SYLLABUS

1. Isolation and estimation of DNA, RNA and protein from animalsource
2. Isolation of plasmid DNA from *E. coli* cells.
3. Agarose gel electrophoresis ofDNA
4. Digestion of DNA with restrictionenzymes.
5. Amplification of a DNA fragment byPCR.
6. Transformation of *E. coli* cells with plasmidDNA.
7. Western Blotting(Demo)

The aim is to isolate the DNA from the given animal tissue

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DEPARTMENT OF BIOCHEMISTRY

PRACTICAL PLAN

SUBJECT NAME: GENETIC ENGINEERING AND BIOTECHNOLOGY PRACTICAL-A
SUB.CODE: 17BCU611A
SEMESTER:VI **CLASS: III B.Sc., BIOCHEMISTRY**

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Referencebook

T1. S. Rajan and R. Selvi Christy (2010). Experimental Procedures in Life Sciences. Anjanaa Book House, Chennai.

SYLLABUS

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Ex No.1

ISOLATION OF DNA

Aim

The aim is to isolate the DNA from the given animal tissue sample.

Principle

Extraction of DNA basically consists of four major steps:

- Preparation of a cellextract
- Purification of DNA from cellextract
- Concentration of DNAsamples
- Measurement of purity and DNAconcentration

Reagents

1. Lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% sodium lauryl sarcosiante, 0.5 μg/ml proteinase K).
2. Ribonuclease A (10 ml, 0.5μg/ml)
3. Phenol (Molecular BiologyGrade)
4. Chloroform
5. IsoamylAlcohol
6. Sodium Acetate (0.5M)
7. Ethanol

Procedure

1. To extract DNA from cells of interest, cells are lysed with 100 –200 μl of lysis buffer [50 mM Tris-HCl (pH 8.0), containing 10 mM EDTA, 0.5% sodium lauryl sarcosinate and 0.5 μg/ml proteinaseK].

2. Incubate for 1 h at50ºC.
3. Add Ribonuclease A (10 µl, 0.5 mg/ml) and incubate for an additional 1 hr at50ºC.
4. Add 1ml of phenol, shake well for 5 to 10 min and then centrifuge at 3000 rpm for 5 min at4ºC.
5. Transfer the supernatant to a new microcentrifuge tube with 500 µl of phenol and 500 µl of chloroform / isoamyl alcohol (24:1), shake it well for 5 to 10 min and centrifuge at 3000 rpm for 5 min at4ºC.
6. After centrifugation, transfer the supernatant to a new tube and add 25 – 50 µl of 3 M sodium acetate (pH 5.2) and 1 ml of ethanol, shake gently till the DNA to precipitate.
7. Then place it under -8^{0}C for 20 minutes and centrifuge at 12000 rpm for 20 min to recover theDNA.
8. Rinse the pellet with 1 ml of 70% ethanol and spin for10min.
9. Discard the supernatant and air dry the pellet at roomtemperature.
10. Dissolve the DNA in 0.5 – 1.0 ml of Millipore water to determine the concentration and purity of DNA by absorbance at 260/280 nm in aUV-spectrophotometer.

Result

The purity of DNAobtained is_____.

ESTIMATION OF DNA

Aim:
To estimate the amount of DNA present in the given unknown solution by diphenylamine method.

Principle:
When DNA is treated with diphenylamine under the acidic condition a bluish green colored complex is formed which has an absorption peak at 595nm.This reaction is given by 2 deoxypentose in general. In acidic solution deoxypentose are converted into a highly reactive β hydroxyl leavulinic aldehyde which reacts with diphenylamine gives bluish green colored complex. The colour intensity was measured using a red filter at595nm.

Reagent required:
1. Stock Standard Solution: 50mg of DNA was dissolved in 50ml of Saline Sodium citrate buffer. Concentration 1mg/ml

2 .Working Standard Solution: 5ml of stock solution solution was diluted to 50ml with distilled water. Concentration 100µg\ml
3. Diphenylamine Reagent: 10g of pure diphenylamine was dissolved with 25ml of concentration sulphuric acid which was made up to 1ml with glacial acidic acid the solution must be preparedfreshly.
4. Buffered Saline ph 7.4: 0.14N Sodium chloride and 0.02M sodiumcitrate.
5. Unknown Solution: The given unknown solution is mad up to 100ml with distilledwater

Procedure:

1. 0.5-2.5ml of working standard solution is pipetted out into 5 test tubes labeled as s1- s5 where concentration ranging from50-250µg.
2. 1ml and 2ml of unknown solution is pipetted out into two test tube u1 andu2.
3. The volume in all test tubes is made up to 3ml with distilled water and 3ml of distilled water alone serve as ablank.
4. 4ml of diphenylamine reagent was added to all the tubes. The tubes were kept in a boiling water bath at 36°C for 20min. The tubes were than cooled and the bluish colour developed is read at595nm.
5. A standard graph is drawn taking concentration of DNA on x-axis and absorption of y-axis. From the standard graph the amount of DNA present in the unknown solution is calculated.

Result:

The amount of DNA present in the given unknown solution is found to be

Ex No.2

ISOLATION OF TOTAL RNA

Aim

To isolate RNA from the given tissue sample.

Principle

Single step guanidium acid phenol method emphasizes on the ability of guanidium isothiocyanate (GITC) to lyse cells, denature protein and inactivate intracellular ribonuclease rapidly. The presence of β mercaptoethanol in the mixture increases the solubilization properties of the GITC extraction buffer. Acid phenol extraction (pH< 5.0) selectivity keeps cellular DNA in the organic phase and help in extraction of proteins and lipids. The addition of chloroform further removes lipids and produce two distinct phases containing the DNA, proteins and lipids and an aqueous phase containing the RNA.

Reagents required

1. TRIR kit has the followingcomponents:
   Phenol, guanidium isothiocyanate, urea, detergents, buffering agents and stabilizers.
2. Chloroform (molecular biologygrade)
3. Isopropanol (molecular biologygrade)
4. 75% ethanol (molecular biologygrade)

Procedure

1. Add 1 ml of TRIR to the homogenised tissue sample and swirl gently for 15 min and then keep at 4°C for 5 min to permit complete dissociation of nucleoprotein complexes.
2. To this, add 0.2 ml chloroform, shake vigorously for 15 sec and place on ice at 4°C for 5min.
3. The lysate is to be then centrifuged at 12,000 x g for 15 min at 4°C to yield lower organic phase containing DNA and proteins and upper aqueous phase containing RNA.
4. The volume of the aqueous phase will be approximately 40-50% of the total volume of thelysate.
5. The aqueous phase to be carefully transferred to a fresh eppendorf micro centrifuge tube without disturbing the interphase. Equal volume of isopropanol to be added, mixed and kept at 4°C for 10 min. It is to be again centrifuged at 12,000 x g for 15 min at 4°C to precipitate theRNA.

6. Discard the supernatant and wash the pellet twice with 75% ethanol and air dried. Dissolve the RNA pellet in 50µl of sterile milliQ water and place in a water bath at 60°C for 10 min to ensure maximum solubility ofRNA.
7. Vortex gently the RNA sample and quantify before storing at-80°C.

Quantification of RNA

Diluted RNA sample to be quantified spectrophotometrically by measuring the absorbance (A) at 260 nm. An absorbance of 1OD is equivalent to RNA concentration of 40 µg/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260 nm with dilution factor and 40 µg. The purity of RNA preparations were assessed by determining the ratio of absorbance of sample at 260 nm and 280nm.

Result

The purity of RNAobtainedis_____.

ESTIMATION OF RNA

Aim:

To estimate the concentration of RNA by orcinol reaction.

Principle:

This is a general reaction for pentoses and depends on the formation of furfural when the pentose is heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour, which can be measured at 665nm.

Requirements:

1. Standard RNA solution- 200µg/ml in 1 N perchloric acid/bufferedsaline.
2. Orcinol Reagent- Dissolve 0.1g of ferric chloride in 100 ml of concentrated HCl andadd 3.5 ml of 6% w/v orcinol in alcohol.
3. Buffered Saline- 0.5 mol/litre NaCl; 0.015 mol/litre sodium citrate, pH7.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled test tubes.
2. Pipette out 1 ml of the given sample in another testtube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as theblank.

4. Now add 2 ml of orcinol reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and heat on a boiling water bath for 20min.
6. Then cool the contents and record the absorbance at 665 nm againstblank.
7. Then plot the standard curve by taking concentration of RNA along X-axis and absorbance at 665 nm alongY-axis.
8. Then from this standard curve calculate the concentration of RNA in the givensample.

Result:
The given unknownsample contains----- µg RNA/ml.

Ex No.3

ISOLATION OF PROTEIN

Aim

To isolate total protein from the given animal tissue sample.

Materials required

RIPA buffer: - RIPA buffer stock sol.(stock at RT): 50 mM Tris-HCl, pH 7.4 - 7.6 (dil. 1M stock); 150 mM NaCl (8.8g/ L). - Add 1% NP-40; 1% Triton X-100; 1% CHAPS; 1 mini tablet Complete (Roche protease inhibitors), 100 µl NaF stock sol. (200 mM, RT) (= phosphatase inhibitor), 100 µl activated Na3VO4 stock sol. (200 mM, 4°C, stored in 50 µl aliquots) to 10 ml RIPA buffer stocksolution.

Procedure

1. Weigh the tissue, then add RIPA buffer at a ratio of 0.4 ml per 0.1 g tissue (1:4 w:v).
2. Homogenize on ice using a tight-fitting glass homogenizer and transfer homogenate into a 2 ml tube.
3. Incubate on ice for 10 min to maximize proteinsolubilization.
4. Centrifuge at 19,000g and 4°C for 10min.
5. Save the supernatant into a clean 2 mltube
6. Snap-freeze the sample in liquidnitrogen.
7. Store at -80°C or useimmediately.

Result

Total protein isolated from the given tissue sample is further estimated for quantification.

ESTIMATION OF PROTEIN

Aim

To estimate the protein using Biuret method.

Principle

The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a purple colour which can be measured at 540 nm.

Reagents Required

1. Biuret Reagent: Dissolve 3 g of copper sulphate (CuSO4.5H2O) and 9 g of sodium potassium tartarate in 500 ml of 0.2 mol/liter sodium hydroxide; add 5 g of potassium iodide and make up to 1 liter with 0.2 mol/liter sodiumhydroxide.
2. Protein Standard: 5 mg BSA/ml. Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter,etc.,

Procedure

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled testtubes.
2. Pipette out 1 ml of the given sample in another testtube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as theblank.
4. Now add 3 ml of Biuret reagent to all the test tubes including the test tubes labeled 'blank' and'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and warm at 37 ºC for 10min.
6. Now cool the contents to room temperature and record the absorbance at 540 nm againstblank.
7. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 540 nm along Y-axis.
8. Then from this standard curve calculate the concentration of protein in the givensample.

Result

The given unknownsamplecontains ----- mg protein/ml.

Ex No.4

ISOLATION OF PLASMID DNA FROM *E. COLI* CELLS

Aim:

To isolate and purify plasmid DNA by alkaline lysis method .

Principle:

This method takes advantage of the physical difference between linear, closed and supercoiled DNA. The bacterial suspension is first exposed to a strong anionic detergent (i.e. SDS) at high pH which helps to rupture the cell wall by hyperlytic osmosis releasing the DNA (chromosomal and plasmid), proteins and other contents which are denatured. The strands of the closed circular plasmid DNA are not completely ruptured in this process as the plasmid has a highly supercoiled confirmation. On addition of neutralization solution, the proteins, polysaccharides and genomic DNA are precipitated, whereas the plasmid DNA remains in the solution. The plasmid DNA is then precipitated by addition of isopropanol. Subsequently, other contaminants are removed by addition of Wash Solution I and II. The pure plasmid is then eluted in Elution Buffer.

Materials required:

1. Control DNA 0.22ml
2. E. coli cells (withplasmid)
3. Resuspension solution5.83ml
4. Lysis solution 6ml
5. Neutralization solution 8ml
6. Isopropanol 22ml
7. Preparation of Luria Bertani broth (10ml):Dissolve 0.25 g of Luria Bertani broth in 10 ml of distilled water and autoclave. Agar Powder, Bacteriological 2g.
8. Preparation of LB (Luria Bertani) agar plates with ampicillin (50 ml):Dissolve 1.25 g of LB media and 0.75 g of agar in 50 ml of sterile distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45^{o}C. Add 50 µl of ampicillin into it and pour on sterile petriplates.

9. Ampicillin Preparation: Dissolve 25 mg of the Ampicillin antibiotic in 500µl of sterile distilled water to prepare a stock concentration of 25 mg/500µl. Store at-20^0C.
10. Collection Tubes, Polypropylene (2.0 ml) 63Nos.
11. 50X TAE 230ml
12. 6X Gel Loading Buffer 0.1ml

Procedure:

Day 1: Revival of Host

1. Pick up a loopful of culture from the E. coli stab and streak onto LB agar plate with ampicillin
2. Incubate overnight at 37^0 C.

Day 2: Inoculation ofculture

1. Pick up a single colony from LB agar plate and inoculate in 10 ml of LB broth containing 10 µlampicillin.
2. Incubate the test tube overnight at 37oC.

Day 3: PlasmidExtraction:

1. HarvestCells

Take 1.5 ml of the overnight grown culture into a micro centrifuge tube and centrifuge the cells at 13,000 rpm for 3 minutes. Discard the supernatant culture medium.

NOTE:For good plasmid yields, the O.D 600 of the culture should be around 3.0 $x10^6$cells/ml.

2. Resuspend Cells

Resuspend the cell pellet in 250 µl of ResuspensionSolution and mix well by gentle vortexing till no cell clumps are visible.

3. LyseCells

Add 250 µl of Lysis Solution to lyse the cells. Mix thoroughly by gently inverting the tube 4-6 times.

NOTE: Do not vortex the tubes as it may result in the shearing of genomic DNA which may contaminate the plasmid DNA. Do not allow this lysis reaction to exceed for more than 5minutes.

4. Neutralize

Add 350 µl of Neutralization Solution and immediately mix thoroughly by inverting the tube gently 4-6 times.

NOTE: On addition of Neutralization Solution, genomic DNA will precipitate out. The mixture should become cloudy and the precipitation should be homogeneous.

5. Centrifuge the sample at 13,000 rpm for 10 minutes to obtain a compact white pellet.

NOTE: A compact white pellet of genomic DNA will form. If the supernatant is not clear, transfer the supernatant to a fresh tube and spin for an additional one minute at 13,000 rpm to remove the interfering salts/precipitates completely.

6. Precipitation of PlasmidDNA

Carefully transfer the supernatant containing plasmid DNA to a new collection tube. Add 1 ml of isopropanol to precipitate plasmid DNA and mix by gentle inversion for 5minutes.

7. Centrifuge at 13,000 rpm for 15 minutes. White pellet of plasmid DNA will be seen, sticking to the side of the tube. Discard the supernatant and invert thevial on blotting paper to drain out leftover
supernatant.

8. First Wash

Resuspend the pellet by adding 500 µl of Wash Solution I and centrifuge at 13,000 rpm for 3minutes.

9. SecondWash

Discard the supernatant and add 700 µl Wash Solution II. Centrifuge at 13,000 rpm for 3 minutes.

Discard the supernatant and air dry the pellet for 10-15 minutes at room temperature.

11. DNA Elution

Resuspend the pellet in 50 µl of Elution Buffer.Centrifuge at 13,000 rpm for 5 minutes to remove insoluble material and transfer the supernatant containing pure plasmid DNA into a new collection tube.

Storage of the eluate with purified DNA: The eluate contains pure plasmid DNA. For short term storage (24-48 hours) of the DNA, 2-8o C is recommended. For long-term storage, -20o C or lower temperature (-80o C) is recommended. Avoid repeated freezing and thawing of the sample which may cause denaturing of DNA. The Elution Buffer will help to stabilize the DNA at thesetemperatures.

Result:

The isolated plasmid from the E.coli culture is continued for further analysis.

Ex No.5

AGAROSE GEL ELECTROPHORESIS

Aim

To identify the purified plasmid DNA molecules from the given sample using Agarose Gel Electrophoresis

Principle

The generated cDNA fragments were resolved in 2% agarose gel under an applied electric field. DNA molecules migrate towards the anode due to negatively charged phosphate along the backbone of DNA. The rate of migration of linear DNA is inversely proportional to its molecular weight. Thus, the larger molecules travel at a much lower speed when compared to smallerone.

Reagents required

1. **TBE buffer 1X: (Tris, Boric acid. EDTA) (pH 8.0):** 3.78 g tris, 1.925 g boric acid and 0.260 g EDTA were dissolved in 350 ml of autoclaved RNAse and DNAse free water and the pH was adjusted to8.0.
2. **1% Ethidium bromide in RNAse and DNAse freewater**
3. **2% Agarose in 1x TBEbuffer**
4. **Gel loadingdye**
   The gel loading dye (6X) was procured commercially in ready to use form.

Procedure

1. Add 1 g of agarose to 50 ml 1X TBE buffer (2%). Then melt it on a microwave oven, make up the volume to 50 ml with TBE buffer and add 10 µl of 1% EtBr, evenly mix and cool to40°C.
2. Then pour the mixed solution into a sealed gel-casting platform and insert the comb after ensuring the absence of airbubbles.
3. Then allow the gel to get harden. Remove the comb after 15 min by taking care of not disturbing the sample wells. Immerse the platform in the electrophoresis buffertank.
4. Mix the 5 µl of PCR products from each reaction tube with 1µl of 6X gel loading dye and load to each well. Load simultaneously of 100 bp molecular weight marker DNA in the first lane to identify the cDNA products.

5. Turn on the power supply and adjust the electricity to 60 mA. Run the gel for 2 h. And then, visualize the resolved cDNA fragments gel for gel documentation.
6. Normalize the band intensity of cDNA fragments of ALP and Collagen with the internal control ß actin and express in OD units relative to GAPDH.

Ex No.6

DIGESTION OF DNA WITH RESTRICTION ENZYMES

Aim

To perform restriction digestion of Lambda (λ)DNA using EcoRI and HindIII enzymes.

Principle

Restriction Digestion involves fragmenting DNA molecules into smaller pieces with special enzymes called Restriction Endonucleases commonly known as Restriction Enzymes(RE). Because of this property the restriction enzymes are also known as molecular scissors. The restriction enzymes are named from the cellular strain from which they are isolated. Restriction enzymes recognize specific sequences inthe double stranded DNA molecule (for example GATATC) and then cut the DNA to produce fragments, called restriction fragments. The target site or sequence which the restriction enzyme recognizes is generally from 4 to 6 base pairs and arranged in a palindromic sequence. Once it is located, the enzyme will attach to the DNA molecule and cut each strand of the double helix. The restriction enzyme will continue to do this along the full length of the DNA molecule which will thenbreak into fragments. The size of these fragments is measured in base pairs or kilobase pairs (1000bases)

Procedure

1. Before starting the experiment, crush ice and placethe vials containing Lambda DNA, Restriction Enzymes and Assay Buffers ontoit.
2. In this experiment Lambda DNA is digested with two restriction enzymes; EcoRI andHindIII.
3. Set up the reaction mixture asfollows:

Reaction 1 (EcoRI digestion)
- Lambda (λ) DNA – 5.0 µl
- 10X Assay Buffer of EcoRI – 2.5µl
- Milli Q water –16.5µl
- EcoRI – 1.0µl

Total 25 µl

Reaction 2 (HindIII digestion)

- Lambda (λ) DNA – 5.0 µl
- 10X Assay Buffer of HindIII – 2.5µl
- Milli Q water –16.5µl
- HindIII – 1.0µl

Total 25 µl

4. After preparing the two reaction tubes, mix the components by gentle pipetting and tapping.
5. Incubate the tubes at 37oC for 1 hour.
6. After 1 hour incubation, immediately place the vials at room temperature (15-25oC) for 10minutes.
7. Run the samples on agarose gel electrophoresis for furtheranalysis.

Observation and Result

Perform Agarose Gel Electrophoresis. Visualize the DNA bands using UV Transilluminator. After running the digested samples on agarose gel, look for the digestion pattern for the two restriction enzymes. Compare the size of each fragment with that of the DNA marker.

Interpretation

Restriction digestion patterns of lambda DNA obtained upon treatment with EcoRI and HindIII are markedly different which demonstrates the fact that each restriction enzyme recognizes and cleaves only a specific base sequence unique to it. The size of the fragments can be determined by comparing with that of the DNA marker ran on the samegel.

Ex No.7

AMPLIFICATION OF A DNA FRAGMENT BY PCR.

Aim

To amplify a specific DNA fragmentby Polymerase Chain Reaction.

Principle

The purpose of a PCR is to amplify a specific DNA or RNA fragment. PCR comprises of three basic steps: Denaturation, Annealing and Primer extension.

Procedure

1) Preparation of master mix forPCR

To a PCR tube add all the following ingredients in order

Ingredients for PCR

1 Molecular Biology Grade Water 30.5µl
2 10X Assay Buffer 5 µl
3 Template DNA2µl
4 Forward Primer (10 nM) 1µl
5 Reverse Primer (10 nM) 1 µl
6 25 mM Mg Cl2 5µl
7 2.5 mM dNTP Mix 5µl
8 Taq DNA Polymerase 0.5 µl

Total volume50µl

2) Tap the tube for 1 – 2 seconds to mix the contentsthoroughly.
3) Add 25 µl of mineral oil in the tube to avoid evaporation of thecontents.
4) Place the tube in the thermocycler block and set the program to get DNA amplification.

NOTE:It is not essential to add mineral oil if the thermocycler is equipped with a heating lid.

Observation and Result

After completion of the PCR, perform agarose gel electrophoresis. Compare the amplified product with the ladder and determine its size.

Ex No.8

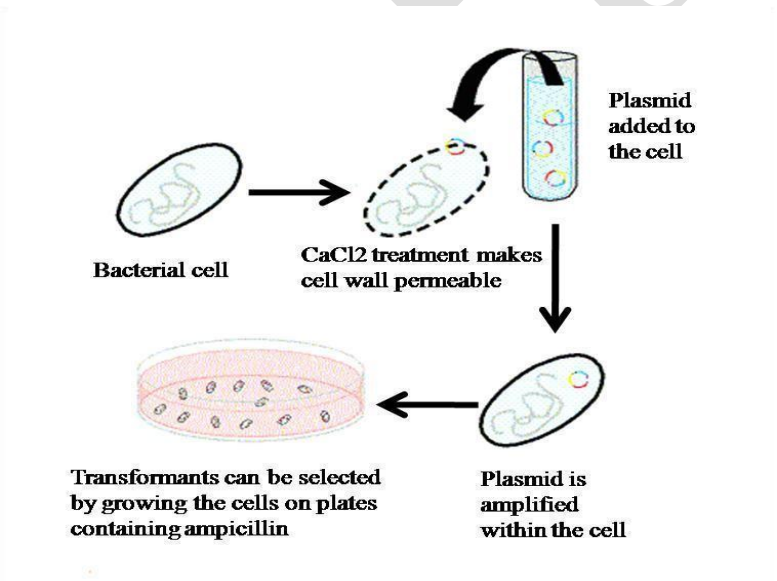
TRANSFORMATION OF *E. COLI* CELLS WITH PLASMID DNA

Aim

To prepare competent cell, transform plasmid DNA in E.Coli cells.

Principle

For the incorporation of plasmid into a cell, bacteria must first be made "competent". This process includes the treatment of cells with bivalent calcium ions in ice-cold condition. As a result small pores are formed on the cell membrane, which makes it permeable.



Reagents preparation

1. The entire process needs to be carried out under sterileconditions.
2. **Preparation of 0.1M Calcium chloride(sterile):**
   To prepare 1000 ml of 11.1 gram of Calcium chloride was weighed and dissolved in700 ml of sterile distilled water and made upto 1000 ml. This solution can be stored at 2-8^{o}C.
3. Pre-chill the tubes before competent cell preparation, 0.1M Calcium chloride solutionand centrifuge tubes. Set the centrifuge at 4^{o}C and water bath at 42^{o}C.
4. 50 ng/µl of plasmid is used fortransformation.
5. **Preparation of LB (Luria Bertani) broth (55 ml):** Dissolve 1.38 g of LB media in 55 ml of distilled water. The solution is to be made sterile byautoclaving.
6. **Preparation of LB (Luria Bertani) agar plates (20 ml):** 0.5 g of LB media and 0.3 g of agar are dissolved in 20 ml of sterile distilled water. The solution is to be made sterile by autoclaving.
7. **Preparation of Ampicillin:** Dissolve 30 mg of ampicillin powder in 600 µl of sterile double distilled water to prepare 50 mg/ml ampicillinsolution.
8. **Preparation of LB (Luria Bertani) Agar plates containing Ampicillin, X-Gal and IPTG (100ml):**
   Dissolve2.5gofLBmediaand1.5gofagarin100mlofdistilledwater.Sterilizeby autoclaving and allow the media to cool down to 40-45^{o}C. Add 100 µl of ampicillin,200 µl of X-Gal and of 100 µl IPTG to 100 ml of autoclaved LB agar media, mix well and pour on sterile petriplates.
9. Transformation to be carried out as soon as possible after the competent cells are prepared. Storage of competent cells decreases the transformationefficiency.
10. **Preparation of LB (Luria Bertani) agar plates (20 ml):** Dissolve 0.5 g of LB media and 0.3 g of agar in 20 ml of sterile distilled water. Sterilize by autoclaving and pour on sterilepetriplate.
11. **Preparation of LB (Luria Bertani) Agar plates containing Ampicillin, X-Gal and IPTG (100ml):**
    Dissolve2.5gofLBmediaand1.5gofagarin100mlofdistilledwater.Sterilizeby autoclaving and allow the media to cool down to 40-45^{o}C. Add 100 µl of ampicillin, 200 µl of X-Gal and of 100 µl IPTG to 100 ml of autoclaved LB agar media, mix well and pour on sterile petriplates.

Procedure

Day 1:

1. Open the bottle containing culture and reconstitute the pellet with 0.25 ml of LBbroth.
2. Pick up a loopful of culture and streak onto LB agar plate and incubate overnight at 37^{0}C .

Day 2:

1. Inoculate a single colony from the revived plate in 1 ml LB broth and incubate overnight at 37^{0}C.

Day 3:

1. Take 50 ml of LB broth in a sterile flask. Transfer 1 ml of overnight grown culture into the flask containing 50 ml of LB broth and incubate at 37^{0}C in a shaker set at 3000 rpm for fourhours.

A) Preparation of Competent Cells:

Note: Competent cells are to be prepared within 3 days of reviving the strain.

1. Transfer the above culture into a pre-cooled 50 ml polypropylenetube.
2. Incubate the culture at 4 ^{o}C for 10 minutes onice.
3. Centrifuge at 5000 rpm for 10 minutes at 4 ^{o}C in a coolingcentrifuge.
4. Decant the medium completely leaving no traces ofmedium.
5. The cell pellet is resuspended in 30 ml pre-chilled sterile 0.1 M Calcium chloride solution and incubate for 30 minutes onice.
6. Centrifuge at 5000 rpm for 10 minutes at4^{o} C.
7. Discard the calcium chloridesolution.
8. The pellet is resuspended in 2 ml pre-chilled sterile 0.1M Calcium chloridesolution.
9. This cell suspension contains competent cells and can be used fortransformation.

B) Transformation ofcells:

1. Take 200 µl of the above cell suspension in two 2.0 ml tubes and label control and transformed. Add 2 µl of plasmid DNA to the tube labeled as transformed and mixwell.
2. Incubate the tubes at 4 ^{o}C for 30minutes.
3. Transfer them to a pre-warmed water bath set at a temperature of42 ^{o}C for 2 minutes to produce heatshock.
4. Rapidly transfer the tubes on ice-bath. Allow the cells to cool for 5minutes.
5. Add 800 µl of LB Broth to both the tubes. Incubate the tubes for 1hour at 37^{o}C to ensure the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid.
6. Take four LB agar plates containing ampicillin, X-Gal, IPTG and label them as control, X, Y and Z. Plate 200 µl of culture from the „control" tube and plate it on the corresponding plate with a sterile spreader. Plate 50 µl, 100 µl and 200 µl of cell cultures from the transformed tube on the plates labeled as X, Y andZ.
7. Store at room temperature till the plates aredry.
8. Incubate the plates overnight at 37^{o}C.

Observation and Result

After incubation observe the plates for the bacterial growth and count the number of visible colonies. Calculate the efficiency of transformation.

Record your observations as follows:

Sr. No.	Plate	Growth	Number of colonies	Transformation Efficiency
1.	Control plate			
2.	Transformed plate (X)			
3.	Transformed plate (Y)			
4.	Transformed plate (Z)			

Denote +ve when you observe bacterial growth, -ve when there is no growth

Calculation of transformation efficiency

Transformation efficiency is defined as the number of cells transformed per microgramof supercoiled plasmid DNA in a transformation reaction**.**

Transformation Efficiency = Number of colonies x 1000 ng/ Amount of DNA plated (ng) = /g.

Interpretation

On transformation of cells with pUC19 plasmid, antibiotic resistance is conferred on the host as this plasmid carries gene for ampicillin resistance. As a result, those cells that grow in presence of ampicillin are transformed cells. The transformed colonies are blue on X-Gal, IPTG plates due to α-complementation.

Ex No.9

WESTERN BLOTTING (DEMO)

Aim:

To learn the technique of Western Blotting for the detection of a specific protein.

Principle:

Western blotting or immunoblotting is a method used for identifying a specific protein in a complex mixture along with determination of its molecular weight. Protein samples are first electrophoresed on SDS-PAGE. In this process proteins migrate through the gel and they are separated according to their size and charge. These separated proteins are electrotransferred onto nitrocellulose/PVDF membrane for further analysis. To detect the protein (antigen) blotted on the membrane it is incubated with an antibody (primary) specific for the protein of interest. The membrane is then incubated with a second antibody (secondary) which is specific for the first antibody. The secondary antibodies are covalently attached to an enzyme, e.g. alkaline phosphatase or horseradish peroxidase. These enzymes form a coloured precipitate upon reacting with a chromogenic substrate. As a result a visible band can be seen on the membrane where the primary antibody is bound to theprotein.

Preparation of reagents:

1. Preparation of 10% APS Solution: Before starting the experiment, dissolve 0.15 g of Ammonium persulphate in distilled water to make a final volume of 1.5 ml. Store at2-8o C. Use within 3 months.
2. Preparation of 1X Tris-Glycine-SDS Gel Running Buffer: To prepare 500 ml of 1X Tris-Glycine-SDS Gel Running buffer, take 100 ml of 5X Tris-Glycine-SDS Gel Running Buffer and add 400 ml sterile distilled water*. Store at 2-8o C. Mix well before use. The 1X Tris-Glycine-SDS Gel Running Buffer can be reused 4-5times.
3. Thaw all refrigerated samples beforeuse.
4. Clean the entire apparatus with detergent and then with distilled water*. Ensure that the plates are free ofdetergent.
5. Preparation of 1X Assay Buffer: To prepare 50 ml of 1X Assay Buffer, take 5 ml of 10X Assay Buffer and add 45 ml of sterile distilledwater*.
6. Preparation of 1X Transfer Buffer: To prepare 1000 ml of 1X Transfer Buffer, take 100 ml of 10X Transfer Buffer, add 200 ml of methanol and 700 ml of sterile distilled water*. Store at 2-8o C. Mix well beforeuse.
7. Preparation of Blocking Buffer: To prepare 20 ml of Blocking Buffer, take 0.2 g of Blocking Agent and add 20 ml of Diluent Buffer.
8. Preparation of 1X Wash Buffer: To prepare 1000 ml of 1X Wash Buffer, take 100 ml of 10X Wash Buffer and add 900 ml of sterile distilled water

Procedure:

Day 1: SDS- PAGE

1. Assemble the electrophoresis unit such that the glass plates are clamped to the unit along with the spacers placed in-between them at two verticaledges.

2. Prepare 1% agarose (0.05g in 5ml of distilled water). Boil to dissolve the agarose and poura thin horizontal layer at the lower edge of the plates to seal the assembly. Let it solidify by allowing it to cool down for 5-10minutes

3. Preparation of 12% Separating Gel- To prepare separating gel, add the components asfollows:
30% Acrylamide-bisacrylamide Solution - 6ml
Distilled water* - 3 ml
2.5X Tris-SDS Buffer (pH 8.8) - 6 ml
10% APS Solution - 125 µl
TEMED - 18 µl
Pour the gel in-between the plates and allow it to solidify for an hour. Immediately after the gel is poured, add distilled water to level the gel.
4. After an hour pour off the water by inverting the castingassembly.
5. Preparation of 5% Stacking Gel- To prepare stacking gel, add the components as follows:30% Acrylamide-bisacrylamide Solution - 1.3ml
Distilled water* - 5.1 ml
5X Tris-SDS Buffer (pH 6.8) - 1.6 ml
10% APS Solution - 75 µl
TEMED - 10 µl
After addition of TEMED gently mix all the components by swirling the beaker. Pour the stacking gel on top of the separating gel and immediately place the comb avoiding air bubbles. Allow it to solidify for 30 minutes.
Note: Acrylamide is a potential neurotoxin and should be treated with great care. Always wear an face mask and use gloves.
6. Pour 1X Tris-Glycine-SDS Gel Running Buffer in the unit such that the buffer connectsthe two electrodes, and hence completes the flow of current. Remove the comb from the Stacking Gelcarefully.
7. Sample Preparation: Take 40 µl of protein sample in a tube and add 8 µl of 5X Sample Loading Buffer to it. Boil the tube containing protein sample at 100o C in a boiling waterbath. Do not boil the tube containing Prestained ProteinLadder.
8. Load samples in alternative wells asfollows:
Lane 1: Prestained Protein Ladder – 5 µl
Lane 3: Protein Sample – 20µl
Lane 5: Protein Sample – 20µl

9. Connect the power cord to the electrophoretic power supply according to the conventions: Red-AnodeandBlack-Cathode.Electrophoreseat120voltsand90mAuntildyefrontreaches 0.5 cm above the sealing gel.
10. Carefully remove the gel from in-between the plates using spatula into the plastic tray containing distilled water. Wash the gel for 1 minute. Discard the water & proceed for blotting and staining destaining procedure. * Molecular biology grade water is recommended (Product code:ML024).
11. To the gel pieces of lane no. 1 and 3 add 20 ml of water and proceed for staining destaining procedure.
12. Cut the Gel along lane no. 4. Transfer lane no. 5 i.e. protein sample in 10 ml of cold Transfer buffer. Incubate at Room Temperature for 10 minutes and proceed withelectroblotting.

Staining and Destaining of Gel:

1. After removing water, add 50 ml of Staining Solution in the tray containing gel, till the bands are visible. Sometimes the gel may have to be kept overnight in the staining solution for visualization of thebands.
2. Remove gel from the Staining Solution. The Staining Solution can be re-used 2-3times.
3. Wash the gel by rinsing with distilled water till a considerable amount of stain leaches out from the gel. Keep changing the distilled water for 3-4times.
4. Add 50 ml of Destaining Solution to the gel. Destaining should be carried out with constant moderateshaking.
5. Continue destaining till clear, distinct bands areobserved.
6. Remove gel from the Destaining Solution. The Destaining Solution can be re-used 2-3times.

Electroblotting:
1. Assemble the gel with nitrocellulose membrane and filter papers, blotting sandwich is placed within the blotting cassette. Try to avoid air bubble between gel and nitrocellulose membrane by rolling a glass tube on the membrane. Note: Take out the transparent sheets carefully while using the nitrocellulosemembrane.
2. Insert this cassette into the gel transfer apparatus filled with cold transfer buffer and then connect the transfer unit to power supply as perconventions.
3. Electrophoreses the sample at 150V, 300 mA for 2 hours forblotting.
4. Remove the nitrocellulose membrane after electrophoresis from the blotting cassette and place the membrane (with protein side up) in 20 ml of 1X Blocking Buffer taken in petridish.
5. Keep it overnight at 40 C.

Day 2: Immunodetection:
1. Discard off the blockingbuffer.
2. Wash the membrane with 20 ml of 1X Wash Buffer for 5 minutes. Repeat the washonce.
3. Immerse the membrane in 20 ml of 1X Assay Buffer. Add 4 µl of primary antibody solution and mix gently for an hour on a gel rocker. After that discard the primary antibodysolution.
4. Wash the blot with 20 ml of 1X Wash Buffer for 5 minutes. Repeat the wash once. Discard the buffer each time.
5. Immerse the blot in 20 ml of 1X Assay Buffer. Add 2 µl of HRP labeled secondary antibody. Mix gently for an hour. Discard the HRP labeled antibodysolution.
6. Do a quick washing of the blot with 20 ml of 1X Wash Buffer. Wash the blot with 20 ml of 1X Wash Buffer for 10 minutes. Repeat the wash. Discard the buffer eachtime.
7. Immerse the washed blot in 3 ml of TMB/H2O2 (substrate) solution, mix gently for 5-10 minutes, within this time coloured band will appear.
8. Remove the blot; wash with distilled water, discard anddry.
9. Compare the SDS-Polyacrylamide gel with the developedmembrane

Observation and Result
The following lanes are appeared in the membrane
Lane 1: Prestained Protein Ladder Lane 3: Protein Sample Lane 4: Immunodetection on the blotted membrane.

Possible viva questions

1. How might you optimize the purity ofDNA?
2. Why must you handle DNA gently in the extractionprocedure?
3. What are the importance of DNAisolation?
4. What are DNA? Mention itstypes?
5. What are the principles of DNAisolation?
6. Mention the uses of alcohol in isolation ofDNA.
7. What is the need of cell lysis in DNAisolation?
8. What are the action of lysozyme in DNAisolation?
9. How acetate purify DNA during DNAisolation?
10. What are chemicals need for DNAisolation?
11. What is the end use of isolatedDNA?
12. What is the importance ofRNA?
13. How do you detect RNA insolution?
14. What are the difference between plasmid and chromosomalDNA?
15. What is single digestion and doubledigestion?
16. Mention properties ofagarose.
17. What iselectrophoresis?
18. What are the different types ofelectrophoresis?
19. What are the uses of ethidium bromide DNAseperation?
20. What are the factors associated with nucleic acid mobility inelectrophoresis?
21. What are the purposes of using buffer in electrophoresisunit?
22. What are the principles of DNAamplification?
23. Who inventedPCR?
24. What isprimer?
25. Explain the principle beyond lac operoninduction.
26. Mention the principle of affinity chromatography in isolation ofmRNA.