AVIT
AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY

VINAYAKA MISSION'S RESEARCH FOUNDATION
(Deemed to be University under section 3 of the UGC Act 1956)

Accredited by NAAC

Approved by AICTE

DEPARTMENT OF BIOTECHNOLOGY

B.TECH

MOLECULAR BIOLOGY LAB MANUAL
(17BTCC85)

HOD/BIOTECH

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17BTCC85-MOLECULAR BIOLOGY LAB

LIST OF EXPERIMENTS

Expt: No	Name of the Experiment
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2.	QUANTITATIVE ANALYSIS OF DNA
3.	QUALITATIVE ANALYSIS OF DNA
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5.	ISOLATION OF MITOCHONDRIA FROM YEAST CELL
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7.	ISOLATION OF DNA FROM ANIMAL
8.	AGAROSE GEL ELECTROPHORESIS

GENERAL GUIDELINES FOR WORKING IN MOLECUALR BIOLOGY LAB

PRECAUTIONARY MEASURES

1. Never mouth – pipette any solution (acids, phenols, peroxides organic solvents, culture of organisms, etc.)
2. Wear a laboratory coat (use gloves when necessary)
3. Aseptic techniques should be followed rigorously at all times.
4. All microbial cultures are handled and treated as potential biohazards and dispose them of after autoclaving or after treating with 10%formalin for 30 minutes.
5. Dispose of all the wastes properly.

PREPARATION OF REAGENTS

1. Store chemicals/solvents at appropriate temperatures as mentioned on the label.
2. The highest purity chemicals and double distilled water should be used for preparation of reagents
and solutions. (Weight of the substance, brand, batch N0.of the substance, type of water used,
amount of alkali or acid used to dissolve should be recorded then and there).
3. Label all reagents. The label should contain the name of the solution/reagent, concentration
(%or molar), date of preparation of the reagent and initials of the preparation of the reagent and
initials of person who prepared.
4. Store the reagents in appropriate bottles and at appropriate temperatures. (Light sensitive
reagents and solutions should be stored in brown /dark bottles).

EXPERIMENTS AND RECORD KEEPING

1. Make sure the laboratory and laboratory benches are clean.
2. Make sure that the balance is cleaned after your weighing.
3. Make sure that the glassware's, plastic wares, media, etc., are ready for the experiment (if needed sterilize them previous day itself).
4. Label all the plates, tubes cultures etc., correctly before starting the experiment.
5. All calculations should be done step by step and recorded neatly.

SAFETY MEASURES

1. Never do direct mouth pipetting of infectious or toxic fluids; use a pipetting device.
2. Plug pipettes with cotton.
3. Do not blow infectious material out of pipettes.
4. Develop the habit of keeping your hand away from your mouth, nose eyes and face while handling the chemicals.

Experiment No. 1

ISOLATION OF GENOMIC DNA FROM *E. COLI*

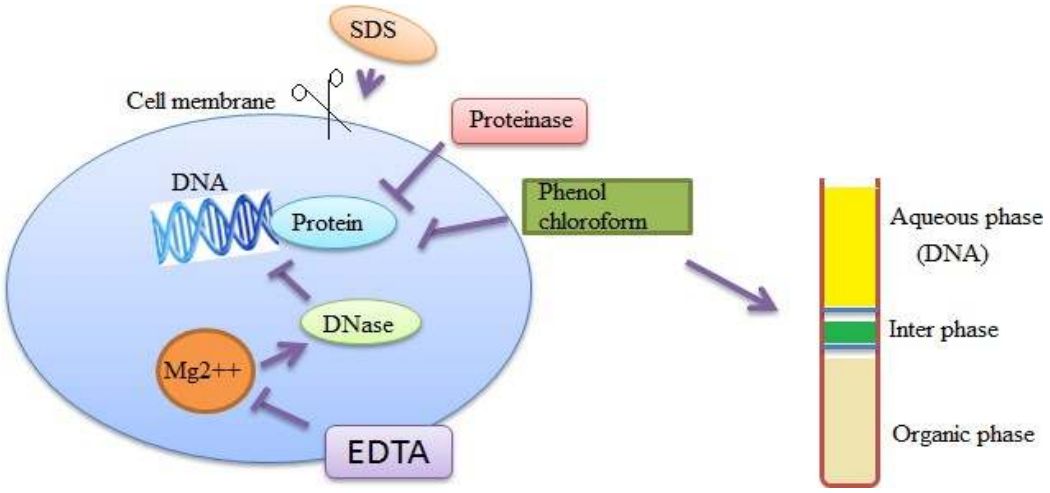
Aim: To isolate the genomic DNA from ***E .coli*** **DH5α** cells.

Principle:

The isolation and purification of DNA from cells is one of the most common procedures in contemporary molecular biology and embodies a transition from cell biology to the molecular biology (from *in vivo* to *in vitro*). The isolation of DNA from bacteria is a relatively simple process. The organism to be used should be grown in a favorable medium at an optimal temperature, and should be harvested in late log to early stationary phase for maximum yield.

The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) is used to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form 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.

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



Materials Required:

- LB Broth
- *E. coli* DH5α cells
- Reagents
- TE buffer (pH 8.0)
- 10% SDS
- Proteinase K
- Phenol-chloroform mixture
- 5M Sodium Acetate (pH 5.2)
- 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. **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: The pH is very important. For RNA purification, the pH is kept around pH 4, which retains RNA in the aqueous phase preferentially. For DNA purification, the pH is usually 7 to 8, at which point all nucleic acids are found in the aqueous phase. 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 with dilute acetic acid (pH 5.2).

6. ISOPROPANOL

7. 70% ETHANOL

PROCEDURE:

- 2 ml overnight culture is taken and the cells are harvested by centrifugation for 10 minutes
- 875 µl of TE buffer is added to the cell pellet and the cells are resuspended in the buffer by gentle mixing.
- 100 µl of 10% SDS and 5 µl of Proteinase K are added to the cells.
- The above mixture is mixed well and incubated at 37º C for an hour in an incubator.
- 1 ml of phenol-chloroform mixture is added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4º C.
- The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube.
- The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
- 100 µl of 5M sodium acetate is added to the contents and is mixed gently.
- 2 ml of isopropanol is added and mixed gently by inversion till white strands of DNA precipitates out.

- The contents are centrifuged at 5,000 rpm for 10 minutes.
- The supernatant is removed and 1ml 70% ethanol is added.
- The above contents are centrifuged at 5,000 rpm for 10 minutes.
- After air drying for 5 minutes 200 µl of TE buffer or distilled water is added.
- 10 µl of DNA sample is taken and is diluted to 1 or 2 ml with distilled water.
- The concentration of DNA is determined using a spectrophotometer at 260/280 nm.
- The remaining samples are stored for further experiments.

PRECAUTIONS:

- Cut tips should be used so that the DNA is not subjected to mechanical disruption.
- Depending on the source of DNA the incubation period of Proteinase K should extended.
- 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:

Experiment No. 2

QUANTITATIVE ANALYSIS OF DNA

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

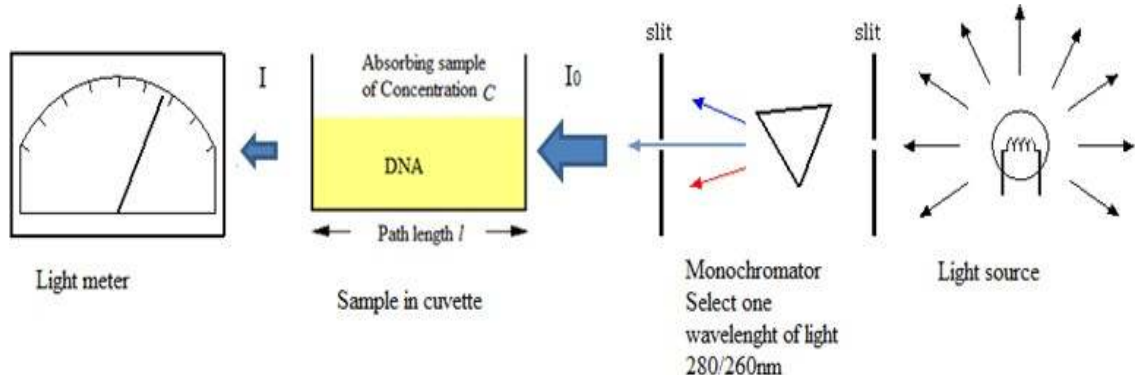
Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by following expression:

$$A = \varepsilon \times c \times l$$

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

Concentration and quality of a sample of DNA is 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.

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

Total DNA (ug) = (A260) (50 ug/ml/A260) (100) (0.1 ml)

where 100 is the dilution factor and 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 and Discussion:

Experiment No. 3

QUALITATIVE ANALYSIS OF DNA

Aim: To separate and visualise 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.

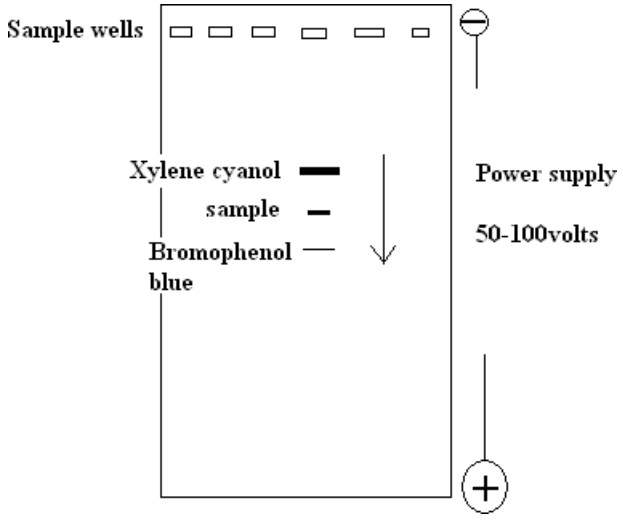
Principle:

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 in to 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 colour 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 xyanol gives a greenish blue colour while bromophenol blue provides 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 gel.

2. Loading of Samples

- Carefully transfer the gel to the electrophoresis tank filled with 1x TAE buffer.
- Prepare your samples [8 ul of DNA 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 3/4 th 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 visualising the bands, avoid direct contact and exposure to eyes.

Results and Discussion:

Experiment No. 4

PLASMID DNA ISOLATION

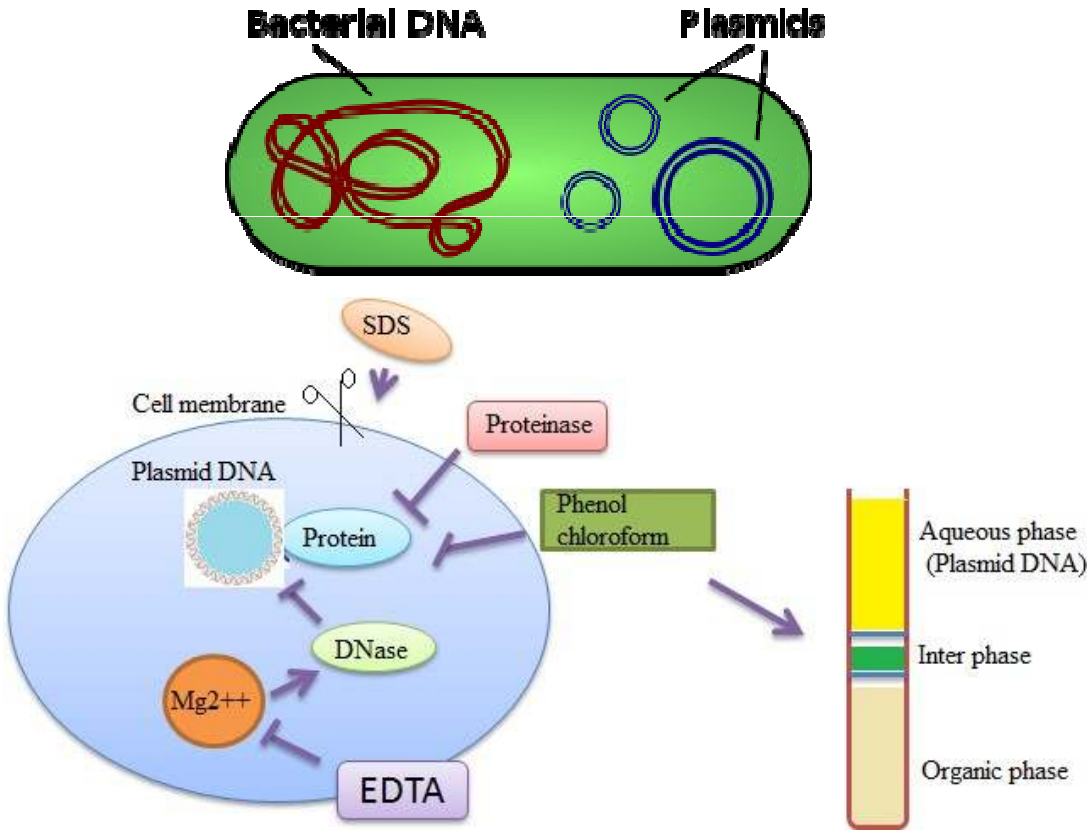
Aim: To isolate plasmid DNA from bacterial cells.

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, 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 cell membrane, Tris is a buffering agent used to maintain a constant pH8. Plasmid 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. 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 neutralise 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 plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and 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 ($0^{o}C$) ethanol or isopropanol. The precipitate is usually redissolved in buffer and treated with phenol or organic solvent to remove the last traces of protein, followed by reprecipitation with cold ethanol.

Schematic diagram of principle of Plasmid DNA Isolation



Materials Required:

- Luria Broth
- Bacterial cells containing plasmid
- Reagents
- TE buffer(pH 8.0)
- Solution I
- Solution II
- 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. **Solution I:** Lysis solution
3. **Solution II:** Denaturing solution
3. **Solution III:** Neutralizing solution
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 in dark.
5. **ISOPROPANOL**

PROCEDURE:

- Take 2 ml 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 fresh tube.
- Add 400 µl of 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 cut 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.
- After air drying for 5 minutes, add 100 µl of TE buffer or autoclaved distilled water to the pellet to resuspend the plasmid DNA. The contaminated salt in the DNA pellet can be removed with 70% ethanol washing.
- 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 plasmid to obtain pure plasmid.
- DNase free plastic wares and reagents should be used.

Results and Discussion

Experiment No. 5

ISOLATION OF MITOCHONDRIA FROM YEAST CELL

Aim: To isolate mitochondria from yeast cells.

Principle: Isolation of mitochondria involves cell disruption and centrifugation. The process of cell disruption involves breaking open of cell so as to spill out the contents within the cell. Centrifugation is the process by which mixtures of cell components are separated by centrifugal force. The more dense particles migrate away from the axis, while less dense components of the mixture migrates towards the axis of centrifuge. The centrifugal technique which is used to separate the cell components from whole cell is called differential centrifugation. Differential centrifugation gives only a crude extract.

Materials Required

1. Yeast culture.
2. Refrigerated Centrifuge.
3. 15ml centrifuge tubes.
4. Sodium Chloride (0.9%).
5. Micropipette.
6. Ice cold lysis buffer.
7. Shaker.
8. Mitochondria storage buffer.
9. Refrigerator.
10. 15ml micro centrifuge tube.

Procedure

1. Aseptically transfer the overnight yeast culture into two 15ml centrifugation tubes.
2. Centrifuge it at 500g for 10 minutes at 4^{o}C.
3. Carefully remove the supernatant without disturbing the pellet.
4. Carefully rinse the pellet in 1ml sodium chloride (0.9%) using a micropipette.
5. The sodium chloride from the centrifugation tube is discarded using a micropipette.
6. Resuspend the pellet in 1ml of ice cold lysis buffer and mix well using a micropipette.
7. Incubate it at 4^{o}C on a shaker for 10 minutes.
8. Centrifuge it at 1000g for 10 minutes at 4^{o}C and carefully remove the supernatant.
9. Resuspend the cell pellet in 1.5 ml ice cold disruption buffer and complete cell disruption by using the blunt end of a needle.
10. Centrifuge the lysate at 1000g for 10 minutes at 4^{o}C.

11. Transfer the supernatant to a fresh 15mL tube and also mix the supernatant obtained from the step 7.
12. Centrifuge it at 6000g for 10 minutes at 4^{o}C and discard the supernatant.
13. Wash the pellet with mitochondria storage buffer.
14. Centrifuge it at 6000g for 20 minutes at 4^{o}C.
15. Resuspend the pellet in mitochondria storage buffer and store at -20^{o}C.

RESULTS AND DISCUSSION:

Experiment no:6

ELUTION OF DNA FROM AGAROSE GEL ELECTROPHORESIS

Aim:

To extract specific bands of DNA from agarose gels in which they are separated through electrophoresis.

Principle:

DNA isolation is a critical step in molecular biology. It is necessary to obtain a specific DNA fragment from the extracted DNA in molecular biology techniques. After isolating plasmids it may contain some chromosomal DNA contamination it will interrupt the further processing of cloning. So it is better to recover the plasmid DNA by eluting it from agarose gels (extraction). The first step in extracting DNA is identifying the DNA band which is to extract, by illuminating under UV light. The desired band is then carefully cut by a Scalpel blade. There are several methods for extracting DNA from the agarose gels. Recovery of DNA from agarose gels by electrophoresis onto DEAE-cellulose membrane is one of the rapid and effective methods. Electroelution is also a good method for DNA recovery especially for larger DNA fragments. Several kit methods are also used in laboratories. In electro elution, the gel fragment of desired DNA band is placed into a dialysis bag with buffer. The bag is then placed into a gel box containing buffer and subjected to an electric current. The DNA extracted is precipitated from the solution. In another recovery method using DEAE cellulose membrane, the gel piece is slide into the slit of DEAE cellulose paper which will bind the DNA. Then an electric current is applied in order to move the band in the paper. DNA is washed off from the paper and is precipitated with ethanol. Freeze-thaw method of extraction is a commonly used advantageous DNA recovering method which will supports the common laboratory facilities. It is very simple and easy to perform with good yield.

Most of the molecular biology laboratories use Low melting point agarose for the separation of DNA from agarose. Low melting point agarose melts at a lower temperature than standard agarose and this temperature does not denature double stranded DNA. It is better to extract DNA fragments in a TAE buffered gel than TBE buffered gel because borate present in the buffer interferes with purification methods.

Materials Required

- Elution buffer
- Scalpel blade
- UV transilluminator
- Agarose
- Micro pipettes
- Micro pipette tips
- Dry bath incubator
- Microfuge tubes
- Centrifuge
- N-Butanol
- Cryo box
- Cyclomixer
- 70% Ethanol
- 95% Ethanol
- TE buffer
- -20^{o}C freezer
- -70^{o}C freezer

Procedure

1. Visualize the low melting point agarose gel with DNA bands under a UV transilluminator and locate the desired DNA band to cut.
2. Carefully cut around the desired DNA band using a scalpel blade.
3. Transfer the gel piece into a microfuge tube.
4. Add elution buffer into the microfuge tube until the level of buffer is just above the level of gel slice.
5. Heat the gel slice at 65^{o}C until it melts.
6. Freeze the melted gel with DNA by placing in a -70^{o}C freezer for10minuts.
7. After freezing, centrifuge for 10minutes and transfer the supernatant into a new microfuge tube.
8. Again add half amount of elution buffer that you added in the previous step into the pellet.
9. Heat at 65^{o}C until the agarose melts.

10. Freeze the melted gel with DNA by placing in a -70^{o}C freezer for10minuts.
11. Centrifuge the tube again for 10 minutes and transfer (pool) the supernatant into the previous tube with supernatant.
12. Discard the tube with pellet.
13. Add an equal volume of n-Butanol to the supernatant and mix the contents well.
14. Vortex the tube for 15 minutes in order to remove the Ethidium bromide.
15. Discard the upper phase of butanol and repeat the process by adding n-butanol again for one or more times.
16. Add 2 times volume of 95% ethanol and mix thoroughly.
17. Keep for precipitation in -70^{o}C freezer for 30minutes to overnight.
18. After precipitation, centrifuge for 15 minutes.
19. Discard the supernatant into a waste beaker and add 200µl of 70% ethanol to the pellet.
20. Centrifuge for 5minutes and discard the supernatant again.
21. Allow the pellets to dry well.
22. Suspend the pellets in 20µl of TE buffer. (If you want to confirm the recovered DNA, run (1µl) it on a gel.
23. The recovered DNA can be now used for further process of cloning otherwise can stored in -20^{o}C freezer.

Result and observation:

Experiment no:7

ISOLATION OF DNA FROM ANIMAL

AIM

To isolate the genomic DNA from the given animal tissue.

PRINCIPLE

To analyze the complex genome of the eukaryotes, it is necessary to prepare the pure high molecular weight DNA. The main principle involved is the breakage of cells to release the nuclei and subsequent treatments with detergents and enzymes to degrade most of the contaminating proteins. The digest is deproteinised by successive phenol, chloroform or isoamyl alcohol extraction and the DNA is recovered by ethanol precipitation.

MATERIALS REQUIRED

Animal tissue sample
Homogenizer
Centrifuge tubes
Agarose gel unit

BUFFERS

Homogenization Buffer
Sucrose - 0.25mM
Sodium chloride – 12.5mM
potassium chloride – 12.5mM
Magnesium chloride – 5mM
Tris Cl – 50mM

Suspension Buffer
Sodium chloride – 10mM
Magnesium chloride – 3mM
Tris Cl – 10mM
Ammonium acetate – 7.5mM
Ethanol – 100%
Phenol, chloroform, isoamyl alcohol – 25:24:1

PROTOCOL

- ✓ Weigh 200mg of tissue sample and grind it in about 5ml of saline buffer to free from blood. Quickly add 8 volumes of ice cold homogenizing buffer.
- ✓ Filter the homogenate through three layers of cheese cloth to remove the clumps.
- ✓ Centrifuge the filtrate at 4ºC for 10 minutes at 3000 rpm.
- ✓ Discard the supernatant and suspend the nuclear pellet in desired volume of suspension buffer.
- ✓ Adjust the nuclear pellet with NaCl and 0.5% of SDS.
- ✓ Centrifuge the sample at 4ºC for 10 minutes at 10,000 rpm.
- ✓ Extract sample thoroughly with a volume of phenol/chloroform/isoamyl alcohol mix.
- ✓ Centrifuge the sample at 4ºC for 10 minutes at 10,000 rpm.Separate the organic extraction by several times.
- ✓ Transfer the aqueous phase to another fresh tube and add 0.5 volume of ammonium acetate, 2 volumes of 100% ice cold ethanol.
- ✓ Centrifuge the sample at 4ºC for 10 minutes at 5,000 rpm.
- ✓ Rinse the pellet with 70% ethanol and then air dry it.
- ✓ Suspend the pellet in 100µl of TE buffer until it dissolve.

OBSERVATION AND RESULT

Experiment no:8

AGAROSE GEL ELECTROPHORESIS

AIM

To separate the DNA fragments based on their Molecular weight.

PRINCIPLE

Agarose gel electrophoresis is the easiest and most popular way of separating and analyzing DNA. Here DNA molecules are separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide.

Agarose is a polysaccharide obtained from the red algae Porphyra umbilicalis. Its systematic name is **(1 4)-3, 6-anhydro-a-L-galactopyranosyl-(1 3)-β-D-galactopyranan.** Agarose makes an inert matrix. Most agarose gels are made between 0.7% and 2% of agarose. A 0.7% gel will show good separation for large DNA fragments (5-10kb) and a 2% gel will show good resolution for small fragments with size range of 0.2-1kb. Low percentage gels are very weak (Note:- it may break when you lift them) but high percentage gels are usually brittle and do not set evenly. The volume of agarose required for a minigel preparation is around 30-50ml and for a larger gel, it is around 250ml.

Materials Required:

Buffers and Solutions:

Agarose solutions.
Ethidium bromide.
Electrophoresis buffer.

Nucleic Acids and Oligonucleotides:
DNA samples.
DNA Ladders.

(Samples of DNAs of known size are typically generated by restriction enzyme digestion of a plasmid or bacteriophage DNA of known sequence).

The equipment and supplies necessary for conducting agarose gel electrophoresis are relatively simple and include:

- An electrophoresis chamber and power supply.
- **Gel casting trays,** which are available in a variety of sizes and composed of UV-transparent plastic.
- **Sample combs,** around which molten agarose is poured to form sample wells in the gel.
- **Electrophoresis buffer,** usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- **Loading buffer,** which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- **Ethidium bromide,** a fluorescent dye used for staining nucleic acids.
- **Transilluminator** (an ultraviolet light box), which is used to visualize ethidium bromide-stained DNA in gels.

NOTE: Always wear protective eyewear when observing DNA on a Transilluminator to prevent damage to the eyes from UV light.

For this we take 2ml of TAE stock solution in an Erlenmeyer flask and make the volume to 100ml by adding 98ml of distilled water. The 1x working solution is 40 mM Tris-acetate/1 mM EDTA

It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel preparation.

For this usually 2 grams of agarose is added to 100ml of electrophoresis buffer.

Agarose Concentration in Gel (% [w/v])	**Range of Separation of Linear DNA Molecules (kb)**
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0-2-3
2.0	0.1-2

PROCEDURE:

1. **Prepare a 50x stock solution of TAE buffer in 1000m of distilled H_2O:**
   For this weigh 242 g of Tris base in a chemical balance. Transfer this to a 1000ml beaker.
   Prepare EDTA solution (pH 8.0, 0.5M) by weighing 9.31g of EDTA and dissolve it in 40ml distilled water. EDTA is insoluble and it can be made soluble by adding sodium hydroxide pellets. Check the pH using pH meter. Make the solution 100ml by adding distilledwater.
   Pipette out 57.1 ml of glacial acetic acid.
   Mix the Tris base, EDTA solution and glacial acetic acid and add distilled water to make the volume to 1000ml

2. **Prepare sufficient electrophoresis buffer (usually 1x TAE) to fill the electrophoresis tank and to cast the gel:**
3. **Prepare a solution of agarose in electrophoresis buffer at an appropriate concentration:**
4. Loosely plug the neck of the Erlenmeyer flask. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves. The agarose solution can boil over very easily so keep checking it. It is good to stop it after 45 seconds and give it a swirl. It can become superheated and NOT boil until you take it out whereupon it boils out all over you hands. So wear gloves and hold it at arm's length. You can use a Bunsen burner instead of a microwave - just remember to keep watching it.

5. Use insulated gloves or tongs to transfer the flask/bottle into a water bath at 55°C. When the molten gel has cooled, add 0.5µg/ml of ethidium bromide. Mix the gel solution thoroughly by gentle swirling.

   (For the preparation of ethidium bromide adds 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the 10 mg/ml solution to a dark bottle and store at room temperature.)

6. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel.

7. Pour the warm agarose solution into the mold.
8. Allow the gel to set completely (30-45 minutes 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. Mount the gel in the electrophoresis tank.
9. Add just enough electrophoresis buffers to cover the gel to a depth of approx. 1mm.

10. Mix the samples of DNA with 0.20 volumes of the desired 6x gel-loading buffer.
11. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette or an automatic micropipettor or a drawn-out Pasteur pipette or a glass capillary tube. Load size standards into slots on both the right and left sides of the gel.

12. 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). If the electrodes are 10cm apart then run the gel at 50V. It is fine to run the gel slower than this but do not run it any faster. Above 5V/cm the agarose may heat up and begin to melt with disastrous effects on your gel's resolution. If the leads have been attached correctly, bubbles should be generated at the anode and cathode.

13. Run the gel until the bromophenol blue and xylenecyanol FF have migrated an appropriate distance through the gel.

    (The presence of ethidium bromide allows the gel to be examined by UV illumination at any stage during electrophoresis).

14. The gel tray may be removed and placed directly on a transilluminator. When the UV is switched on we can see orange bands of DNA.