18BTC105J- MOLECULAR BIOLOGY LABORATORY

B.Tech (Biotechnology)

II YEAR/ IV SEMESTER

Name :
Register no :

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIOENGINEERING
FACULTY OF ENGINEERING AND TECHNOLOGY
SRM INSTITUTE OF SCIENCE AND TECHNOLOGY
KATTANKULATHUR- 603 203

20 - 20

SRM
INSTITUTE OF SCIENCE & TECHNOLOGY
(Deemed to be University u/s 3 of UGC Act, 1956)

BONAFIDE CERTIFICATE

Register no:

Certified to be the bonafide record of work done by_____of B.Tech. Biotechnology Degree Course in the **18BTC105J - Molecular biology laboratory** at **SRM Institute of Science and Technology,** Kattankulathur, during the academic year 20 -20 .

Lab Incharge

Date: **Head of Department**

Submitted for University Examination held in _____

at **SRM Institute of Science and Technology**, Kattankulathur.

Date: **Examiner-1** **Examiner-2**

Name of the student :
Register no :

INDEX

Date:

Experiment No. 1

Isolation of Genomic DNA from *E. Coli* (DH5α)

Aim: To isolate the genomic DNA from ***E .coli*** **(DH5α)** cells.

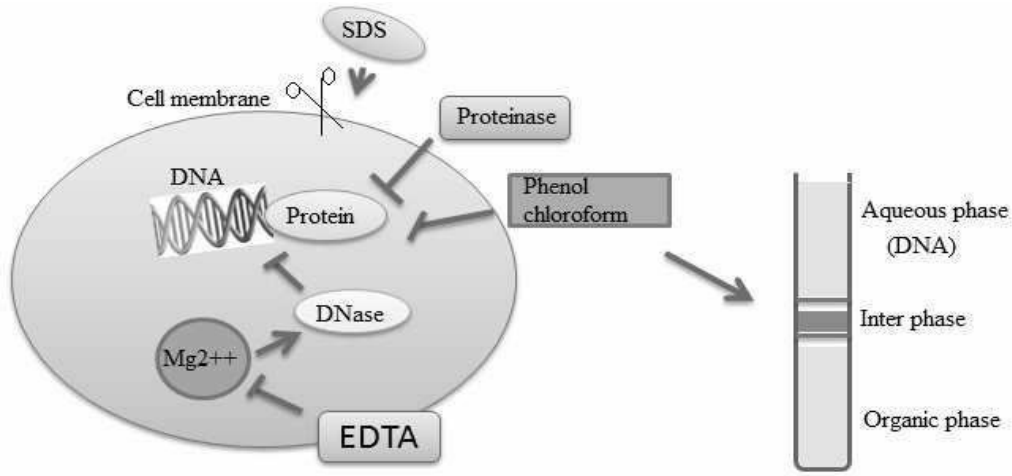
Background:

DNA was first isolated in 1869 by Friedrich Miescher at the University of Tübingen. He obtained DNA from human leukocytes washed from pus-laden bandages amply supplied by surgical clinics in the time before antibiotics usage, and he referred DNA as nuclein. He continued to study DNA and switched from leukocytes to salmon sperm as his starting material. Meisher's choice of starting material was based on the knowledge that leukocytes and sperm have large nuclei relative to cell size. DNA isolated from salmon sperm and from (bovine) lymphocytes is still available commercially.

Principle:

In prokaryotes, DNA is double-stranded and circular and is found throughout the cytoplasm. The cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl or lauryl sulfate) is used to disrupt the cell membrane. DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for most nucleases. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol/ chloroform is used to denature and separate
protein from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and a pure phenol layer. Proteins can also be removed by salting out proteins by sodium acetate. The denatured protein forms a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol. This method of DNA isolation gives good results for most of the Gram-negative bacteria. A few *E. coli* strains contain high molecular weight polysaccharides that may interfere with DNA. It needs to consider the genotype of *E. coli* strain to know whether we need to modify any steps to get a good outcome.

Schematic diagram of the principle of isolation of genomic DNA from *E. coli*



Materials Required:

1. LB Broth
2. *E. coli* DH5α cells
3. Reagents
4. TE buffer (pH 8.0) (10 mM Tris, 1mM EDTA)
5. 10% SDS
6. Proteinase K
7. Phenol-chloroform mixture
8. 5M Sodium Acetate (pH 5.2)
9. Isopropanol
10. 70% ethanol
11. Autoclaved Distilled Water
12. Eppendorf tubes 2 ml
13. Micropipettes
14. Microtips or pipette tips
15. Microfuge

Preparation of Reagents:

1.**TE BUFFER (pH 8.0):** 10 mm Tris HCl (pH 8.0) 1 mm EDTA (pH 8.0)
2. **10% SDS:** Dissolve 10 g of SDS in 100 ml autoclaved distilled water.
3.**PROTEINASE K:** Dissolve 10 mg of Proteinase K in 1 ml Autoclaved distilled water.
4.**PHENOL/ CHLOROFORM MIXTURE:** Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE 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.
5. 5M SODIUM ACETATE: Dissolve 41 g of sodium acetate in 100 ml distilled water and adjust pH to 5.2 with diluted acetic acid.
6. ISOPROPANOL
7. 70% ETHANOL

Procedure:

- 2 ml overnight culture was taken and cells were harvested by centrifugation for 5 minutes (2500*g*).
- 875 µl of TE buffer was added to the cell pellet and cells were resuspended in the buffer by gentle mixing.
- 100 µl of 10% SDS and 5µl of Proteinase K were added to the cells.
- The above mixture was mixed well and incubated at 37º C for 20 min in an incubator.
- 1ml of the phenol-chloroform mixture was added to the contents, mixed well by inverting and the samples were incubated at room temperature for 5 minutes.
- The contents were centrifuged at 10000 rpm for 10 minutes at 4º C.
- The highly viscous jelly-like supernatant was collected using cut tips and was transferred to a fresh tube.
- The process was repeated once again with the phenol-chloroform mixture and the supernatant was collected in a fresh tube.
- To this 100 µl of 5M sodium acetate was added and mixed gently.
- 1.5 ml of isopropanol was added and mixed gently by inversion till white strands of DNA precipitated.
- The contents were centrifuged at 5000 rpm for 10 minutes.
- The supernatant was removed and 1ml 70% ethanol was added.
- The above contents were centrifuged at 5000 rpm for 10 minutes.
- After air-drying for 5 minutes, 50 µl of TE buffer or distilled water was

added.

- The DNA samples were stored for further experiments.

Precautions:

- Cut tips should be used so that the DNA is not subject to mechanical disruption.
- Depending on the source of DNA the incubation period of Proteinase K should be optimized.
- The phenol/chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA.
- DNase free plastic wares and reagents should be used.

Results and Discussion:

Date:

Experiment No. 2 (I)

Qualitative Analysis of Genomic DNA

Aim: To separate and visualize Genomic DNA bands by Agarose gel electrophoresis.

Introduction:

Agarose gel electrophoresis is a powerful and widely used method that separates molecules on the basis of electrical charge, size, and shape. The method is particularly useful in separating charged biologically important molecules such as DNA (deoxyribonucleic acids), RNA (ribonucleic acids), and proteins. Agarose forms a gel- like consistency when boiled and cooled in a suitable buffer.

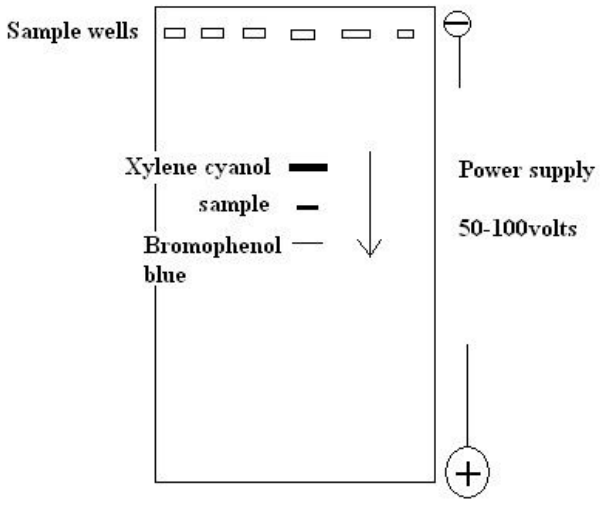
Principle:

The agarose gel contains molecule-sized pores, acting like molecular sieves. The pores in the gel control the speed that molecules can move. DNA molecules possess a negative charge in their backbone structure due to the presence of $-PO^4$ groups thus this principle is exploited for its separation. Smaller molecules move through the pores more easily than larger ones. Conditions of charge, size, and shape interact with one another depending on the structure and composition of the molecules, buffer conditions, gel thickness, and voltage. Agarose gels are made with between 0.7% (provides good resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1 kb fragments).

The gel setup provides wells for loading DNA into it. The loaded DNA molecules move towards the positively charged electrode (anode) and get separated along the length of the gel. Ethidium bromide (EtBr), a chromogen is added to the gel to visualize the separated DNA under UV trans-illumination. EtBr intercalates between the bases and glows when UV radiation is passed through the gel.

Purpose of gel loading buffer

The loading buffer gives color and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. The gel loading dye possesses bromophenol blue and xylene cyanol. Density is provided by glycerol or sucrose**.**



Xylene cyanol gives a greenish-blue color while bromophenol blue provides a bluish colored zone. The successful DNA run is determined by the presence of both the colored dye in the gel.

Materials Required:

- Electrophoresis buffer: 1x TAE buffer
- Agarose ultra-pure (DNA graded)
- electrophoresis tank, gel tray, sample comb, and power supply
- Plastic or insulation tape
- Ethidium bromide: 10 mg /ml stock solution
- 5x Gel loading dye
- DNA marker solution, DNA sample, and gloves.

Procedure:

1. **Making a 1% Agarose Gel**

- Weigh 0.5 g agarose and dissolve it in 50 mL of 1x TAE 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.)
- Heat the solution over a hot plate to boiling constituency marked with a clear solution

- Leave the solution to cool and add 2µl of EtBr solution mix it well by gentle swirling.
- 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
- Allow the solution to cool and harden to form a gel.

2. Loading of Samples

16. Carefully transfer the gel to the electrophoresis tank filled with a 1x TAE buffer.
17. Prepare your samples [8 ul of DNA sample (0. 1 ug to 1 ug) and 2 ul of 5x gel loading dye]
18. Remove the comb and load the samples into the well.
19. Connect appropriate electrodes to the power pack and run it at 50- 100volts for 20min.
20. Monitor the progress of the gel with reference to tracking dye (Bromophenol blue). Stop the run when the marker has run 3/4 th of the gel.

2. Examining the gel

1. Place the gel on the UV-transilluminator and check for orange colored bands in the gel.

Precautions:

- Wear gloves during the addition of EtBr and while handling the casted gel (EtBr is a potent carcinogen).
- Handling the gel should be careful as the gel may break due to improper handling.
- While performing the UV-trans illumination for visualizing the bands, avoid direct contact and exposure to eyes.

Results and Discussion:

Date:

Experiment No. 2 (II)
Quantitative Analysis of Genomic DNA

Aim: To determine the amount, concentration and purity of the given DNA sample.

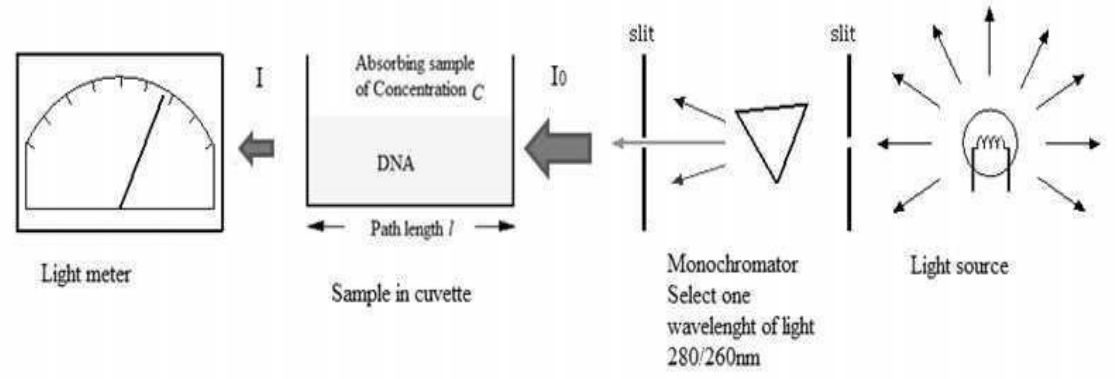
Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by the following expression:

$$A = \varepsilon * c * l$$

The device UV spectrophotometer works on this principle and used to find the concentration of the sample.

The concentration and quality of a sample of DNA are measured with a UV spectrophotometer.

A standard graph can be drawn using different concentrations of DNA and OD (optical density) values.



The diagram above shows that a beam of monochromatic radiation (Io) is directed to a sample solution. Absorption takes place by the sample and the beam of radiation is leaving out (I).

Materials Required:

- DNA sample
- TE buffer
- UV spectrophotometer

Procedure:

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

Total DNA (µg) = 50 µg/ml x Absorbance at A260 x dilution factor x 0.05 ml

Where 0.05 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 an OD260/OD280 between 1.8 and 2.0

Results and Discussion:

Date:

Experiment No. 3
Plasmid DNA Isolation

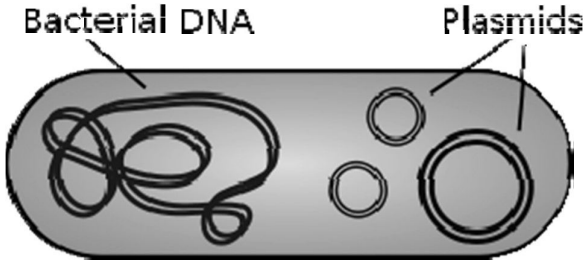
Aim: To isolate plasmid DNA from bacterial cells.

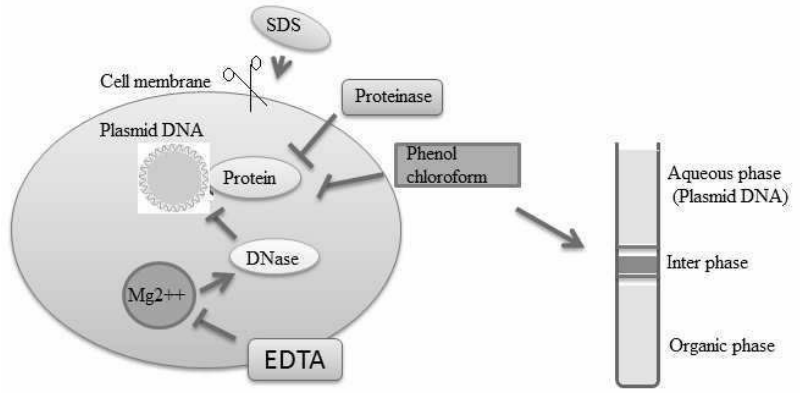
Principle:

When bacteria are lysed under alkaline conditions both DNA and proteins are precipitated. After the addition of acetate-containing neutralization buffer, the large and less supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids can renature and stay in solution.

In prokaryotes, the plasmid is double-stranded, circular, and is found in the cytoplasm. The cell membranes must be disrupted in order to release the plasmid in the extraction buffer. Solution 1 contains glucose, Tris, and EDTA. Glucose provides osmotic shock leading to the disruption of the cell membrane, Tris is a buffering agent used to maintain a constant pH 8. The plasmid can be protected from endogenous nucleases by chelating Mg2++ ions using EDTA. Mg2++ ion is considered as a necessary cofactor for most nucleases. Solution II contains NaOH and SDS and this alkaline solution is used to disrupt the cell membrane and NaOH also denatures the DNA into single strands. Solution III contains acetic acid to neutralize the pH and potassium acetate to precipitate the chromosomal DNA, proteins, along with the cellular debris. Phenol /chloroform is used to denature and separate proteins from the plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and a pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. Once the plasmid DNA is released, it must be precipitated in alcohol. The plasmid DNA in the aqueous phase is precipitated with cold (0oC) ethanol or isopropanol. The precipitate is usually redissolved in the buffer and treated with phenol or an organic solvent to remove the last traces of protein, followed by reprecipitation with cold ethanol.

Schematic diagram of the principle of Plasmid DNA Isolation





Materials Required:

- Luria Broth
- Bacterial cells containing plasmid
- Reagents
- TE buffer(pH 8.0)
- Solution I , Solution II, and Solution III
- Phenol-chloroform mixture
- Isopropanol
- 70% ethanol
- Autoclaved Distilled Water
- Eppendorf tubes 2 ml
- Micropipette
- Microtips
- Microfuge

Preparation of Reagents:

1. **TE BUFFER (pH 8.0):** 10 mm Tris HCl (pH 8.0) 1 mm EDTA (pH 8.0)
2. **Resuspension solution (Solution-I):**50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0), Store at 40 °C.
3. **Lysis solution (Solution-II)** - 0.2 N NaOH, 1% SDS, Store at room temperature.
4. **Neutralizing solution (Solution-III)** - 3 M KOAc (pH 6.0) - For 100 ml solution, 60 ml 5 M potassium acetate (49.07 g potassium acetate in 100 ml H2O). 11.5 ml glacial acetate and 28.5 ml H2O, store at room temperature.

5. **Phenol – Chloroform Mixture:** Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE 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 in dark.
6. **Isopropanol**

Procedure:

- Take 2 ml of overnight culture and harvest cells by centrifugation for 5 minutes. Discard the supernatant carefully.
- Add 100 µl of solution I to the cell pellet and resuspend the cells by gentle mixing.
- Incubate the above mixture at room temperature for 5 minutes.
- Add 200 µl of solution II to the mixture and mix by inverting the tubes for 5 minutes.
- Incubate for 5-10 minutes at room temperature.
- Add 500µl of ice-cold solution III to the mixture and mix by inverting the tube.
- Incubate on ice for 10 minutes.
- Centrifuge at 10,000 rpm for 5 minutes.
- Transfer the supernatant into the fresh tube.
- Add 400 µl of the phenol-chloroform mixture to the contents, mix well by inverting and incubate them at room temperature for 5 minutes.
- Centrifuge at 10000 rpm for 5 minutes.
- Collect the supernatant (viscous) using micro tips and transfer to a fresh tube.
- Add 0.8 ml of isopropanol and mix gently by inversion. Incubate for 30 min at room temperature.
- Centrifuge the contents at 10,000 rpm for 10 minutes.
- Discard the supernatant after centrifugation.
- The contaminated salt in the DNA pellet can be removed with 70% ethanol washing.
- After washing air-drying for 5 minutes, add 100 µl of TE buffer or autoclaved distilled water to the pellet to resuspend the plasmid DNA.
- Take 10 µl of plasmid sample and dilute to 1 ml with distilled water for spectrometric analysis.
- The concentration of plasmid is determined using a spectrophotometer at 260/280 nm.
- An aliquot of plasmid DNA is used for agarose electrophoresis for quantitative and qualitative analyses.

Precautions:

- Cut tips should be used so that the plasmid is not subjected to mechanical disruption.
- The phenol-chloroform extraction should be repeated depending on the source of the plasmid to obtain pure plasmid.
- DNase free plastic wares and reagents should be used.

Results and Discussion:

Date:

Experiment No. 4 (I)

Qualitative Analysis of Plasmid DNA

Aim: To separate and visualize Plasmid DNA bands by Agarose gel electrophoresis.

Introduction:

Agarose gel electrophoresis is a powerful and widely used method that separates molecules on the basis of electrical charge, size, and shape. The method is particularly useful in separating charged biologically important molecules such as DNA (deoxyribonucleic acids), RNA (ribonucleic acids), and proteins. Agarose forms a gel- like consistency when boiled and cooled in a suitable buffer.

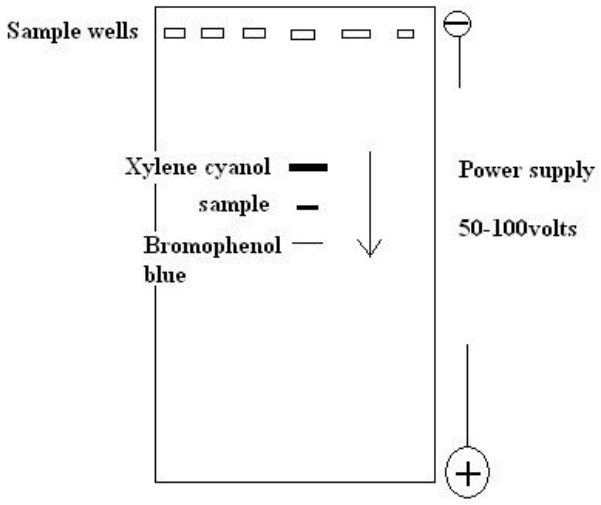
Principle:

The agarose gel contains molecule-sized pores, acting like molecular sieves. The pores in the gel control the speed that molecules can move. DNA molecules possess a negative charge in their backbone structure due to the presence of $-PO^4$ groups thus this principle is exploited for its separation. Smaller molecules move through the pores more easily than larger ones. Conditions of charge, size, and shape interact with one another depending on the structure and composition of the molecules, buffer conditions, gel thickness, and voltage. Agarose gels are made with between 0.7% (provides good resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1 kb fragments).

The gel setup provides wells for loading DNA into it. The loaded DNA molecules move towards the positively charged electrode (anode) and get separated along the length of the gel. Ethidium bromide (EtBr), a chromogen is added to the gel to visualize the separated DNA under UV trans-illumination. EtBr intercalates between the bases and glows when UV radiation is passed through the gel.

Purpose of gel loading buffer

The loading buffer gives color and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. The gel loading dye possesses bromophenol blue and xylene cyanol. Density is provided by glycerol or sucrose**.**



Xylene cyanol gives a greenish-blue color while bromophenol blue provides a bluish colored zone. The successful DNA run is determined by the presence of both the colored dye in the gel.

Materials Required:

- Electrophoresis buffer: 1x TAE buffer
- Agarose ultra-pure (DNA graded)
- electrophoresis tank, gel tray, sample comb, and power supply
- Plastic or insulation tape
- Ethidium bromide: 10 mg /ml stock solution
- 5x Gel loading dye
- DNA marker solution, DNA sample, and gloves.

Procedure:

2. **Making a 1% Agarose Gel**

- Weigh 0.5 g agarose and dissolve it in 50 mL of 1x TAE 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.)
- Heat the solution over a hot plate to boiling constituency marked with a clear solution
- Leave the solution to cool and add 2µl of EtBr solution mix it well by gentle swirling.
- 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

- Allow the solution to cool and harden to form a gel.

2. Loading of Samples

2. Carefully transfer the gel to the electrophoresis tank filled with a 1x TAE buffer.
3. Prepare your samples [8 ul of DNA sample (0. 1 ug to 1 ug) and 2 ul of 5x gel loading dye]
4. Remove the comb and load the samples into the well.
5. Connect appropriate electrodes to the power pack and run it at 50- 100volts for 20min.
6. Monitor the progress of the gel with reference to tracking dye (Bromophenol blue). Stop the run when the marker has run 3/4 th of the gel.

3. Examining the gel

1. Place the gel on the UV-transilluminator and check for orange- colored bands in the gel.

Precautions:

- Wear gloves during the addition of EtBr and while handling the casted gel (EtBr is a potent carcinogen).
- Handling the gel should be careful as the gel may break due to improper handling.
- While performing the UV-trans illumination for visualizing the bands, avoid direct contact and exposure to eyes.

Results and Discussion:

Date:

Experiment No. 4 (II)
Quantitative Analysis of Plasmid DNA

Aim: To determine the amount, concentration and purity of the given plasmid DNA sample.

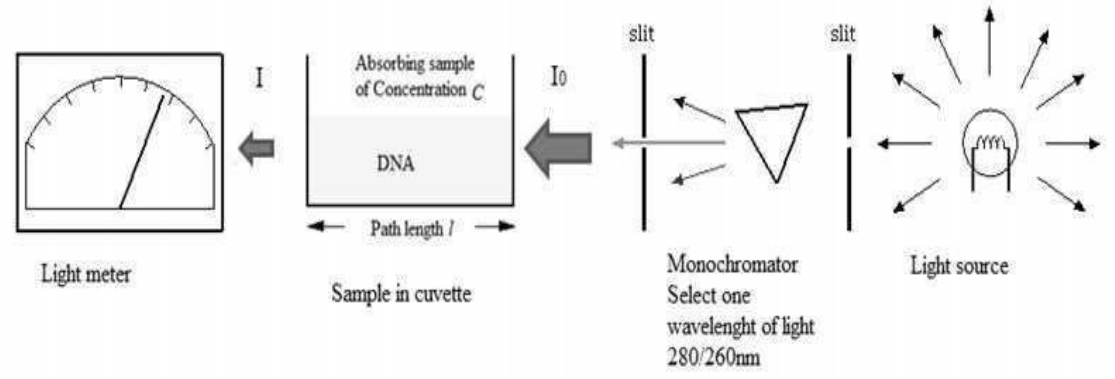
Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by the following expression:

$$A = \varepsilon * c * l$$

The device UV spectrophotometer works on this principle and used to find the concentration of the sample.

The concentration and quality of a sample of DNA are measured with a UV spectrophotometer.

A standard graph can be drawn using different concentrations of DNA and OD (optical density) values.



The diagram above shows that a beam of monochromatic radiation (Io) is directed to a sample solution. Absorption takes place by the sample and the beam of radiation is leaving out (I).

Materials Required:

- DNA sample
- TE buffer
- UV spectrophotometer

Procedure:

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

Total DNA (µg) = 50 µg/ml x Absorbance at A260 x dilution factor x 0.05 ml

Where 0.05 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 an OD260/OD280 between 1.8 and 2.0

Results and Discussion:

Date:

Experiment No. 5

Total RNA isolation from Bacterial Cells

Aim: To isolate total RNA from the given bacterial culture.

Principle: Total RNA is isolated and separated from DNA and protein after extraction with a solution called as Trizol (Sigma). Trizol is an acidic solution containing guanidinium thiocyanate (GITC), phenol and chloroform. GITC irreversibly denatures proteins and RNases. This is followed by centrifugation. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol. RNase enzymes can be inactivated by including diethylpyrocarbonate (DEPC).

Materials Required:

- Bacterial culture
- Trizol
- Chloroform
- Isopropanol solution
- TAE buffer
- 70% ethanol

Procedure:

2. Take 1ml of bacterial culture in a fresh Eppendorf and centrifuge at 6000 RPM for 10 mins.
3. Discard the medium and resuspend the pellet in 800 µL of TE buffer.
4. To this add 160 µL of Trizol ($1/5^{th}$ of TE buffer volume).
5. The solution was mixed well by pipetting several times. To this add 32 µl of chloroform ($1/5^{th}$ volume of trizol).
6. Incubate for 2 to 5 minutes and centrifuge at 12000 rpm for 15 minutes at 4° C.
7. Transfer the aqueous phase into a new tube and add an equal volume of isopropanol. Mix well.

- Centrifuge at 10000 rpm for 10 minutes at 4° C.
- Discard the supernatant and resuspend the pellet in 70% ethanol.

- Again centrifuge at 10000 rpm for 10 minutes at 4° C.
- Discard the supernatant. Air-dry the pellet at 37° C for 10-15 minutes.
- Resuspend the pellet in 30 µL of TE buffer.
- Analyze the RNA sample quantitatively and qualitatively.

Results And Discussion:

Date:

Experiment No.6 (I)
Qualitative Analysis of RNA

Aim: To separate and visualize RNA bands by Agarose gel electrophoresis.

Introduction:

Agarose gel electrophoresis is a powerful and widely used method that separates molecules on the basis of electrical charge, size, and shape. The method is particularly useful in separating charged biologically important molecules such as DNA (deoxyribonucleic acids), RNA (ribonucleic acids), and proteins. Agarose forms a gel- like consistency when boiled and cooled in a suitable buffer.

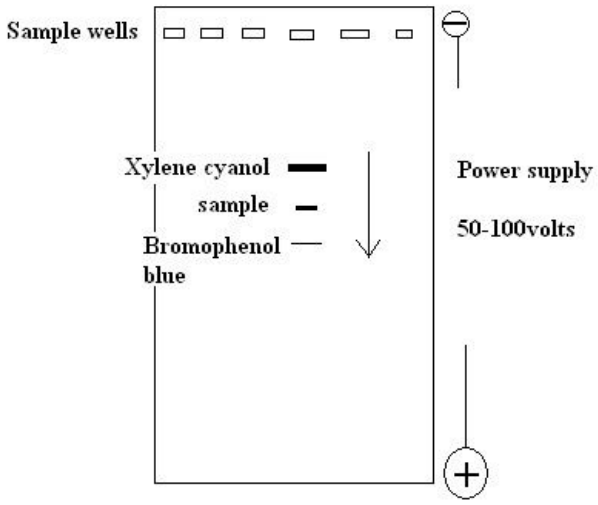
Principle:

The agarose gel contains molecule-sized pores, acting like molecular sieves. The pores in the gel control the speed that molecules can move. DNA molecules possess a negative charge in their backbone structure due to the presence of $-PO_4$ groups thus this principle is exploited for its separation. Smaller molecules move through the pores more easily than larger ones. Conditions of charge, size, and shape interact with one another depending on the structure and composition of the molecules, buffer conditions, gel thickness, and voltage. Agarose gels are made with between 0.7% (provides good resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1 kb fragments).

The gel setup provides wells for loading DNA into it. The loaded DNA molecules move towards the positively charged electrode (anode) and get separated along the length of the gel. Ethidium bromide (EtBr), a chromogen is added to the gel to visualize the separated DNA under UV trans-illumination. EtBr intercalates between the bases and glows when UV radiation is passed through the gel.

Purpose of gel loading buffer

The loading buffer gives color and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. The gel loading dye possesses bromophenol blue and xylene cyanol. Density is provided by glycerol or sucrose**.**



Xylene cyanol gives a greenish-blue color while bromophenol blue provides a bluish colored zone. The successful DNA run is determined by the presence of both the colored dye in the gel.

Materials Required:

- Electrophoresis buffer: 1x TAE buffer
- Agarose ultra-pure (DNA graded)
- electrophoresis tank, gel tray, sample comb, and power supply
- Plastic or insulation tape
- Ethidium bromide: 10 mg /ml stock solution
- 5x Gel loading dye
- DNA marker solution, RNA sample, and gloves.

Procedure:

1. **Making a 1% Agarose Gel**

- Weigh 0.5 g agarose and dissolve it in 50 mL of 1x TAE 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.)

- Heat the solution over a hot plate to boiling constituency marked with a clear solution

- Leave the solution to cool and add 2µl of EtBr solution mix it well by gentle swirling.

- 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

- Allow the solution to cool and harden to form a gel.

2. Loading of Samples

- Carefully transfer the gel to the electrophoresis tank filled with a 1x TAE buffer.
- Prepare your samples [8 ul of RNA sample (0. 1 ug to 1 ug) and 2 ul of 5x gel loading dye]
- Remove the comb and load the samples into the well.
- Connect appropriate electrodes to the power pack and run it at 50- 100volts for 20min.
- Monitor the progress of the gel with reference to tracking dye (Bromophenol blue). Stop the run when the marker has run 3/4th of the gel.

3. **Examining the gel**

- Place the gel on the UV-transilluminator and check for orange- colored bands in the gel.

Precautions:

- Wear gloves during the addition of EtBr and while handling the casted gel (EtBr is a potent carcinogen).
- Handling the gel should be careful as the gel may break due to improper handling.
- While performing the UV-trans illumination for visualizing the bands, avoid direct contact and exposure to eyes.

Results and Discussion:

Date:

Experiment No. 6 (II)

Quantitative Analysis of RNA

Aim: To determine the amount, concentration and purity of the given RNA sample.

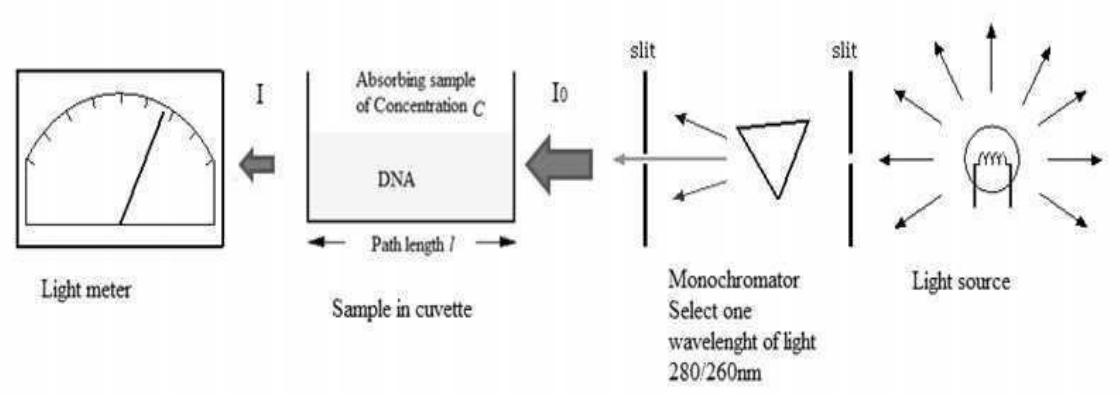
Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by the following expression:

$$A = \varepsilon * c * l$$

The device UV spectrophotometer works on this principle and used to find the concentration of the sample.

The concentration and quality of a sample of RNA are measured with a UV spectrophotometer.

A standard graph can be drawn using different concentrations of RNA and OD (optical density) values.



The diagram above shows that a beam of monochromatic radiation (Io) is directed to a sample solution. Absorption takes place by the sample and the beam of radiation is leaving out (I).

Materials Required:

RNA sample, TE buffer, UV spectrophotometer

Procedure:

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

Total RNA (µg) = 40 µg/ml x Absorbance at A260 x dilution factor x 0.03 ml

Where 0.03 ml is the total volume of the RNA.

Quality: RNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure RNA. If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm.

Results and Discussion:

Date:

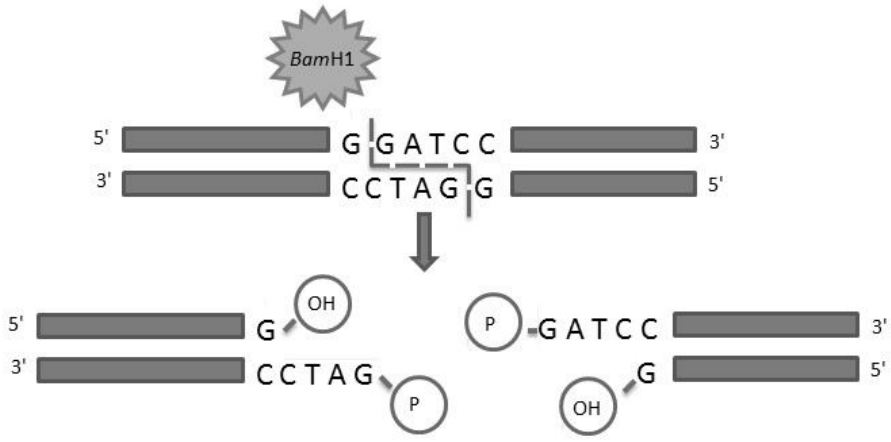
Experiment No. 7

Restriction Enzyme Digestion

Aim: To digest the pUC18/λ DNA with *EcoRI/Hind III* enzyme

Principle:

Restriction endonucleases are the class of enzymes that are used to cleave DNA at specific sites called Restriction sites. Every restriction enzyme has a specific restriction site at which it cuts a DNA molecule. For example restriction sequence for *Bam*HI is GGATCC (type II restriction enzyme. The most abundantly used restriction enzymes are type II restriction enzymes which cleave at specific restriction site only. These endonucleases function adequately at pH 7.4 but different enzymes vary in their requirements for ionic strength usually provided by sodium chloride and magnesium chloride. It is also advisable to add a reducing agent such as dithiothreitol (DTT) which stabilizes the enzymes and prevents their inactivation. Any variation in the concentration of Na or Mg can lead to changes in the specificity of the enzyme so that it can cleave at additional or non-standard restriction sequences. The phosphodiester bond is cleaved between specific bases, one on each DNA strand, no matter the source of the DNA. The restriction endonucleases produce either sticky or blunt ends upon cleavage. Also based on the number of sequences identified for cleavage they can be tetracutter (4), hexacutter (6) or octacutter (8).



Materials Required For Restriction Enzyme Digestion:

- pUC18 DNA / λ DNA
- *EcoRI/Hind III* enzyme
- 10X buffer
- 1Kb Ladder
- Sterile water
- Agarose
- 6X loading dye
- 1.5 ml Sterile Vials
- Ethidium Bromide
- 1X TAE buffer

Procedure:

- Take 1 µg of _____DNA (____µl) in a fresh Eppendorf.
- To this, add_____µl of sterile water followed by____µl of 10X buffer.
- Add_____µl of _____ enzyme (1 unit) and incubate the mixture at 37˚C for 2 hrs.
- Prepare 0. 7% agarose gel and load the samples including 1 Kb DNA ladder, undigested _____DNA and _____ digested _____ DNA.
- Run the gel at 50 V for 1 hr.
- Visualize the gel under UV illuminator.
- ____ul of the sample and 2ul of the dye were mixed
- Load____µl of this into the gel

Reaction Mixture:

DNA	:	µl (1 ug)
Sterile water	:	µl
10X buffer	:	µl
Restriction enzyme ( )	:	µl

Total	:	µl

(Incubate at 37˚ C for 1-2 hrs)

Results and Discussion: (make it appropriate for the DNA and restriction enzyme used in your study)

Date:

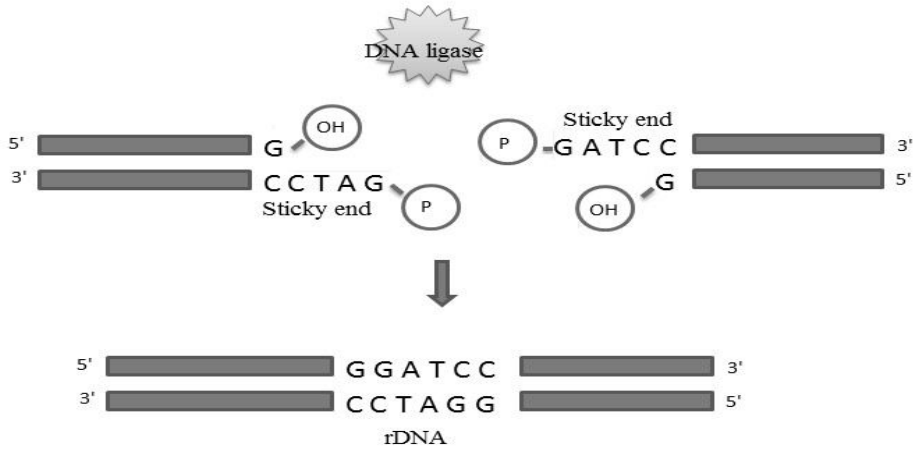
Experiment No. 8

Ligation of DNA fragments

Aim: To perform the ligation of linearized lambda DNA fragments or the ligation of any restriction enzyme digested DNA fragments, that have undergone single digestion.

Principle:

The basic strategy in molecular cloning is to insert a DNA fragment of interest (a segment of DNA) into a DNA molecule (called a vector) that is capable of independent replication in a host cell. The result is a recombinant molecule composed of the DNA insert linked to vector DNA sequences. Construction of these recombinant DNA molecules is dependent on the ability to covalently seal single-stranded nicks in DNA. This process is accomplished both *in vivo* and *in vitro* by the enzyme DNA ligase. DNA ligation is the process of joining together two DNA molecules ends (either from the same or different molecules). The enzyme that joins the DNA fragments is called DNA ligases. The DNA ligase seals the nicks in DNA by the formation of a phosphodiester bond between adjacent 3' hydroxyl and 5' phosphate termini. The enzyme extensively used in joining DNA fragments is the T4 DNA ligase. The ligase joins both cohesive ends as well as blunt-ended DNA. It is a single polypeptide with an M.W of 68,000 Dalton requires ATP as a cofactor. The maximal activity pH range is 7.5-8.0. The enzyme exhibits 40% of its activity at pH 6.9 and 65% at pH 8.3.



Materials Required:

- Sample fragmented Lambda DNA.
- T4 DNA ligase
- Ligation buffer
- Nuclease free distilled water (autoclaved)
- Agarose
- Gel loading dye
- Ethidium Bromide
- Micropipettes
- Micro tips
- Microfuge
- 50x TAE buffer
- Electrophoresis unit and power supply
- Microwave oven/heater
- UV transilluminator

Procedure:

- _____ µl of lambda DNA fragment sample (approximately 1 ug) is added to a microcentrifuge tube. (Sample has gone through single digest or double digested by Restriction enzyme)
- _____µl of 10X Ligation buffer is added.
- _____µl of sterile water is added.
- _____µl of T4 DNA Ligase (1 U) is added to the above contents.
- The total volume in the tube is_____µl. Incubation is carried out for 1 hr at 37º c or incubate in 16º C for overnight.
- _____ul of the DNA gel loading dye (6X) is added to the samples (digested lambda DNA fragments and ligated lambda DNA).
- The samples can be analyzed on 0.7% agarose gel by running at 100V for an hour.

Reaction Mixture:

DNA fragments	:	µl (1 ug)
Sterile water	:	µl
10X buffer	:	µl
T4 DNA Ligase	:	µl

Total	:	µl

Results and Discussion: (make it appropriate for the DNA fragments used in your study for ligation)

Date:

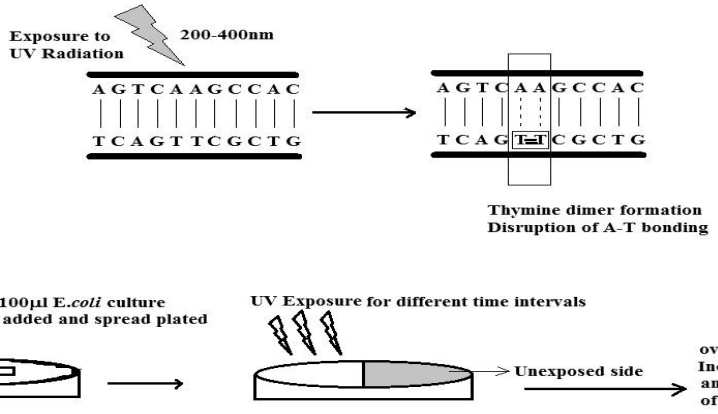
Experiment No. 9

Effect of UV Radiation on Bacterial Growth

Aim: To study the effect of UV exposure on the growth of bacterial cells.

Principle:

Mutations are a heritable change in the base sequence of DNA. Such mutations can be neutral or beneficial to an organism, but most are actually harmful because the mutation will often result in the loss of an important cellular function. Mutagens can be in the form of a chemical (nicotine) or in the form of electromagnetic radiation. Again there are two forms of electromagnetic radiation that are mutagenic; ionizing radiation and non-ionizing radiation. Ionizing radiation (x-ray, gamma radiation) has the potential to remove electrons from molecules in a cell. These electrons called free radicals that damage most other molecules in a cell, such as DNA or RNA, by oxidizing them. Nonionizing radiation (UV) causes the formation of pyrimidine dimers in the DNA molecule i.e. adjacent pyrimidine units are ionized forming highly reactive free radicals. These ionized pyrimidines interlink to form the dimers. The dimerization occurs by the formation of cyclobutane ring and this ring inhibits the replication process, thus hindering the normal functioning of the cell.



Materials Required:

Luria Broth (LB)-nutrient agar plates

Procedure:

- LB-nutrient agar plates are prepared.

- Overnight culture of *E.coli* is taken and 0.1 ml of it was surface plated.
- The plate is exposed to direct UV light for time intervals of 5, 10, 15, 30 minutes, by covering half of the plate with a glass plate.
- After exposure plates are incubated overnight at 37^{o} C.
- The number of bacterial colonies formed is counted in exposed and covered areas and percentage of reduction according to the respective time of exposure is determined using the given relation:-

$$\% \text{ of Reduction} = \frac{\text{colonies in (covered area- exposed area)} \times 100}{\text{colonies in a covered area}}$$

Observation:

1) For 5 min. exposure

   Colonies in covered area=

   Colonies in exposed area=

   % of Reduction=

2) For 10 min. exposure

   Colonies in covered area=

   Colonies in exposed area=

   % of Reduction=

3) For 15 min. exposure

   Colonies in covered area=

   Colonies in exposed area=

   % of Reduction=

4) For 30 min. exposure

   Colonies in covered area=

   Colonies in exposed area=

   % of Reduction=

Results and Discussion:

Date:

Experiment No. 10

Poly Acrylamide Gel Electrophoresis (PAGE)

Aim:

To separate DNA through 12% PAGE.

Principle:

Polyacrylamide gels are synthetic gels and are tougher than agar and agarose gels. Polyacrylamide gels are optically clear (including UV transparency) and electrically neutral due to the absence of any charged groups, in contrast to the presence of COO- and SO3- groups in agar and agarose gels. Moreover, Polyacrylamide gels can be prepared with a wide range of pore size by the relative proportions of Acrylamide to bisacrylamide. Therefore, Polyacrylamide gels are widely used to resolve the mixture of peptides, proteins and small molecular weight of nucleic acids.

Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N, N'- methylenebisacrylamide. The reaction is a free radical polymerization, usually carried out with ammonium persulfate as the initiator and N, N, N', N'- tetramethylethylenediamine (TEMED) as the catalyst. Although the gels are generally more difficult to prepare and handle, involving a longer time for preparation than agarose gels, they have major advantages over agarose gels. They have a greater resolving power, can accommodate larger quantities of DNA without significant loss in resolution and the DNA recovered from polyacrylamide gels is extremely pure (Guilliatt, 2002). Moreover, the pore size of the polyacrylamide gels can be altered in an easy and controllable fashion by changing the concentrations of the two monomers. Anyway, it should be noted that polyacrylamide is a neurotoxin (when unpolymerized), but with proper laboratory care, it is no more dangerous than various commonly used chemicals (Budowle & Allen, 1991). Some advantages and disadvantages of using polyacrylamide gels for DNA electrophoresis are depicted in Table 2 (Stellwagen, 1998). The choice of acrylamide concentration is critical for optimal separation of the molecules,(Hames, 1998). Choosing an appropriate concentration of acrylamide and the cross-linking, agent, methylenebisacrylamide, the pore sized in the gel can be controlled. With increasing the total percentage concentration (T) of monomer (acrylamide plus cross-linker) in the gel, the pore size decreases in a nearly linear relationship. Higher

percentage gels (higher T), with smaller pores, are used to separate smaller molecules. The relationship between the percentage of the total monomer represented by the cross-linker (C) is more complex.

Researchers have settled on C values of 5% (19:1 acrylamide/bisacrylamide) for most forms of denaturing DNA and RNA electrophoresis, and 3.3% (29:1) for most proteins, native DNA and RNA gels. For optimization, 5% to 10% polyacrylamide gels with variable crosslinking from 1% to 5% can be used. Low cross-linking (below 3% C) yields "long fiber gels" with increased pore size (Glavač & Dean, 1996). Moreover, it should be pointed out that at low acrylamide/bisacrylamide concentrations the handling of the gels is difficult because they are slimy and thin. Table 4 gives the recommended acrylamide/bisacrylamide ratios and gel percentages for different molecular size ranges.

Polyacrylamide gel electrophoresis is two types viz., Denaturing and non - denaturing (native) PAGE. In denaturing PAGE the protein is treated with the anionic detergent, SDS and β-mercaptoethanol or DTT. The SDS molecules bind to protein by strong hydrophobic interactions and it has been estimated that 1.4g SDS binds per gram of proteins. Since each SDS carries a negative charge, a protein molecule with a molecular weight of 50,000(i.e) approx.450 amino acids acquire 225 net negative charges. In order to break intra disulfide bonds, the protein is treated with β-mercaptoethanol.

Advantages

- Stable chemically cross-linked gel Sharp bands
- Good for separation of low molecular weight fragments
- Stable chemically cross-linked gel

The volume of Reagents Used to Cast Polyacrylamide Gels

Gel %	30% Acrylamide	Water	5x TBE	APS	TEMED
8 %	3.2 ml	6.4 ml	2.4 ml	200 µl	10 µl
10%	4.0 ml	5.6 ml	2.4 ml	200 µl	10 µl
12%	4.8 ml	4.8 ml	2.4 ml	200 µl	10 µl

Materials required:

- Mini gel Apparatus (Vertical)
- D.C power supply with Cord
- Test tubes
- Eppendorf's
- Micropipettes and tips
- Gel storage bag

Reagents required:

- Acrylamide stock (30%)
- Separating Gel Buffer (1.5M Tris, pH 8.8)
- Ammonium persulfate (10% APS)
- N, N, N', N'- tetramethyl ethylene diamine (TEMED)
- Electrophoresis buffer

Preparation of reagents:

- Acrylamide stock (30%) – Acrylamide- 29.2gm, Bisacrylamide- 0.8gm,

Dissolve the salts in 100 ml of water.

- Separating Gel Buffer (1.5M Tris, pH 8.8) - Weigh 18.17gm of Tris in 90 ml of distilled water adjust the pH was adjusted to 8.8 using 1N hydrochloric acid and final volume make upto 100 ml

- Ammonium persulfate (10% APS) - Weigh 1gm of APS and dissolve in10ml of distilled water.

- 10X TBE Stock Solution (Tank Buffer) – (1X = 89 mM Tris base, 89 mM Boric acid, 2 mM EDTA) - 108.0 g Tris base, 55.0 g Boric acid, 7.44 g $Na_2EDTA \cdot 2H_20$, Adjust volume to 1 liter with distilled water, Filter through a 0.45 µm filter, pH adjustment is not necessary

Procedure :

- Clean the glass plates by soaking in detergent and wash thoroughly with water and keep in a hot air oven.
- Assemble the plates with spacers using petroleum ether (Vaseline) and clamp it with the help of clips.

- Prepare the gel mixture as shown in the table and immediately pour it into the assembled plate.
- Insert the comb gently in between the glass plates on the top of the gel mixture.
- Allow the gel to get polymerized for at least 20 min.
- When the polymerization of the gel gets over, remove the comb and the lower spacer strip carefully.
- Remove excess vaseline from the bottom of the gel by wiping it with a piece of tissue paper.
- Remove the comb carefully and clean the wells using filter paper.
- Fill the lower reservoir and upper reservoir of the electrophoresis apparatus with the required volume of the tank buffer.
- Fix the gel plate to the electrophoresis tank carefully with appropriate clips and clamps.
- Load the DNA samples in the wells and equalize with the level electrophoresis buffer in the upper chamber by filling with electrophoresis buffer.
- Raise the level of buffer in the upper reservoir.
- Connect the electrodes to the power pack and the switch on the current and observe for the formation of bubbles which indicates the passage of current
- Keep 15-20mA/75-100V for separating gel.
- Turn off the power supply when the tracking dye reached the bottom of the gel and transfer the gel to the staining solution for 1-2 hours.
- Later transfer the gel to the destaining solution until the clear bands are visible.

Results and Discussion:

Date:

Experiment No. 11

Polymerase Chain Reaction

Aim: To carry out the polymerase chain reaction (amplification) of the given DNA sample.

Principle:

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium ***Thermus aquaticus*.** This DNA polymerase enzymatically assembles a new DNA strand from, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides called DNA primers, which are required for initiation of DNA synthesis. It has the following steps:

- Initial denaturation
- Denaturation
- Primer annealing
- Extension of DNA
- Final Extension

The initial denaturation and final extension occur once (1 cycle). The other three steps i.e. denaturation, primer annealing and extension of DNA occur in every cycle (30 cycles).

Materials Required:

- 10X Assay buffer (100 mM Tris-Cl (pH 9.0), 50 mM KCl, 15 mM MgCl2, and 0.1% of gelatin)
- PCR tubes
- Nuclease free water

- Gel loading buffer
- 10 mM dNTP mix
- Agarose
- 100 bp ladder
- Forward & Reverse primers
- Ethidium Bromide
- Template DNA
- UV-Transilluminator
- Taq DNA polymerase, Thermocycler
- Tips, Gloves & Micropipettes
- TAE buffer

Procedure:

Materials	**Volume**
Sterile Water	38µl
10X assay buffer	5 µl
10 mM dNTP mix	3 µl (0.6mM)
Template DNA	1 µl (100ng)
Forward primer	1 µl (100ng)
Reverse primer	1 µl (100ng)
Taq DNA polymerase	1 µl (3 Units)
Total Volume	**50 µl**

- The contents were mixed gently and then spun.

PCR Amplification Cycles:

Initial Denaturation	94' C, 1 minute - 1 cycle	
Denaturation	**94º C, 30 seconds** ----------	
Annealing	**58º C, 30 seconds**----------	30 cycles
Extension	**72º C, 1 minute**----------	
Final extension	72º C, 2 minute – 1 cycle 4º C, hold	

- Following PCR amplification, 5 µl of gel loading dye was added in each of the PCR tubes.
- The mix was tapped thoroughly.
- 15 µl of the reaction mixture was carefully pipetted out and was loaded onto 1.5% of agarose gel.
- 10 µl of 100 bp ladder was loaded. The order in which the samples had been loaded was noted.
- The samples were run at 100 volts for 1-2 hours till the tracking dye (Bromophenol blue) had reached 3/4th of the length of the gel.
- The gel was viewed under UV- transilluminator.

Observation:

The amplified band was compared with the 100 bp ladder and the size of the fragment was noted. Also, it was observed for the presence of any other band other than the amplified product.

Preparation of Agarose Gel:

- 1.5% of the 1X TAE buffer was prepared.
- 1.5 g agarose was weighed and added into 100 mL of 1X TAE buffer resulting in 1.5% of agarose gel.
- It was boiled till agarose dissolved completely and clear solution results.
- Meanwhile, the combs of electrophoresis were placed such that it was approximately 2 cm away from the cathode.
- Ethidium bromide (final concentration 0.5µg/mL) was added, and the agarose solution was poured in the central part of the tank when the temperature reaches approximately 60ºC.generation of any air bubbles

was avoided. The thickness of the gel should be around 0.5 cm to 0.8 cm. The gel was allowed to solidify.

- 1X TAE buffer was poured onto the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
- The combs were lifted gently ensuring the wells remain intact.

Results and discussions: