16MBU413 RECOMBINANT DNA TECHNOLOGY – PRACTICAL (3H – 2C)

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**
End Semester Exam: 9 Hours

SCOPE

This paper imparts the concepts of rDNA technology and their applications and Acquire knowledge on the applications of genetic engineering.

OBJECTIVES

- To learn the basic tools in recombinant technology
- To understand the various concepts of cloning vectors and cloning strategies
- To emphasize the knowledge in biotechnology and techniques.

EXPERIMENTS

1. Preparation of competent cells for transformation.
2. Demonstration of Bacterial Transformation and calculation of transformation efficiency.
3. Digestion of DNA using restriction enzymes and analysis by agarose gel electrophoresis
4. Ligation of DNA fragments.
5. Cloning of DNA insert and Blue white screening of recombinants.
6. Interpretation of sequencing gel electropherograms.
7. Designing of primers for DNA amplification.
8. Amplification of DNA by PCR.
9. Demonstration of Southern blotting.

Suggested reading

1. Brown TA. (2010). Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
2. Clark DP and Pazdernik NJ. (2009). Biotechnology: Applying the Genetic Revolution. Elsevier Academic Press, USA.
3. Primrose SB and Twyman RM. (2006). Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
4. Sambrook J and Russell D. (2001). Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Press
5. Wiley JM, Sherwood LM and Woolverton CJ. (2008). Prescott, Harley and Klein's Microbiology. McGraw Hill Higher Education
6. Brown TA. (2007). Genomes-3. Garland Science Publishers

Primrose SB and Twyman RM. (2008). Genomics: Applications in human biology. Blackwell Publishing, Oxford, U.K.

EX.NO: ISOLATION OF TOTAL GENOMIC DNA FROM BACTERIAL CELLS

Aim: To isolate total genomic DNA from the bacterial cells and visualizing the same DNA by gel electrophoresis.

Principle:

Genomic DNA preparation differs from the plasmid DNA preparation. Genomic DNA is extracted from bacterial cells by immediate and complete lysis whereas plasmid DNA is isolated by slow-cell lysis to form a sphaeroplast.

The procedure of genomic DNA extraction can be divided into 4 stages:

1. A culture of bacterial cell is grown and harvested.
2. The cells are broken open to release their contents.
3. The cells extracted are treated to remove all components except the DNA.
4. The resulting DNA is then purified.

Materials required:

1. TE buffer (pH 8.0)
2. 10% (w/v) sodium dodecyl sulfate (SDS)
3. 20 mg/mL proteinase K
4. Phenol/chloroform (50:50)
5. Isopropanol
6. 70% ethanol
7. 3M sodium acetate pH 5.2

Procedure:

1) *E. coli* culture was grown overnight in Luria broth.
2) The overnight culture was transfered into 1.5 mL to a microcentrifuge tube and centrifuged at 10000 rpm for 5 min. The supernatant was decanted and repeated with another 1.5 mL of cells.
3) The bacterial pellet was resuspended in 467 µL TE buffer by repeated pipetting. 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K was added, mixed, and incubated for 1 hr at 37°C.
4) Equal volume of buffer saturated phenol : chloroform : isoamyl alcohol (25:24:1) or chloroform:Isoamyl alcohol (24:1) was added and mixed well by inverting the tube until the phases are completely mixed. (Caution: Phenol causes severe burns. Wear gloves, goggles, and a lab coat, and keep tubes capped tightly)
5) Centrifuged at 12000 rpm for 5 min.

6) The upper aqueous layer phase was transferred to a new 1.5 ml eppendorf tube and equal volume of chloroform:Isoamyl alcohol (24:1) was added, Again mixed well by inverting and transfered to a new tube and centrifuged at 12000 rpm for 5 minutes.
7) The upper aqueous phase was again transferred to a new 1.5 ml eppendorf tube.
8) To the aqueous phase, 1/10 volume of 3M sodium acetate (pH 5.2) was added.
9) Then 0.6 volume of isopropanol was added and mixed gently until the DNA gets precipitates.
10) Centrifuged at 12000 rpm for 10 min and the supernatant was discarded.
11) The DNA pellet was washed by adding 200 µL of 70% ethanol (Centrifuge at 10000 rpm for 3 min)
12) Finally the DNA pellet was suspended in a 100–200 µL TE buffer. Complete resuspension may take several days.
13) After DNA has dissolved, the purity of the DNA was checked by electrophoresis and spectrophotometric analysis.
14) 0.8 % agarose gel was prepared using 1X TAE buffer.
15) Isolated DNA samples were loaded into the wells, recording which samples are loaded into which wells as lane 1, 2, etc. The power supply was started after sample loading, with the voltage set to 50 V.
16) Ran the gel until the second dye from the well has reached 3/4 th of the gel.
17) After the run was completed, the gel was observed under UV transilluminater.
18) Then DNA was stored at 4°C short term, –20°C or –80°C long term

Result and Observations:

EX. NO: **AGAROSE GEL ELECTROPHORESIS**

Aim: To separate and analyze the isolated DNA sample from the plant/animal/bacterial source.

Principle:

Electrophoresis is the migration of charged molecules in response to an electric field. Their rate of migration depends on the strength of the electric field; on the net charge, size and shape of the molecules, and also on the ionic strength, viscosity, and temperature of the medium in which the molecules are moving.

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1–10 mg of DNA can be detected by direct examination of the gel in ultraviolet light. If necessary, these bands of DNA can be recovered from the gel and used for a variety of cloning purposes. Movement of the DNA in the gel depends on its molecular weight, conformation, and concentration of the agarose, voltage applied, and strength of the electrophoresis buffer.

Materials required:

1) Submarine gel apparatus, including glass plate, comb, gel tray and surround.
2) Agarose.
3) Stock solutions –
    (i) 50 X TAE buffer (0.04 M tris-acetate, 0.001 M EDTA, pH 8.0)
        For 1 litre – 242 g Tris base
        57.1 mL glacial acetic acid
        100 mL 0.5 M EDTA (pH 8.0)
    (ii) Ethidium bromide: 10 mg/mL
    (iii) 6 X gel-loading buffer – containing 0.25 % bromophenol blue and 40 % sucrose in water.
4) Agarose solution in 0.5 X TBE or 1 X TAE buffer (generally 0.7%–1%).
5) 1X TAE or 0.5 X TBE (same buffer as in agarose).
6) UV- transilluminator

Protocol:

Preparation of Agarose Gels

1. The ends of gel tray was sealed with tape.
2. The comb was placed in the gel tray about 1 inch from one end of the tray and the comb was positioned vertically, so that the teeth are about 1–2 mm above the surface of the tray.
3. 1X TAE was prepared by diluting the appropriate amount of 50X TAE buffer with distilled water.
4. 1 gm of agarose was added to the prepared 100 mL of 1X TAE in a 250-mL conical flask and boiled to dissolve agarose.
5. When the agarose gel solution temperature was around 60°C, 10 µl of Ethidium bromide stock solution was added to get a final concentration of 0.5 µg/mL and mixed thoroughly.
6. Then the cooled agarose solution was poured in the gel tank without air bubbles and was 0.5–0.9 cm thick.The set was kept undisturbed until the agarose solidifies.
7. Once the gel was solidified, 1X TAE buffer poured slowly into the gel until the buffer level stands at 0.5 to 0.8 cm above the gel surface.
8. The wells were formed by gently lifting the comb.

Electrophoresis

1. To run, the comb and the sealed tapes were removed gently, the agarose gel along with tray was placed in an electrophoresis chamber, and covered (just until wells are submerged) with 1 X TAE electrophoresis buffer (the same buffer used to prepare the agarose).
2. To prepare samples for electrophoresis, 1 µL of 6X gel loading dye was added for every 5 µL of DNA solution, and mixed well. 5–12 µL of DNA per well was loaded (for minigel).
3. The electrophoresis was ran at 50–150 volts, until dye markers have migrated an appropriate distance, depending on the size of the DNA to be visualized.
4. After electrophoresis, the agarose gel was placed onto the UV trans-illuminator platform and visualized the DNA bands under shortwave UV light.

Note: If the gel was not stained with ethidium during the run, stain the gel in 0.5 µg/mL ethidium bromide until the DNA has taken up the dye and is visible under shortwave UV light.

Result and observations:

EX.NO: ISOLATION OF PLASMID DNA FROM BACTERIAL CELLS

Aim: To isolate plasmid DNA from the bacterial cells by alkaline lysis miniprep method and visualizing the same DNA by gel electrophoresis.

Principle:

Plasmids are extrachromosomal, double-stranded, closed-circular DNA present in many microorganisms. Plasmid DNA needs to be extracted (from bacterial hosts, mostly *E.coli*) almost routinely in cloning experiments. Many methods have been described for successful extraction of plasmid DNA; however, the alkaline miniprep method is most useful for quick extraction of plasmids, mostly for analytical use. In alkaline lysis miniprep method, the bacterial cells are lysed followed by SDS, NaOH treatment. The high pH of NaOH denatures the bacterial DNA but not the covalently closed-circular plasmid DNA. Neutralization of the high pH by sodium or potassium acetate makes the bacterial DNA to precipitate. The plasmid DNA is then purified by organic solvent.

Materials required:

1. Luria Broth - containing Trypton, 1%; Yeast extract, 0.5% NaCl, 1%
2. Antibiotics - Ampicillin, 50 mg/mL
3. Solution I - containing

    50 mM glucose

    25 mM Tris-CI (pH 8.0)

    10 mM EDT A (pH 8.0)

Solution I can be prepared in batches of approximately 100 mL autoclaved for 15 minutes at 10 lb/sq and stored at 4°C). add RNase A (100mg/ml) to get 100 ug/ml of solution I.

4. Solution II -containing

    0.2 N NaOH (freshly diluted from 10 N stock)

    1% SDS

5. Solution III - containing

    5 M potassium acetate 60 mL

    Glacial acetic acid 11.5 mL

    Distilled Water 28.5 mL

    The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

6. 50 % PEG 6000 solution
7. 5M NaCl
8. TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0
9. 70% and 100% ethanol.

Procedure:

Harvesting and Lysis of Bacteria

Harvesting - A single bacterial colony was inoculated into 2 mL of LB medium containing the appropriate antibiotic in a loosely capped 15-mL tube.

1) the culture was incubated for overnight at 37°C with vigorous shaking.
2) 1.5 mL of the culture was transferred into a centrifuge tube.
3) Centrifuged at 12000 g of 5 min using microcentrifuge.
4) the medium was removed by decantation, leaving the bacterial pellet as dry as possible.

Lysis by Alkali

1) The bacterial pellet was resuspended in 300 µL of ice-cold Solution I by vigorous vortexing.
2) 300 µL of freshly prepared Solution II was added.
3) The tube was closed tightly and the contents were mixed by inverting the tube rapidly for 5 minutes. (Make sure that the entire surface of the tube comes in contact with Solution II. Do not vortex). Then 300 µL of ice-cold Solution III was added.
4) the content was invert mixed and incubated on ice for 3–5 minutes.
5) After incubation, centrifuged at 14000 rpm for 10 minutes in a microcentrifuge. Then the supernatant was transferred to a fresh tube.
6) To the supernatnant, 166 µl of 50 % PEG6000 and 118 µl of 5M NaCl were added and mixed thoroughly by vortexing. Then centrifuged at 14000 rpm for 10 minutes in a microcentrifuge,
7) The supernatant was removed by gentle aspiration. The tube was kept in an inverted position on a paper towel to allow all of the fluid to drain away. Any drops of fluid adhering to the walls of the tube was also removed.
8) The pellet of DNA was rinsed with 200 µL of 70% ethanol.
9) Centrifuged at 12000 rpm for 10 minutes in a microcentrifuge.
10) The supernatant was removed as described in previous step, and the pellet of DNA was allowed to dry in the air for 1 minute.
11) The DNA pellet was resuspended in 50 µL of TE (pH 8.0). Vortexed briefly.
12) The isolated plasmid DNA was analyzed in 1.2 % agarose gel electrophoresis.

Results and Observations:

EX.NO: RESTRICTION ENZYME DIGESTION OF DNA

Aim: To perform restriction digestion of isolated plant genomic DNA with *EcoR* I and *Hind* III enzymes and to analyze the restriction pattern by agarose gel electrophoresis.

Principle:

Type II restriction enzymes hydrolyse the backbone of DNA between deoxy ribose and phosphate groups within the recognition sites and generate two different types of ends. This leaves a phosphate group on the 5' ends and a hydroxyl group on the 3' ends of both strands. The 5' or 3' overhangs generated by enzymes that cut asymmetrically are called sticky or cohesive ends, because they will readily stick or anneal with their complementary sequences by base pairing (Example: *EcoR* I). Some enzymes cut at precisely opposite sites in two strands of DNA and generate blunt ends without overhangs called blunt ends (Example – *Hae* III).

Materials required:

1. 10X restriction enzyme buffer (see manufacturer's recommendation)
2. DNA
3. restriction enzymes – *EcoR* I and *Hind* III
4. sterile water
5. phenol:chloroform (1:1)

Procedure

1. The following contents were added to a microfuge tube:

2 µL of appropriate 10X restriction enzyme buffer, 0.1 to 5 mg DNA, and sterile water to a final volume of 19 µL (Note: These volumes are for analytical digests only. Larger volumes may be necessary for preparative digests or for chromosomal DNA digests.)

2. 1 to 2 µL (3 to 20 units) enzyme was added, mixed gently and centrifuged for a few seconds in the microfuge.
3. Incubated at the appropriate temperature (usually 37°C) for 1 to 2 hours.
4. Meanwhile, 1 % agarose gel was prepared for electrophoresis.
5. After an restrict digestion incubation, 3 µL of 6x gel loading buffer was added to the samples.
6. The digested samples, 10 µL of control DNA, 10 µL of DNA size marker were loaded, (note down the order of loading).
7. Electophoresis was performed at 50 – 100 V for 1-2 hours.
8. After electrophoresis, the gel was observed under UV transilluminator.

(Note) If the DNA is to be used for another manipulation, heat-inactivate the enzyme (if it is heat-labile) at 70°C for 15 min, phenol/chloroform extract, and ethanol precipitate, or purify on DNA purification column.

EcoR I restriction site:

Hind III restriction site

Results and Observations:

EX.NO: **LIGATION OF DNA**

Aim: To perform ligation of λ/*EcoR* I digest using T4 DNA ligase and to anlayze the ligated sample by agarose gel electrophoresis.

Principle:

Construction of recombinant DNA molecule is dependent on the ability to covalently seal single stranded nicks in DNA. This process is performed both invitro and invivo by the enzyme called DNA ligase. It catalyses the formation of phosphodiester bond between 5' phosphate and 3' hydroxyl terminals of double stranded DNA. Thereby joins the double stranded DNA restriction fragments having either blunt end or homologus cohesive ends.

E.coli ligase and T4 DNA ligase are the two DNA ligases used in recombinant DNA technology. T4 DNA ligase has the unique ability to join cohesive and blunt ended fragments. T4 DNA ligase produced from bacteriophage T4 and requires ATP as energy source. Cohesive end ligation is carried out at 12 to 16º C to maintain a good balance between annealing of ends and enzyme activity. If reaction is set at higher temperatures, annealing of the ends become difficult, while lower temperatures reduces the ligase activity.

Materials required:

1. λ/*EcoR* I digest
2. 2X ligase assay buffer
3. T4 DNA ligase
4. Sterile distilled water
5. Agarose
6. 50X TAE buffer
7. 6X gel loading buffer

Protocol:

1. Ligase assay buffer and λ/*EcoR* I digest were thawed.
2. T4 DNA ligase vial was placed on ice 1
3. To the tube, labeled as ligated sample, 10 µL of λ/*EcoR* I digest was added to it.
4. Then 10 µL of 2X ligase assay buffer and 1 µL of T4 DNA ligase were also added and mixed by tapping.
5. It was then incubated at 16ºC for 2 hrs in waterbath.
6. Meanwhile, 1% agarose gel was prepered for electrophoretic analysis.
7. After incubation, 2 µL of 6X gel loading buffer was added to the ligated sample and the control sample containing the 10 µL of λ/*EcoR* I digest alone.

8. Both the samples were loaded onto the wells

9. Electrophoresis was ran at 50 – 100V for 1-2 h.

10. After electrophoresis, the gel was viewed under UV-transilluminator.

11. The bands of ligated λ DNA was compared with the λ/*EcoR* I digest.

Results and Observations:

EX.NO: TRANSFORMATION OF PLASMID DNA USING CALCIUM CHLORIDE METHOD

Aim: To prepare chemically competent E.coli cells and to perform transformation of plasmid DNA into the competent E.coli cells.

Principle:

Bacteiral transformation is a process in which the bacterial cells managed to uptake free or external DNA from the environment or surrounding medium. The transformation ability by most bacteria is limited in nature. However, bacterial cells can be artificially induced to take up DNA by treating them with calcium chloride.

Culture of bacterial cells that are capable of taking up the DNA is said to be competent. During the process of competence, the bacterial cell wall changes and receptors of some kind are either formed or activated on the cell wall which are responsible for initial binding of the DNA. Then the competent cells are briefly exposed to a temperature of 42ºC (Heat shock), wherein pores are created and DNA is taken up. Further the immediate chilling on ice ensures closure of pores. These are then said to be transformed and are then screnned for transformants or recombinants.

Materials required:

1. E.coli starin
2. Luria broth
3. Ampicillin – 100 mg/ml stock solution
4. 0.1 M $CaCl_2$
5. Plasmid DNA
6. X – Gal
7. IPTG
8. Luria agra plate

Protocol:

Day 1: Revival of bacterial strain

1. The lyophilized vial was opened, to that 0.1 ml of LB medium was added.
2. A loopful of suspension was streaked onto LB plates and incubated at 37ºC for overnight.

Day 2:

3. A single colony was inoculated into 5 ml of LB medium and incubated at 37ºC for overnight.

Preparation of competent cells:

1. One ml of overnight grown culture was inoculated to 100 ml of LB medium and incubated at 37 ºC for 2-3 hrs, until OD A_{600} reaches to 0.23 - 0.26.

2. Then the culture flask was chilled on ice for 20 mins (To arrest the bacterial growth).

3. The culture was aseptically transferred into sterile centrifuge tubes and centrifuged at 6000 rpm for 5 min at 4 ºC.

4. The supernatant was discarded and 15 ml of cold 0.1 M $CaCl_2$ solution was added to the cell pellet aseptically. The cell pellet was gently suspended in the solution using a pre-chilled pipette (Care should be taken not to remove the tubes from ice during resuspension).

5. The tube was placed on ice for 30 min. Then centrifuged at 6000 rpm for 5 min at 4 ºC.

6. The supernatant was discarded and resuspended gently in 0.6 ml cold 0.1M $CaCl_2$ solution.

7. 100 µl aliquots of competent cells were aseptically transferred to 6 pre-chilled 1.5 ml sterile microfuge tubes and placed on ice.

Transformation procedure:

8. 5 µl (10 ng) of plasmid DNA was added to 5 aliquots of 100 µl of competent cells. Gently tapped and incubated on ice for 20 mins. One aliquot of competent cell was used without transformation as control.

9. After incubation, the cells were subjected to 42 ºC for 2 min in water bath and then the vials were kept in ice for 5 mins.

10. Then 1 ml of LB medium was added to the tubes aseptically and incubated at 37 ºC for an hour.

11. Meanwhile, LB agar plates with ampicillin (100 µg/ml of medium) and X- Gal and IPTG were prepared.

12. After 1 h growth of transformed cells, 100 µl were spreaded onto the plates using a spreader.

13. The control plate (LB agar alone) was prepared by plating 100 µl of competent cell alone that has not been transformed (to check the cell contamination).

14. All plates were incubated for overnight at 37 ºC.

Transformation efficiency is expressed as Number of transformants/ µg of plasmid DNA

Results and Observations:

EX.NO: DNA AMPLIFICATION BY THE POLYMERASE CHAIN REACTION (PCR) METHOD

Aim: To amplify the given sample of DNA using PCR.

Principle: Polymerase chain reaction (PCR) is a very simple method for in vitro DNA amplification using Taq polymerase.

A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the 4 nucleotides, large quantities of the primer sequence, and DNA polymerase. The polymerase is the Taq polymerase, isolated from *Thermus aquaticus*.

The 3 parts of the polymerase chain reaction are carried out in the same vial, but at different temperatures. The first part of the process separates the two DNA chains in the double helix. This is done simply by heating the vial to 90°C–95°C for 30 seconds. But the primers cannot bind to the DNA strands at such a high temperature, so the vial is cooled to 55°C. At this temperature, the primers bind or "anneal" to the ends of the DNA strands. This takes about 20 seconds. The final step of the reaction is to make a complete copy of the templates. Since the Taq polymerase works best at around 75°C (the temperature of the hot springs where the bacterium was discovered), the temperature of the vial is raised.

The Taq polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on, to the end of the DNA strand. This completes 1 PCR cycle.

The 3 steps in the polymerase chain reaction—the separation, of the strands, annealing the primer to the template, and the synthesis of new strands—take less than two minutes. Each is carried out in the same vial. At the end of a cycle, each piece of DNA in the vial has been duplicated. But the cycle can be repeated 30 or more times. Each newly synthesized DNA piece can act as a new template, so after 30 cycles, 1 million copies of a single piece of DNA can be produced. Taking into account the time it takes to change the temperature of the reaction vial, 1 million copies can be ready in about 3 hours.

Thus, this technique involves DNA synthesis in 3 simple steps.

Step 1. Denaturation of the template into single strands.

Step 2. Annealing of primers to the template.

Step 3. Extension of new DNA.

Materials Required

1. *DNA template:* Between 1 and 5 ng of cloned DNA or between 40 and 100 ng of genomic DNA should be used per reaction. It is convenient to dilute template stocks to an appropriate concentration, e.g., 5 ng/mL in dH_2O for cloned DNA.
2. *Primers:* Primers should be resuspended in dH_2O at 100 ng/mL. Each primer should be used at ~100 ng per reaction.
3. *Buffer:* Buffer should be prepared as a 10X stock.

   10X PCR buffer: 100 mM Tris. HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$.This buffer can be prepared containing 0.1% gelatin.
4. *Taq DNA polymerase:* Taq should be used at 2.5 U per reaction.
5. *Magnesium:* Extra magnesium can be added to the PCR reaction.
   - If using the buffer above, a final Mg^{2+} concentration of 1.5 mM will be obtained. If necessary, magnesium can be titrated to obtain an optimal concentration.
   - Suggested concentrations for this would be 1.5, 3.0, 4.5, 6.0 and 10 mM.
   - Magnesium can be prepared as $MgCl_2$ at 25 mM and autoclaved.
   - Increasing the magnesium concentration has the same effect as lowering the annealing temperature.
6. *Nucleotides:* dNTPs should be prepared from 100 mM commercial stocks as a 10X stock at 2 mM of each dNTP. This is most easily done by adding 2 µL of each dNTP to 92 µL dH_2O in an eppendorf tube.
7. *Water:* Water should be autoclaved and used solely for PCR.

   Milli-Q water is fine for PCR or "water for injection" if the distilled water is in doubt. It can be aliquotted into 1-mL volumes and kept separate from DNA and other sources of contamination. Each aliquot should be discarded following a single use.
8. *Paraffin oil:* In some instruments, paraffin oil must be added to prevent evaporation of the sample.
9. PCR machine.

Procedure

1. Add 38 µL of sterile milliQ water (or autoclaved double distilled water) to a sterile microfuge.
2. Add 5 µL of 10 X Taq polymerase assay buffer with $MgCl_2$ to the microfuge.
3. Add 3 µL of 2.5 mm dNTP mixed solution to the microfuge.
4. Add 1 µL of control template DNA.

5. Add 1 mol each of forward and reverse primers.

6. Add 1–2 units (0.5–0.7 µL) of Taq DNA polymerase.

7. Gently mix.

8. Layer the reaction mixture with 50 µL of mineral oil to avoid evaporation (if required).

9. Carry out the amplification using the following reaction conditions:

10. Initial denaturation at 94°C for 1 min.

11. Denaturation at 94°C for 30 sec.

12. Annealing at 48°C for 30 sec.

13. Extension at 72°C for 1 min.

14. Final extension at 72°C for 2 min.

15. Meanwhile, prepare a 1 % agarose gel for electrophoresis.

After PCR, add 3 µL of 6x gel loading dye to the PCR products.

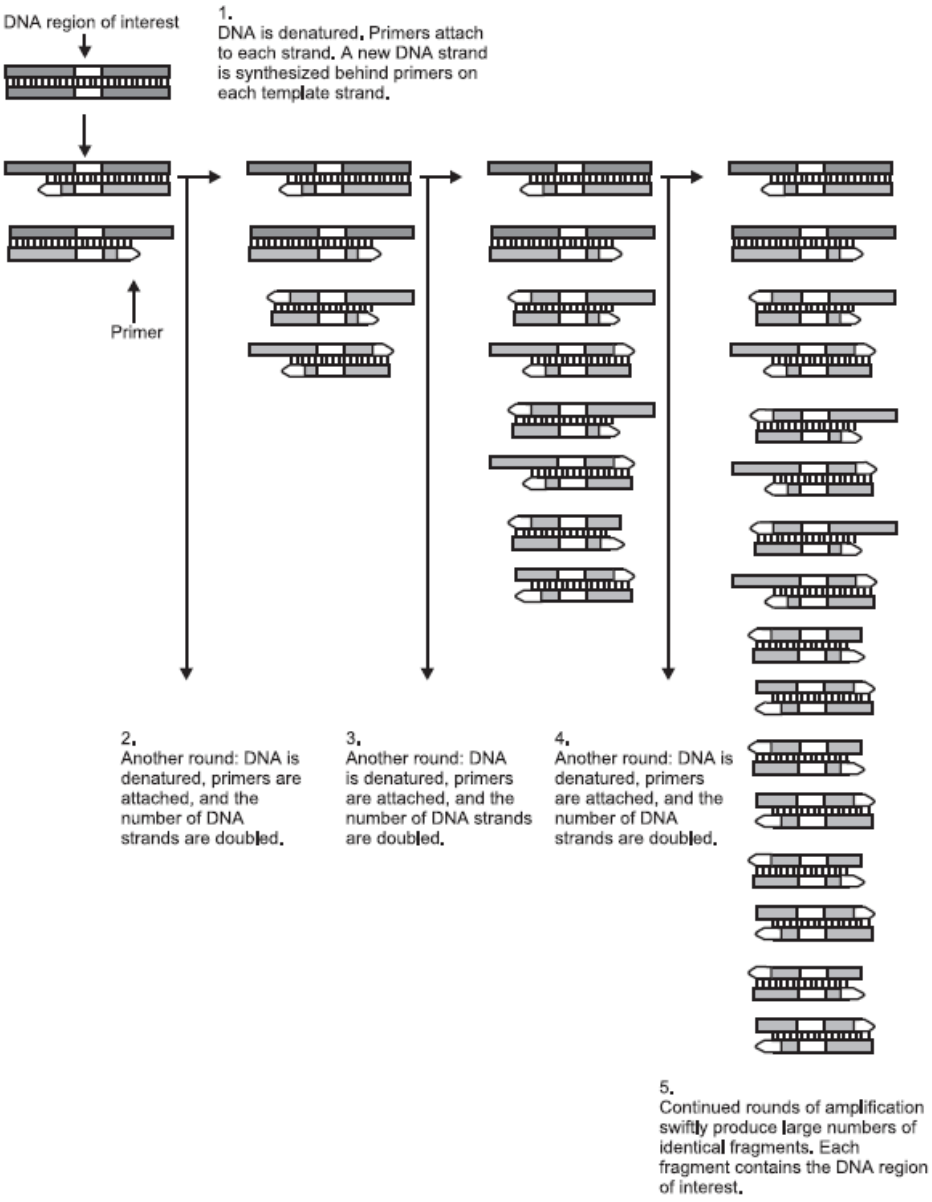
6. Load the PCR products along with DNA size marker, note down the order of loading.

7. Electrophorese the samples at 50 – 100 V for 1-2 hours.

8. After electrophoresis, observe the gel under UV transilluminator.

Result and observation:

The specific segment of DNA was amplified by PCR and analyzed using agarose gel electrophoresis.


DNA region of interest
1.
DNA is denatured. Primers attach to each strand. A new DNA strand is synthesized behind primers on each template strand.
Primer
2.
Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.
3.
Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.
4.
Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.
5.
Continued rounds of amplification swiftly produce large numbers of identical fragments. Each fragment contains the DNA region of interest.

EX.NO: **SDS -Polyacrylamide Gel Electrophoresis of Proteins**

Aim: To analyze the isolated total protein from bacterial cells using 12 % SDS-PAGE.

Principle:

SDS-PAGE is the most widely used method for qualitatively analyzing protein mixtures. It is particularly useful for monitoring protein purification and to determine the relative molecular weight of proteins because the method is based on the separation of proteins according to size. The method can

Formation of Polyacrylamide Gels

Crosslinked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of *N,N*'-methylene-*bis*-acrylamide (normally referred as "*bis*-acrylamide") and is used as a crosslinking agent. Acrylamide monomer is polymerized in a head-to-tail fashion into long chains, and occasionally a *bis*-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way, a cross linked matrix of fairly well-defined structure is formed. The polymerization of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulfate and the base *N,N,N',N*'-tetramethylenediamine (TEMED). TEMED catalyzes the decomposition of the persulfate ion to give a free radical (i.e., a molecule with an unpaired electron):

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\bullet}$$

If this free radical is represented as R• (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule, then the polymerization can be represented as follows:

$$R^\bullet + M \rightarrow RM^\bullet$$
$$RM^\bullet + M \rightarrow RMM^\bullet$$
$$RMM^\bullet + M \rightarrow RMMM^\bullet, \text{ and so forth}$$

In this way, long chains of acrylamide are built up, being cross linked by the introduction of the occasional *bis*-acrylamide molecule into the growing chain.

Use of Stacking Gels

For both SDS and buffer gels samples may be applied directly to the top of the gel in which protein separation is to occur (the separating gel). However, the sharpness of the protein bands produced in the gel is limited by the size (volume) of the sample applied to the gel. Basically the separated bands will be as broad (or broader, owing to diffusion) as the sample band applied to the gel. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel, thus giving sharper protein bands in

the separating gel. This modification allows relatively large sample volumes to be applied to the gel without any loss of resolution. The stacking gel has a very large pore size (4% acrylamide) which allows the proteins to move freely and concentrate, or stack under the effect of the electric field. Sample concentration is produced by isotachophoresis of the sample in the stacking gel. The band-sharpening effect (isotachophoresis) relies on the fact that the negatively charged glycinate ions (in the reservoir buffer) have a lower electrophoretic mobility than the protein–SDS complexes. which in turn, have lower mobility than the Cl– ions if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that [Cl–] > [protein-SDS] > [glycinate]. There are only a small quantity of protein–SDS complexes, so they concentrate in a very tight band between the glycinate and Cl– ion boundaries. Once the glycinate reaches the separating gel, it becomes more fully ionized in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8 and that of the separating gel is 8.8.) Thus, the interface between glycinate and the Cl– ions leaves behind the protein–SDS complexes, which are left to electrophorese at their own rates.

SDS-PAGE

Samples to be run on SDS-PAGE are first boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces any disulfide bridges present that are holding together the protein tertiary structure. SDS is an anionic detergent and binds strongly to, and denatures, the protein.

Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely masked by the SDS molecules. The sample buffer also contains an ionizable tracking dye usually bromophenol blue that allows the electrophoretic run to be monitored, and sucrose or glycerol which gives the sample solution density, thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein, the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance owing to the sieving effect of the gel. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the

bottom of the gel the current is turned off and the gel is removed from between the glass plates, shaken in an appropriate stain solution (usually Coomassie brilliant blue) for a few hours, and then washed in destain solution overnight. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background.

Materials

1. Stock acrylamide solution:

   30% acrylamide, 0.8% *bis*-acrylamide. Filter through Whatman No. 1 filter paper and store at 4°C.

2. Buffers:

   1.875 *M* Tris-HCl, pH 8.8.

   0.6 *M* Tris-HCl, pH 6.8.

3. 10% Ammonium persulfate. Make fresh.

4. 10% SDS.

5. TEMED.

6. Electrophoresis buffer:

   Tris (12 g), glycine (57.6 g), and SDS (2.0 g). Make up to 2 L with water. No pH adjustment is necessary.

7. Sample loading buffer

   0.6 *M* Tris-HCl, pH 6.8 - 5.0 mL

   SDS 0.5 g

   Sucrose 5.0 g

   β-Mercaptoethanol - 0.25 mL

   Bromophenol blue, 0.5% stock - 5.0 mL

   Make up to 50 mL with distilled water.

8. Protein stain:

   0.1% Coomassie brilliant blue R250 in 50% methanol, 10% glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the final solution through Whatman No. 1 filter paper if necessary.

9. Destain: 10% methanol, 7% glacial acetic acid.

10. Microsyringe for loading samples.

    Micropipet tips that are drawn out to give a fine tip are also commercially available.

Protocol

1. Samples to be run are first denatured in sample buffer by heating to 95–100°C for 5 min.

2. Clean the internal surfaces of the gel plates with detergent or methylated spirits, dry, then join the gel plates together to form the cassette, and clamp it in a vertical position. The exact manner of forming the cassette will depend on the type of design being used.

3. Mix the following in a 250-mL Buchner flask or beaker

	For 15% gels	For 10% gels
1.875 *M* Tris-HCl, pH 8.8	8.0 mL	8.0 mL
Water	11.4 mL	18.1 mL
Stock acrylamide	20.0 mL	13.3 mL
10% SDS	0.4 mL	0.4 mL
Ammonium persulfate (10%)	0.2 mL	0.2 mL

4. "Degas" this solution under vacuum for about 30 s.

5. Add 14 μL of TEMED, and gently swirl the flask to ensure even mixing. The addition of TEMED will initiate the polymerization reaction and although it will take about 15 min for the gel to set, this time can vary depending on room temperature, so it is advisable to work fairly quickly at this stage.

6. Using a Pasteur (or larger) pipet transfer this separating gel mixture to the gel cassette by running the solution carefully down one edge between the glass plates. Continue adding this solution until it reaches a position 1 cm from the bottom of the comb that will form the loading wells. Once this is completed, dispose the excess gel solution remaining in flask in an appropriate waste container **not** down the sink.

7. To ensure that the gel sets with a smooth surface **very carefully** run distilled water down one edge into the cassette using a Pasteur pipet. (Because of the great difference in density between the water and the gel solution the water will spread across the surface of the gel without serious mixing). Continue adding water until a layer of about 2 mm exists on top of the gel solution.

8. The gel can now be left to set. As the gel sets, heat is evolved and can be detected by carefully touching the gel plates. When set, a very clear refractive index change can be seen between the polymerized gel and overlaying water.

9. While the separating gel is setting prepare the following stacking gel (4°C) solution. Mix the following in a 100-mL Buchner flask.

0.6 *M* Tris-HCl, pH 6.8	1.0 mL
Stock acrylamide	1.35 mL

Water	7.5 mL
10% SDS	0.1 mL
Ammonium persulfate (10%)	0.05 mL

Degas this solution as before.

10. When the separating gel has set, pour off the overlaying water. Add 14 μL of TEMED to the stacking gel solution and use some (~2 mL) of this solution to wash the surface of the polymerized gel. Discard this wash, and then add the stacking gel solution to the gel cassette until the solution reaches the cutaway edge of the gel plate. Place the well-forming comb into this solution, and leave to set. This will take about 20 min. (Refractive index changes around the comb indicate that the gel has set). It is useful at this stage to mark the positions of the bottoms of the wells on the glass plates with a marker pen to facilitate loading of the samples.

11. Carefully remove the comb from the stacking gel, and then rinse out any nonpolymerized acrylamide solution from the wells using electrophoresis buffer. Remove any spacer from the bottom of the gel cassette, and assemble the cassette in the electrophoresis tank. Fill the top reservoir with electrophoresis buffer, and look for any leaks from the top tank. (If there are no leaks fill the bottom tank with electrophoresis buffer, and then tilt the apparatus to dispel any bubbles caught under the gel).

12. Samples can now be loaded onto the gel. Place the syringe needle through the buffer and locate it just above the bottom of the well. Slowly deliver the sample into the well. Five- to 10-μL samples are appropriate for most gels. The dense sample buffer ensures that the sample settles to the bottom of the loading well. Continue in this way to fill all the wells with unknowns or standards, and record the samples loaded.

13. Connect the power pack to the apparatus, and pass a current of 30 mA through the gel (constant current) for large format gels, or 200 V (constant voltage) for minigels.

14. Continue electrophoresis until the bromophenol blue reaches the bottom of the gel. This will take 2.5–3.0 h for large format gels (16 μm × 16 μm) and about 40 min for minigels (10 μm × 7 μm)..

14. Dismantle the gel apparatus, pry open the gel plates, remove the gel, discard the stacking gel, and place the separating gel in stain solution.

15. Staining should be carried out with shaking, for a minimum of 2 h. When the stain is replaced with destain, stronger bands will be immediately apparent, and weaker bands will appear as the gel destains.

Results and observations:

SOUTHERN BLOTTING (FIRST METHOD)

Introduction

This method to know the presence or absence of a particular fragment in genomic DNA was first developed by E. D. Southern in 1975. The advent of Southern blotting technique was a turning point in the field of molecular biology. It involves the capillary transfer of DNA fragments from an agarose gel to various types of membranes. Restriction Fragment Length Polymorphisms can be analyzed using the technique, wherein DNA fragments are separated on agarose gels denatured in situ and transferred onto membranes for analysis.

Materials

- Denaturation solution: NaCl, 1.5 M and NaOH, 0.5 M
- Neutralization solution: NaCl, 1.5 M; Tris-Cl (pH 7.5), 0.5 M and EDTA (pH 8.0), 1 mM
- 20X SSC: NaCl, 1.5 M and trisodium citrate, 0.1 M
- Depurinization solution: 0.25 N HCl
- Nylon or nitrocellulose membrane

Procedure

- After agarose gel electrophoresis, photograph the gel and soak it in 0.25 N HCl for 15 minutes at room temperature, with gentle shaking.
- Decant the acid solution and denature the DNA by soaking the gel in several volumes of denaturation solution for 30 minutes at room temperature, with constant shaking.
- Neutralize the gel by shaking in several volumes of neutralization solution for 30 minutes at room temperature, with shaking.
- Wrap a piece of Whatman 3-mm paper around a glass plate. Place the wrapped support on a large plastic tray with the ends of the 3-mm paper dipping into the 20X SSC solution in tray.
- Invert the gel and place it on a damp 3-mm paper on the support. Make sure that there are no air bubbles between the 3-mm paper and the gel.
- Cut a piece of nylon membrane slightly bigger than the gel. Use gloves and forceps to handle the membrane.
- Float the membrane on 20X SSC until it wets completely.
- Place the wet nylon membrane on top of the gel. Remove all the air bubbles that are trapped between the gel and tile membrane.

Results and observations: