Semester – IV

17MBU413 RECOMBINANT DNA TECHNOLOGY – PRACTICAL (4H – 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
7. Primrose SB and Twyman RM. (2008). Genomics: Applications in human biology. Blackwell Publishing, Oxford, U.K.

EX.NO:1 & 2

PREPARATION OF COMPETENT CELLS FOR TRANSFORMATION AND DEMONSTRATION OF BACTERIAL TRANSFORMATION AND CALCULATION OF TRANSFORMATION EFFICIENCY

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 is 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

Observation and Result:

EX.NO:3

DIGESTION OF DNA USING RESTRICTION ENZYMES AND ANALYSIS BY AGAROSE GEL ELECTROPHORESIS

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 µ L 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 µ L (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 µ L of 6x gel loading buffer was added to the samples.
6. The digested samples, 10 µ L of control DNA, 10 µ L of DNA size marker were loaded, (note down the order of loading).
7. Electophoresis was performed at 50 – 100 V for 1-2 h.
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.

Observation and Result:

EX.NO: 4 **LIGATION OF DNA FRAGMENTS**

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 µ L 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 16 ºC for 2 h in water bath.
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.

Observation and Result:

EX.NO: 5

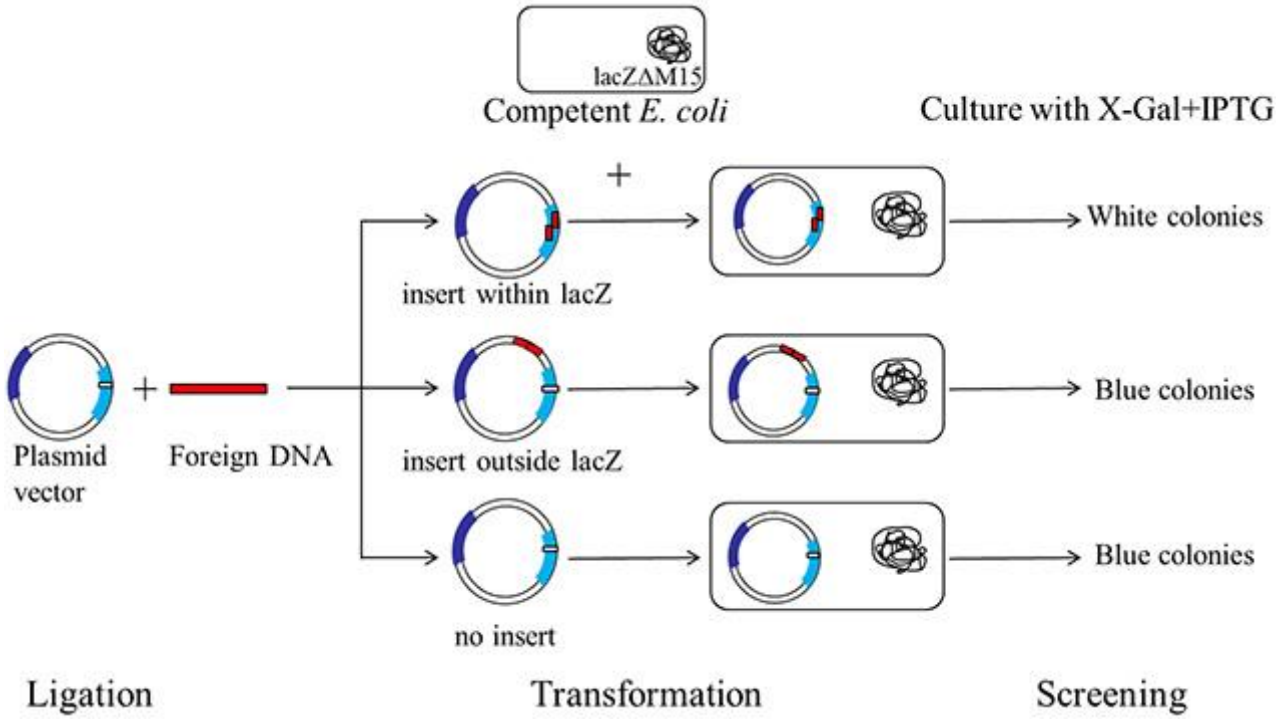
CLONING OF DNA INSERT AND BLUE WHITE SCREENING OF RECOMBINANTS

Aim: To identify the recombinant bacterial cells using blue and white screening technique.

Introduction: Blue-white screening of bacterial colonies is a popular and effective molecular biology tool often used to detect recombinant bacteria in cloning experiments. Central to this technique is the enzymatic activity of β-galactosidase, a tetrameric enzyme encoded by the lacZ α gene in E. coli that metabolizes lactose to form glucose and galactose. Alternatively, β-galactosidase can hydrolyze a different substrate, X-Gal, resulting in 5-bromo-4-chloro-indoxyl, which dimerizes to form a blue pigment.

Principle: For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizable analog of galactose that induces the expression of lacZ gene. It should be noted that IPTG is not a substrate for β-galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.



Materials required:

- X-Gal
- Dimethylformamide (DMF)

- Isopropyl β-D-1-thiogalactopyranoside, IPTG
- Screening antibiotic of choice
- Agar media

Procedure:

Method Preparation of X-Gal and IPTG

X-Gal and IPTG can be incorporated into agar media before pouring into plates or added onto pre-made plates.

1. Prepare 20 mg/ml X-Gal in DMF
2. Prepare 100mM IPTG solution in dH_2O or dilute from a 1M IPTG solution.

Screening on agar media containing IPTG and X-Gal (recommended)

1. Autoclave the growth media agar, then cool to 50 °C.
2. Add 10 µl of 20 mg/ml X-Gal solution per 1 ml of media or 2 µl of 100 mg/ml X-Gal solution per 1 ml of media
3. Add 10 µl IPTG (100mM) per 1 ml of media for a final concentration of 1mM.
4. Add the screening antibiotic.
5. Pour plates and allow them to cool to room temperature before use. This usually takes at least 30 minutes.
6. Spread transformed competent cells as desired.

Note: Blue-white selection plates are generally stable for only 1 week if stored at 4°C in clear sleeves, but may be stored in the dark, or in dark sleeves, at 4°C for up to 1 month.

Screening on pre-made agar plates lacking IPTG and X-Gal

1. Pour autoclaved growth media containing screening antibiotic on media plates and dry in a laminar flow hood.
2. Add 40 µl 100mM IPTG and 120 µl X-Gal (20 mg/ml) to the surface of each plate and spread over the entire surface.

Note: The plate edges are difficult to spread evenly and may give false positives. We advise picking colonies in the middle of the plate, if possible, for best results.

3. Dry X-Gal/IPTG-coated media in a laminar flow hood for approximately 30 minutes before use.
4. Spread transformed competent cells and incubate inverted at either 37 °C until blue colonies form (usually ~24 hours).

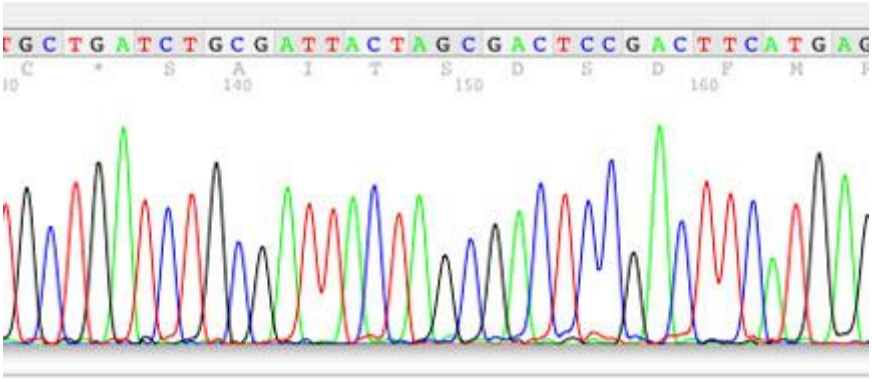
Observation and Result:

EX.NO: 6

INTERPRETATION OF SEQUENCING GEL ELECTROPHEROGRAMS

Aim: To interprete gel electropherograms.

Principle: An electropherogram is a plot of results from an analysis done by electrophoresis automatic sequencing. An electropherogram shows a sequence of data that is produced by an automated DNA sequencing machine.



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

Procedure:

Step 1: A DNA sequence of interest is first amplified using the polymerase chain reaction (PCR).

1. Add to reaction tube:
    a. Double-stranded DNA sequence of interest
    b. Taq DNA polymerase (a heat-resistant enzyme that catalyzes the addition of deoxynucleotides)
    c. A primer (a short single-stranded DNA sequence that base pairs to the DNA sequence of interest and serves as a site from which to add complementary bases.)
    d. A supply of the four deoxynucleotides
2. Heat to separate DNA strands
3. Cool to allow primers to bind
4. Taq DNA polymerase extends the 3' end of each primer to produce double-stranded DNA molecules
5. Repeat for 20 cycles.

Step 2: The amplified DNA strands are subjected to the DNA sequencing reactions.

1. Add to reaction tubes:

a. Amplified DNA strand from Step 1
b. Taq DNA polymerase
c. Primer
d. All four deoxynucleotides
e. Dideoxynucleotides (human-made nucleotides that cannot form covalent bonds to another nucleotide and are labelled with a fluorescent dye. The addition of a dideoxynucleotide terminates the growing DNA strand)

2. Run through 20 30 cycles of heating and cooling.

Step 3: The results of the sequencing reactions are separated by capillary gel electrophoresis and visualized using fluorescent dyes.

1. Load the contents of the sequencing reactions into capillary tubes. We use capillary tubes made of fused silica (glass) and coated on the outside with polyimide (for strength). The tubes have an internal diameter of 100 micrometers and are 100 cm in length. The tubes contain a gel solution which serves as a sieving matrix. Each of our electrophoresis machines holds 96 capillary tubes.
2. Begin electrophoresis.

- The DNA strands migrate, and therefore separate from each other, according to size. The smallest strand travels the fastest and is detected first.
- A laser beam directed at the bottom of the capillary tubes excites the fluorescent-labelled dideoxynucleotide which is positioned at the end of the DNA strand.
- A photodetector measures the light emitted by each dideoxynucleotide as it passes through the laser beam.
- The computer records each wavelength of light and generates an electropherogram with colored peaks representing each wavelength. The terminal base (the dideoxynucleotides) of the shortest fragment is the first base in the electropherogram. The colors of each base are listed below:
    - Guanine (G) yellow (the tracings on the chart paper use black instead of yellow)
    - Cytosine (C) blue
    - Adenine (A) green
    - Thymine (T) red
    - Unknown base = N

Observation and Result:

EX.NO: 7 & 8

DESIGNING OF PRIMERS FOR DNA AMPLIFICATION AND AMPLIFICATION OF DNA BY POLYMERASE CHAIN REACTION (PCR) METHOD

Aim: To design of primers and 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 complimentary 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 µ L 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 µ L 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 µ L) 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.

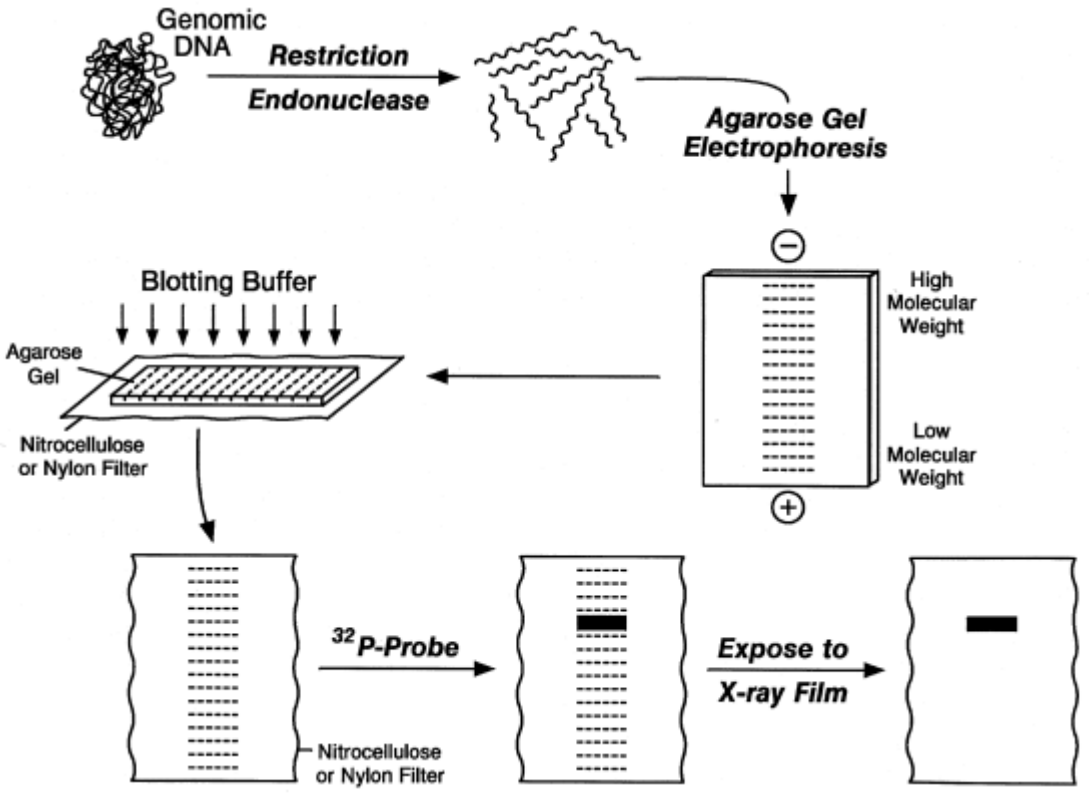
Observation and Result:

EX.NO: 9

DEMONSTRATION OF SOUTHERN BLOTTING

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.

- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.



Materials Required:

- 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

Procedure

Step I: Restriction digest

- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments
- The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis

Step III: Denaturation

- The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.
- DNA strands get separated

Step IV: Blotting

- The separated strands of DNA is then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

- The DNA bound to membrane is then treated with labelled probe
- The labelled probe contains the complementary sequences to the gene of interest
- The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Observation and Result: