPERIMENT NO. 5
ISOLATION OF RNA FROM MAMMLIAN CELLS

AIM:

To isolate RNA from mammalian cells

PRINCIPLE:

TRIzol solubilization and extraction is a relatively recently developed general method for deproteinizing RNA. This method is particularly advantageous in situations where cells or tissues are enriched for endogenous RNases or when separation of cytoplasmic RNA from nuclear RNA is impractical. TRIzol (or TRI Reagent) is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein. After solubilization, the addition of chloroform causes phase separation where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. Therefore, RNA, DNA, and protein can be purified from a single sample. TRIzol extraction is also an effective method for isolating small RNAs, such as microRNAs, piwi associated RNAs, or endogeneous, small interfering RNAs.

MATERIALS REQUIRED

TRIzol Reagent

Chloroform

Isopropanol

DEPC Treated water

70% Ethanol

Mortar & Pestel

Dry ice

Microcentrifuge tubes

Micropipettes

METHODS

1. Snap-freeze the tissue to be extracted. Powder on dry ice.
2. .Add 1 mL of TRIzol per 50-100 mg of tissue
3. Solubilize the samples for 5 min at room temperature.
4. Add 0.2 mL of chloroform per mL of TRIzol. Mix vigorously by hand or vortex for 15 sec. Allow the mixture to stand for 2-3 min at room temperature.
5. Centrifuge at maximum speed in a microcentrifuge or at 14,000 RPM in a centrifuge for 10 min at room temperature
6. Transfer the upper clear phase to a fresh tube, taking care not to disturb the interface
7. Add 0.5 mL of isopropanol per each milliliter of the clear phase. Mix vigorously by rapid shaking or vortexing. Let stand for 10 min.
8. . Collect the precipitated RNA by centrifugation at maximum speed in a microcentrifuge or at 14,000 RPM in a centrifuge for 10 min at 4°C
9. Carefully decant the supernatant and wash with 70% Ethanol
10. Dissolve in 20ul of TE

RESULT:

DATE:

EXPERIMENT NO. 6
cDNA SYNTHESIS AND REAL TIME PCR

AIM:

To Perform Real time PCR for a given RNA sample

PRINCIPLE:

Quantification of gene expression levels is an important aspect of molecular biological laboratories. By measuring the amount of particular mRNA, one is able to determine to what extent that gene is being expressed. Expression of genes change from gene to gene, cell to cell or during various experimental conditions. Quantitative gene expression studies are done as a validation of protein levels; extent of transcription of a gene; to study the difference in expression of a gene in the diseased state compared to the normal state; change in gene expression during cell differentiation or development; change in expression for cells that are exposed to a chemical substance and quantification of noncoding RNA gene expression. RT-qPCR is a combination of three steps: the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, the amplification of the cDNA using the PCR and the calculating the fold change of amplification products in real time. Real-time PCR (qPCR) uses fluorescent reporter dyes as a reporter to combine the amplification and detection steps of the PCR reaction in a single tube format. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle.

MATERIALS REQUIRED:

Reverse transcriptase enzyme for RT-PCR,
0.2 ml tubes,
RNA samples,
RNase free water,
SYBR Green mix,
Primers

POCEDURE:

DNA Removal

1. Prepare the solution as below and incubate at 45°C for 3 Min

RNA (Total 5 ug or mRNA1 ug)	1ul
gDNA Removal	2
Rnase Free water	13 ul

cDNA synthesis

Reagents	Volume (μl)
Tola RNA Mix	15
Reverse Transcriptase Mix	4
Reverse Transcriptase	1
Total	20μl

Temperature	Time
25°C	3 min
45°C	10 min
85°C	5 min

Followed by holding temperature at 4°C

Real Time PCR:

Place the capillaries in the cold block to pre-cool

Spin down enzyme and reaction mix SYBER Green I

Prepare master mix in the aluminum cold block as follows

Reagents	Volume (μ l)
Forward Primer	2.00 μ l
Reverse Primer	2.00 μ l
SYBR GREEN Mix	10.00 μ l
DNA template	2 μ l
RNase free Water	4 μ l
Total	20

Mix gently and aliquot 18 μ l into each capillary

Seal the capillary and centrifuge for 5 seconds at 3000rpm.

Load the sample to the rotor of the light cycler.

Adjust the following PCR reaction conditions in the thermal cycler

Temperature	Time
94°C	15 min
94°C	15 sec
48°C	30 sec
72°C	1 min

RESULT

DATE:

EXPERIMENT NO. 7
PROTEIN SEPARATION BY SDS- PAGE

AIM

To separate protein by SDS PAGE

PRINCIPLE

The separation of macromolecules in an electric field is called *electrophoresis*. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The most commonly used system is also called the Laemmli method after U.K. Laemmli, who was the first to publish a paper employing SDS-PAGE in a scientific study.

SDS is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. In a gel of uniform density the relative migration distance of a protein (R_f , the f as a subscript) is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between R_f and mass can be plotted, and the masses of unknown proteins estimated.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification

procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques such as Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins.

MATERIALS REQUIRED

Vertical Electrophoresis apparatus
Gel plates, Spacers, Comb
Ethanol
Vaseline
Distilled water

BUFFERS AND REAGENTS

Acrylamide (30%)

Dissolve 290g of **acrylamide** and 10g of N,N'-methylbisacrylamide in 600ml of H₂O. Adjust the volume to 1L with H₂O. Sterilize the **solution** by filtration (0.45 micron pore size). Check the pH (should be 7.0 or less). Store in dark bottles at room temperature.

Gel casting Buffer

1.5 M Tris-HCl, pH 8.8 (to prepare resolving gel): Dissolve 18.15 g of Tris base in 80 mL distilled water. Adjust pH to 8.8 using 6N HCl. Make up the final volume to 100 mL with distilled water

0.5 M Tris-HCl, pH 6.8 (to prepare stacking gel): Dissolve 6 g of Tris base in 80 mL distilled water. Adjust pH to 6.8 using 6N HCl. Make up the final volume to 100 mL with distilled water

2X Loading Buffer

Bromophenol blue 0.004%
2-mercaptoethanol 10%
Glycerol 20%
SDS 4%
Tris-HCl 0.125 M

10X Running Buffer

Tris-HCl 25 mM
Glycine 200 mM
SDS 0.1% (w/v)

SDS (10%)

Dissolve 10 g in 100 ml of distilled water.

APS (10%)

Dissolve 100 mg in 1 ml of distilled water (*Need to be prepared fresh for every experiment*)

Staining solution

Stain: Dissolve 0.4g of Coomassie blue R350 in 200 mL of 40% (v/v) HPLC grade methanol in water with stirring as needed. Filter the solution to remove any insoluble material. Add 200mL of 20% (v/v) acetic acid in water. The final concentration is 0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid.

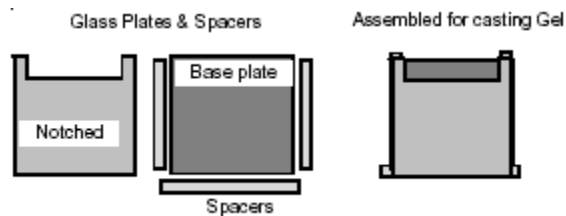
Distaining solution

Add 500mL of HPLC- grade methanol to 300 mL of HPLC grade water. Add 100 mL of reagent grade acetic acid and, after mixing, adjust the total volume to 1000mL with water. The final concentrations are 50% (v/v) methanol in water with 10% (v/v) acetic acid

TEMED

PROCEDURE

1. Assemble the plates for casting gel as shown below.



2. Clamp the assembly of plates to fix it in a gel casting apparatus. Ensure the assembly is leak proof by filling water between the plates. Silicon grease can be applied to spacer to make a water-tight seal.
3. Prepare the separation gel . Mix in the following order:

Gel %	Water (mL)	30% acrylamide (mL)	1.5 M Tris-HCl, pH 8.8 (mL)	10% SDS (μL)	10% APS (μL)	TEMED*(μL)
8%	4.6	2.6	2.6	100	100	10
10%	3.8	3.4	2.6	100	100	10
12%	3.2	4.0	2.6	100	100	10
15%	2.2	5.0	2.6	100	100	10

4. Pour gel, leaving ~2 cm below the bottom of the comb for the stacking gel. Make sure to remove bubbles.
5. Layer the top of the gel with isopropanol. This will help to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out (*In ~30 min, the gel should be completely polymerized.*)
6. Remove the isopropanol and wash out the remaining traces of isopropanol with distilled water.
7. Prepare the stacking gel (4%). Mix in the following order:

H ₂ O	6.1 mL
Acrylamide/bis (30%, 37.5:1)	1.3 mL
Tris-HCl (0.5 M, pH 6.8)	2.5 mL
SDS, 10%	100 μL
TEMED	10 μL
Ammonium persulfate (APS), 10%	100 μL

8. Pour stacking gel on top of the separation gel.
9. Add combs to make wells. In ~30 min, the stacking gel should become completely polymerized.
10. Add 25 μ l of sample loading buffer to protein sample. Add 25 μ l of sample loading buffer to 25 μ l of protein marker. Place it in a boiling water bath for 5 minutes.
11. After the stacking gel has set, carefully remove the comb and the bottom spacer. Wash the wells immediately with distilled water to remove non-polymerized acrylamide. Fill the bottom reservoir with 1X reservoir buffer and carefully fix the plate to the apparatus without trapping any air bubbles between the buffer and the bottom of the gel. Fix the plates to PAGE apparatus. Fill the top reservoir with 1X reservoir buffer.
12. Load samples and molecular mass protein markers into wells for separation by electrophoresis.
13. Set voltage at 100 V and switch on the power supply.
14. When the dye front comes to 0.5 cm above the bottom of the gel, turn off the power. This will take approximately 1 to 1 1/2 hours
15. Remove the gel plates and gently pry the plates apart using a spatula or similar tool, not at the notch. Cut off the wells and the stacking gel and place the gel in a water containing Tray
16. Wash the gel Gently and discard the water
17. Add 100 ml of staining solution and keep it in the rocker for overnight.
18. Remove the staining solution and add 100 ml of destaining solution and change the destaining solution for every one hr till the bands are visible.

RESULTS

DATE:

EXPERIMENT NO. 8 WESTERN BLOTTING

AIM

PRINCIPLE

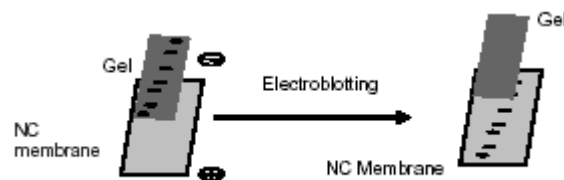
Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: (1) Electrophoresis -separation by size, (2) Blotting - transfer to a solid support, and (3) Immunodetection - marking target protein using a proper primary and secondary antibody to visualize.

Electrophoresis

Mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. (Experiment No 8)

Blotting

Blotting is transfer of resolved proteins from the gel onto a surface of a suitable membrane, done commonly by electrophoresis and referred to as **electroblotting**. The gel is placed in contact with nitrocellulose membrane which is then sandwiched between filter paper, two porous pads and two plastic supports. The entire set up is then placed in an electrophoretic tank containing blotting buffer. The proteins get transferred to the corresponding position on the membrane as resolved on the polyacrylamide gel, forming a mirror image of the gel.



Immunodetection

Protein of interest on the membrane is further located by immunodetection. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present. The electroblotted sample will then be detected using anti-mouse GAPDH IgG (Cell signaling) as primary antibody and corresponding secondary antibody labeled with Horse Radish Peroxidase (HRP). HRP is then detected using hydrogen peroxide as a substrate and Dimethylamino benzidine (DAB) as a chromogen. HRP acts on hydrogen peroxide to release oxygen, which oxidizes the DAB to DAB oxide. The DAB oxide is deposited wherever enzyme is present and appears as a light brown band on the NC membrane

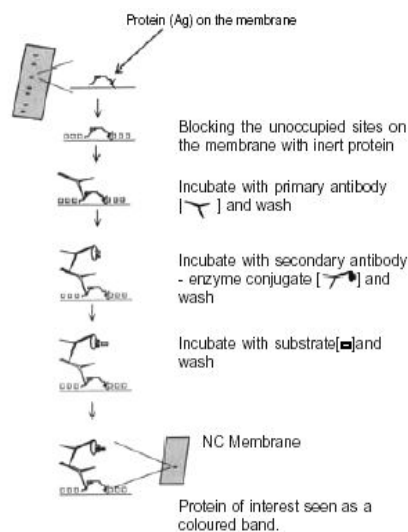


Fig 9.1 Principle of Immunodetection

MATERIALS

Gel Rocker

Conical flask, measuring cylinder, Petri dish, Staining tray.

Distilled water.

Micropipettes, Tips, Water bath.

BUFFERS AND REAGENTS

PBS 10X

1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, and 18 mM KH₂PO₄.

Transfer buffer (1X)

Tris 3 g

Glycine 14.4 g

Methanol 200 ml

Dissolve Tris and Glycine in 700 ml of distilled water, adjust the pH to 8.3 and make it up to 800 ml. *Add methanol before the experiment*

Blocking solution

Skimmed milk powder - 10 g

Tween 20 - 200 μl

Glycerol – 10 ml

PBS – 10 ml

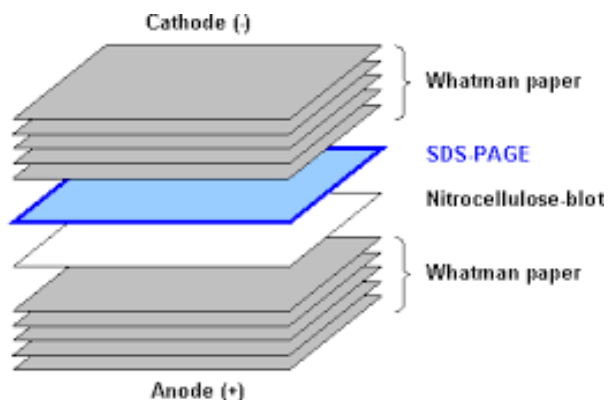
Sodium azide – 20 mg

Dissolve the above contents in 100 ml of millipore water

ASSA

PROCEDURE

1. **Electroblotting:**
2. Assemble the blotting sandwich within the blotting cassette as shown in the figure. Take care to avoid air bubbles between the gel and NC membrane.
3. Insert the cassette into the apparatus filled with blotting buffer and connect blotting unit to power supply as per the convention,
red: anode,
black: cathode
4. Electrophoreses the sample at 50 V for 2 hours for blotting to occur.
5. Remove the NC membrane gently from the cassette and place the membrane in 10 ml of freshly prepared blocking buffer taken in a petri dish. Leave it overnight at 4°C.



Assembly of a horizontal electroblotting apparatus

Day 2 : Immunodetection

6. Discard blocking buffer.
7. Immerse blot in 10 ml of primary antibody solution and mix gently and incubated for 4 to 5 hours in a rocker (gentle). The primary antibody solution can be used atleast 5 to 6 times.
8. Wash the blot by immersing in 10 ml of 1X PBS for 3-5 minutes. Repeat the wash three times. Discard the buffer each time.
9. Immerse the blot in 10 ml of 1X HRP labeled secondary antibody. Mix gently and incubate for 45 minutes. Discard the HRP labeled antibody.

10. Wash the blot by immersing in 10 ml of T-TBS and TBS for 3-5 minutes.
11. Immerse the washed blot in 10 ml of substrate solution, mix gently for 5-10 minutes, within this time coloured band will appear.
12. Remove the blot, wash with distilled water, discard and dry.

Note: Although the colored band fades with time, the rate of color loss can be retarded if the blots are kept in dark.

13. Compare the SDS-Polyacrylamide gel with the developed NC membrane.