SEMESTER IV

18BTU412 RECOMBINANT DNA TECHNOLOGY PRACTICAL 4H - 2C

Total hours/week: L:0 T:0 P: 4 Marks: Internal: 40 External: 60 Total: 100

Practical

1. Isolation of chromosomal DNA from plant cells
2. Isolation of chromosomal DNA from *E.coli*
3. Qualitative and quantitative analysis of DNA using spectrophotometer and agarose gel Electrophoresis.
4. Plasmid DNA isolation
5. Restriction digestion of DNA/ Plasmid DNA
6. Ligation of DNA insert into plasmid vector
7. Preparation of Competent cells
7. Transformation of competent cells.
8. Demonstration of PCR.

References

1. Brown, T.A. (2006). *Gene Cloning and DNA Analysis* (5th ed.). Oxford: UK, Blackwell Publishing.
2. Primrose, S.B., & Twyman, R.M. (2006). *Principles of Gene Manipulation and Genomics* (7th ed.). Oxford: UK, Blackwell Publishing.
3. Sambrook, J., Fritsch, E.F., & Maniatis, T. (2001). *Molecular Cloning-A Laboratory Manual.* (3rd ed.). Cold Spring Harbor Laboratory Press.

EX.NO:1 ISOLATION OF TOTAL GENOMIC DNA FROM PLANT TISSUSE
Date:

Aim: To isolate total genomic DNA from the given plant tissue sample

Principle:

High molecular weight DNA free from protein and RNA should be the basic technique for DNA isolation protocol. The isolated high molecular weight genomic DNA is essential for all molecular biology experiments. The cell wall of plant cell must be broken or digested to release cellular components, which generally performed by grinding with dry ice or liquid nitrogen. The plasma membrane of cell must be disrupted using a detergents (SDS or CTAB). The release DNA must be protected from endogenous nucleases using EDTA. The released cellular components generally emulsified with either chloroform or phenol to denature the proteins. The released DNA must be precipitated using isopropanol. The precipitated DNA then washed with alcohol to remove the salt present in the DNA.

Materials required:

1. 2X CTAB Lysis Buffer - containing
   - 0.1 M Tris HCl (pH 8.0)
   - 20 mM Na EDTA (pH 8.0)
   - 1.4 M NaCl
   - 2% CTAB (W/V)
   - 2% PVP (W/V)
   - 0.3% β-mercaptoethanol or $Na_2S_2O_5$ (add when used).
2. 1X CTAB lysis buffer – dilute 2X CTAB lysis buffer using distilled water (1:1 ratio)
3. Chloroform :isoamyl alcohol (24:1)
4. Ice cold isopropanol
5. 3M sodium acetate (pH 5.2)
6. TE buffer (pH 8.0)

Procedure:

1. 0.1 g of plant tissue was ground using liquid nitrogen to get fine powder
2. The ground fine powder was the transferred into 2 ml eppendorf tube. (Do not allow the sample to thaw)

3. Immediately, 0.9 ml of 2X CTAB lysis buffer was added and vortexed vigorously.

4. Then incubated at 65ºC for 30 min. The contents were mixed frequently during the incubation.

5. Then centrifuged at 12000 rpm for 5 min, the supernatant was transferred into new 2 ml eppendorf tube.

6. To the pellet, 0.3 ml of 1X CTAB lysis buffer was added, mixed well by vortexing and again incubated at 65ºC for 10 min.

7. The contents were centrifuged at 12000 rpm for 10 min and the supernatant was transferred into the 1st supernatant sample.

8. To the supernatant, equal volume of chloroform: isoamyl alcohol (24:1) was added and invert mixed for 15 min, centrifuged at 10000 rpm for 10 min at room temperature.

10. The aqueous layer was transferred into a new 1.5 ml eppendorf tube.

11. 0.7 volume of ice cold isopropanol and 10 µL of 3M sodium acetate (pH 5.2) was added to the aqueous layer, the contents were mixed by invert mixing.

13. Then centrifuged at 10000 rpm for 10 min at room temperature.

14. The supernatant was discarded, and 70 % ethanol was added to the DNA pellet.

15. Again centrifuged at 10000 rpm for 10 min.

16. Then the ethanol supernatant was discarded and the pellet was subjected to air drying.

17. After air drying, the pellet was resuspended in 50 µL TE buffer (pH 8.0).

18. After DNA has dissolved, the purity of the DNA was checked by electrophoresis and spectrophotometric analysis.

19. 0.8 % agarose gel was prepared using 1X TAE buffer.

20. 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.

21. Ran the gel until the second dye from the well has reached 3/4 th of the gel.

22. After the run was completed, the gel was observed under UV transilluminater.

24. Then DNA was stored at 4°C short term, –20°C or –80°C long term.

Results and Observations:

EX.NO:2 ISOLATION OF TOTAL GENOMIC DNA FROM MICROBES (*E.coli*)
Date:

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:3 ESTIMATION OF DNA
Date:

Aim: To estimate the isolated DNA sample quantity and determination of DNA purity.

Principle:

The DNA isolated from living cells is usually contaminated with protein, RNA, and salts used during the isolation process. The purity of DNA may be estimated by utilizing the property of the heterocyclic rings of the nucleotides of absorbing light strongly in the UV range.

DNA absorbs maximum light energy at about 260 nm. An optical density of 1.0 corresponds to approximately 50 mg/mL of double stranded DNA.

The ratio of absorbance viz. A260/A28O and A28O/A260 provides an estimation regarding the purity of DNA. A typically pure preparation of good-quality DNA should exhibit the following spectral properties:

A260/A28O = 1.80

A28O/A260 = 0.55

Materials required:

1. Sample DNA
2. TE buffer: Tris-HCI, 10 mM and EDTA, 1 mM; pH 8.0
3. Spectrophotometer and quartz cuvette

Procedure:

1. To find out the purity of DNA, make the appropriate dilution with TE buffer,and measure the absorbance at 260 nm and 280 nm.

Notes

Do not use glass or plastic cuvettes, as lights in the UV range do not pass through these.

2. Calculate A260/A280 and A280/A260, and check if the values are within the acceptable limit.

3. Calculate the Dt~A concentration as follows:

Concentration of DNA = (A260 × 50 × dilution factor) in mg/mL.

Results and Observations:

EX. NO: 3 AGAROSE GEL ELECTROPHORESIS
Date:

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: 4 ISOLATION OF PLASMID DNA
Date:

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: 5 RESTRICTION DIGESTION OF DNA
Date:

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:6 LIGATION OF DNA
Date:

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:7 Preparation of Competent Cells

Time required:

Day 1: Overnight
Day 2: Overnight
Day 3: 4 hours to grow culture
2 hours to prepare the competent cells

Procedure:

Day 1

1. Streak out the E.coli strain on an LBM plate (no ampicillin!) to isolate colonies and incubate at 37 degrees C overnight (16-20 hours).

Day 2 1. Use a sterile inoculating loop to collect cells from a single colony and inoculate 50 ml sterile 1X LBM Grow at 37 degrees C overnight (16-20 hours) in a shaker incubator. Also place 2 flasks of 250 ml 1X LBM in the incubator to equilibrate the temperature of the medium.

Day 3

1. Add 25 ml of the overnight culture to each 250 ml LBM flask. Place another flask of 150 ml 1X LBM in the incubator to equilibrate the temperature of the medium. Grow the cultures to OD650 = O.2. (not dense approximately 3 hours). Add 75 ml of equilibrated 1X LBM to each flask and continue incubating for 30 minutes.
2. Pellet the cells in chilled autoclaved large centrifuge bottles using the Beckman J-6 centrifuge and JA 10 rotor (must be cold!) at 5000 rpm for 10 minutes. Subsequent resuspensions may be done in the same bottle. Cells must remain cold for the rest of the procedure: Transport tubes on ice and resuspend on ice in the cold room.
3. Decant supernatant and resuspend the cells in 1/4 original volume (87.5 ml) ice cold 100 mM MgCl2. Hold on ice for 5 minutes. Transfer the cells to pre-chilled sterile large centrifuge bottles. Spin in the Beckman J- 6 centrifuge for 10 minutes using the JA-20 rotor 4000 rpm at 4 degrees C.
4. Decant the supernatant and resuspend the cells in 1/20 original volume (17.5 ml) of ice cold 100 mM CaCl2. Hold on ice for 20 minutes. Pellet as above 4000 rpm for 10 minutes.
5. Decant the supernatant and resuspend the cell pellet in 1/100 original volume (3.5 ml) of a solution that is 85% v/v 100 mM CaCl2 and 15% v/v glycerol (100%). For each culture processed chill approximately 15 labeled eppendorf tubes in a dry ice-EtOH bath. Pipet 300 ul cells into each tube and place immediately into the dry ice-EtOH bath. Transfer the frozen competent cell aliquots to -80 degrees C.
6. After the competent cells have been stored for 24 hours check the efficiency of transformation: Use 1 ng 10 ng and 100 ng of any ampicillin resistant plasmid on LBM + Amp plates as per transformation protocol for intact plasmids. Check the background level by plating 50 ul of cells alone on an LBM + Amp plate. Expect yields to be approximately 5x10e7 colonies per ug of supercoiled DNA.
   Solutions:

1. 100 mM MgCl2:
    - 1:10 dilution of lab stock; use sterile ingredients or filter
    - sterilize
2. 100 mM CaCl2:
    - 1:10 dilution of lab stock; use sterile ingredients or filter
    - sterilize
3. 85% 100 mM CaCl2, 15% glycerol:
    - 42.5 ml 100 mM CaCl2
    - 7.5 ml 100% glycerol
    - 50.0 ml total volume; mix well and use sterile ingredients or filter sterilize

Precautions:

Plasmid/cosmid DNA should be considered biohazards and wastes should be disposed of appropriately.

Transformation Protocol

Protocol

1. Thaw competent cells on ice.
2. Chill approximately 5 ng (2 µl) of the ligation mixture in a 1.5 ml microcentrifuge tube.
3. Add 50 µl of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4–5 times to mix the cells and DNA. Do not vortex.
4. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds*. Do not mix.
6. Add 950 µl of room temperature media* to the tube.
7. Place tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 50–100 µl of the cells and ligation mixture onto the plates.
10. Incubate overnight at 37°C.

    * Please note: For the duration and temperature of the heat shock step as well as for the media to be used during the recovery period, please follow the recommendations provided by the competent cells' manufacturer.

EX. NO: 8 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION (PCR)

Date:

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

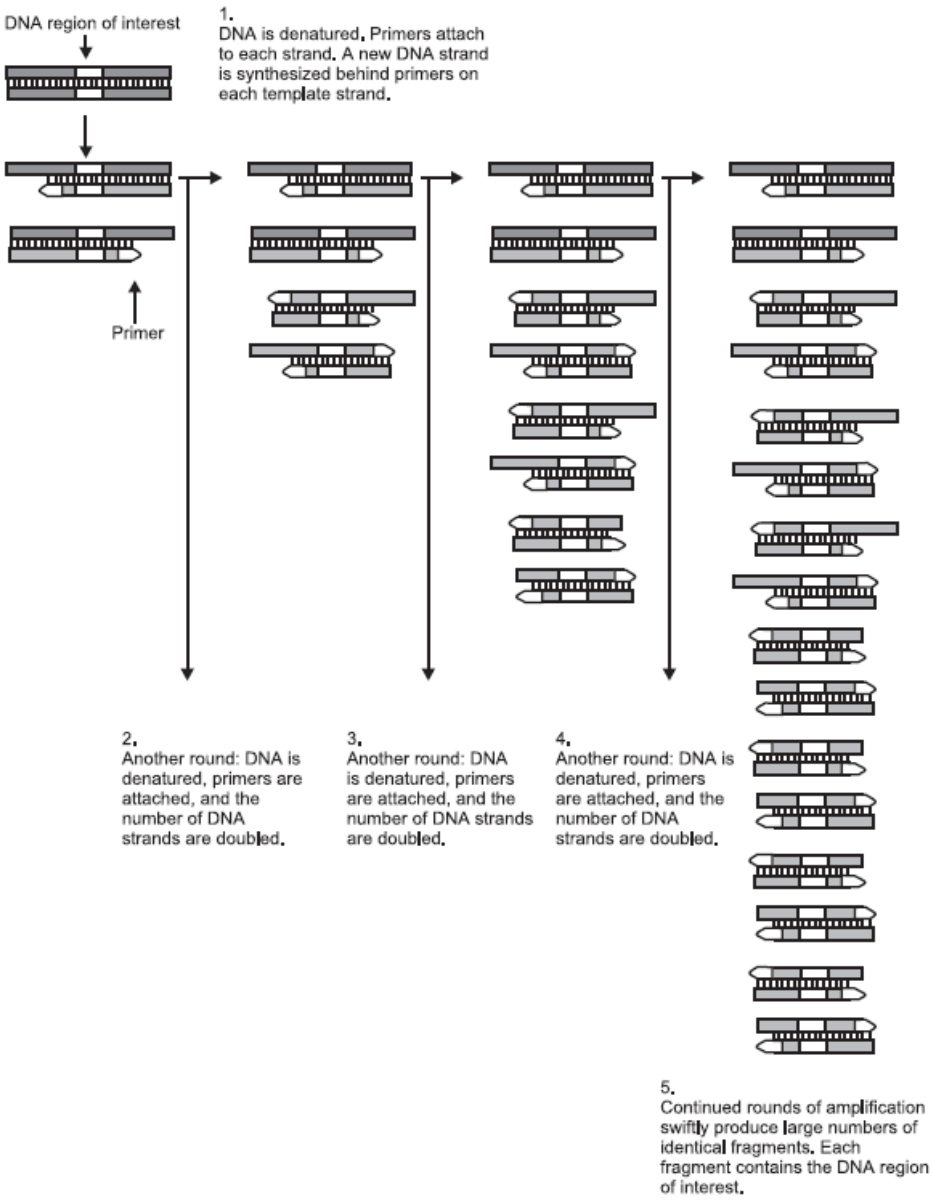
1. Add 38 µL of sterile milliQ water (or autoclaved double distilled water) to a sterile microfuge.
2. Add 5 µL of 10 X Taq polymerase assay buffer with $MgCl_2$ to the microfuge.
3. Add 3 µL of 2.5 mm dNTP mixed solution to the microfuge.
4. Add 1 µL of control template DNA.
5. Add 1 mol each of forward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