Index

P- XXI

Expt. No.	Name	Date	Page No.	Sign
1	Restriction digestion of lambda DNA			
2	Isolation of chromosomal DNA from *E. coli.*			
3	Separation of *E. coli* DNA by agarose gel electrophoresis.			
4	Confirmation and Estimation of DNA by diphenylamine			
5	Measurement of B-galactosidase activity using ONPG.			
6	Selection of recombinant clones on suitable media			
7	Polymerase Chain Reaction (PCR)			

Expt. No. 1 **Date**

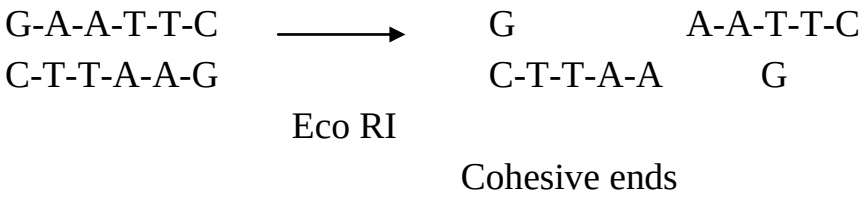
Restriction digestion of lambda DNA

Aim

To carry out digestion of lambda DNA by Restriction endonucleases and analysis of fragments by gel electrophoresis

Theory / Approach

Restriction enzymes (or restriction endonucleases) cleave DNA in a very specific fashion. Type II restriction enzymes, most commonly used for DNA analysis and genetic engineering, each have a unique nucleotide sequence at which it cuts a DNA molecule. A particular restriction enzyme will cleave DNA at that recognition sequence and nowhere else. The recognition sequence is often a six base pair palindromic sequence (the top DNA strand from 5' to 3' is the same as the bottom DNA strand from 5' to 3'), but others recognize four or even eight base pair sequences.



A common use for restriction enzymes is to generate a "fingerprint" of a particular DNA molecule. Because of the sequence specificity of restriction enzymes, these enzymes can cut DNA into discrete fragments which can be resolved by gel electrophoresis. This pattern of DNA fragments generates a "DNA fingerprint," and each DNA molecule has its own fingerprint.

A restriction enzyme reaction contains the DNA to be analyzed, a restriction enzyme, and a restriction enzyme buffer mix. Each restriction enzyme prefers a particular restriction enzyme buffer mix. This buffer mix, commonly supplied at a 10X concentration, contains a buffering agent (usually Tris) to maintain constant pH, salt (usually NaCl or KCl) to provide the correct ionic strength for the digest, and Mg++ (from MgCl2) as a necessary cofactor for enzyme activity.

Commercially available restriction enzymes usually have activities at 10-20 units/µl. A "unit" is usually defined as the amount of enzyme needed to digest 1 µg of bacterial virus lambda DNA in 1 hour in a 50 µl reaction. We generally use 10-20 units (1 µl) of restriction enzyme per reaction. This is

usually far more than needed, but this excess assures that complete digestion will occur. Digestion times are 1.5 hours, but can be lengthened. After the reactions are completed, the samples can be stored in the refrigerator until ready to use. Loading dye is added to the DNA digests, and the samples loaded onto a gel.

Requirements

1. Incubator
2. Electrophoresis units and power supplies
3. 0.8% agarose in 1X TAE (melted)
4. 1X TAE for electrophoresis units
5. Methylene blue solution
6. Staining trays
7. Lambda DNA (0.5 µg/ul)
8. Deionized or distilled H2O
9. 10X restriction enzyme buffer
10. Eco RI (10 units/ ul)
11. Microcentrifuge tubes
12. Micropipets and tips

Procedure

1. Take	Lambda DNA (0.5 µg/ul)	6 µl
	Deionized or distilled H_2O	10 µl
	10X buffer	2 µl
	Eco RI	2 µl

2. Pool the reagents at the bottom of the tube. The tubes can be tapped to collect the fluids at the bottom.
3. Incubate reaction tubes for at least 1.5 hours at 37^0 C in incubator.
4. Pool the liquid from the restriction enzyme reaction at the bottom of the tube. Add 4µl 6X loading dye to each reaction tube. Be sure the loading dye mixes with the DNA sample at the bottom of the tube.
5. Set up the electrophoresis unit by inserting the black casting dams and placing a comb in the slot closest to the black electrode.
6. Pour the melted agarose into gel deck.
7. Remove black casting dams and the comb.
8. Load the DNA samples in the wells NEAR THE BLACK ELECTRODE.
9. Pour the TAE solution into the side farthest from you samples, so that you gels is completely covered plus a little more.
10. Connect electrophoresis unit to power supply. The DNA will migrate through the gel for ~30 minutes.

Methylene blue staining

1. Once the purple dye has migrated approximately 2/3 of the gel, turn off the power and carefully remove the gel.
2. Remove the tray that you poured agarose on to and gently slide the gel into the staining tray.
3. Completely cover the gel with methylene blue for 10 minutes.
4. Pour off the stain and observe the bands.

Observation : DNA bands are observed.

Result: Restriction enzyme digested E. coli DNA and is analyzed by gel electrophoresis.

Expt. No. 02 **Date: -**

Isolation of chromosomal DNA from E. coli

Aim: - To isolate chromosomal DNA from E. coli

Theory / Approach: -

Most of the bacteria contain cell wall polysaccharides, proteins, and nucleic acids. For the isolation of genomic DNA, it is necessary to get rid of cell wall debris, polysaccharides and proteins by lysing the bacterial cells.

The genetic material in the bacteria is not very well organized as compared to the eukaryotic genome. So extraction of bacterial genomic DNA is fairly simple and do not involve elaborate protocols.

Three major types of techniques or combination of them are employed in isolation of nucleic acids— differential solubility, adsorption methods or density gradient centrifugation. Choice of method depends on source of DNA being isolated and the application. Major goal of nucleic acid isolation is removal of proteins, which is accomplished due to their different chemical properties. Most nucleic acid isolation protocols involve—

- Cell lysis step
- Differential solubility (Phenol extraction or adsorption to solid support)
- Precipitation

Cell lysis: -

Nucleic acids (DNA / RNA) must be solubilized from the cells. This solubilization is usually carried out under denaturing conditions such as SDS, Alkali, Boiling or Chaotropic agents. These denaturing conditions efficiently solubilize the nucleic acids and generally do not adversely affect them. In addition, the denaturing conditions promote the removal of proteins during the subsequent steps and inhibit the activity of nucleases that degrade the nucleic acids.

Differential solubility: -

Phenol extraction: - Phenol is an organic solvent used to separate proteins from DNA. It is mixed in equal volumes with the DNA. The two phases are then separated by centrifugation and the upper aqueous phase that contains the nucleic acid is retained. Proteins are seen as flocculent material at the interface.

Precipitation: - DNA is precipitated from dilute solutions with ethanol or isopropanol, in presence of Sodium or Potassium acetate, pH 5 to 5.5 added to the final concentration of 0.3 M Sodium acetate and acidic pH will neutralize the highly charged phosphate backbone and promote hydrophobic interactions. The precipitated DNA is collected by centrifugation or is pooled out with a Pasteur pipette. The pellet is rinsed with 70 % ethanol to remove excess salt, dried and dissolved in appropriate buffer.

i) Isolation and Purification of DNA by phenol extraction method.

Requirements: -

1. MacConkey's broth
2. *E. coli* culture
3. Extraction medium (0.15 M NaCl), (0.1 M EDTA)

   (877 mg NaCl in 100 ml water + 2.92 gm EDTA)

4. SLS (Sodium Lauryl Sulphte 25 %)
5. Phenol
6. TE buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)
7. 24:1 (v/v) chloroform-isoamyl alcohol
8. Ethanol
9. Microcentrifuge

Procedure for Cell lysis and purification of DNA by Phenol extraction method

1. Pure culture of E. coli is inoculated in 100 ml sterile MacConkey's broth and incubated at 37 ^{0} C for 24 hours.
2. Centrifuged at 10,000 RPM for 20 minutes to pellet the cells.
3. Approximately 1 gm of pellet is taken and washed with 20 ml extraction medium (Saline EDTA).
4. 0.1 ml Lysis Solution SLS (Sodium Lauryl Sulphte) is added in it.
5. The mixture is heated at 60^{0}C for 10 minutes and cooled to room temperature.
6. Add an equal volume of phenol to the DNA containing reaction mixture and vortex gently.
7. After cooling equal volume (20 ml) of 24:1 (v/v) chloroform-isoamyl alcohol is added and it is shaken at room temperature for 30 minutes.
8. This mixture is centrifuged at 10000 RPM for 5 minutes.
9. After centrifugation 3 layers are found. Upper layer containing DNA is transferred in a test tube by micropipette.
10. To it double volume of ethanol is added on top and stirred slowly with glass rod for few minutes. The DNA will precipitate as threads.

Expt. 03 **Date**

Separation of *E. coli* DNA using agarose gel electrophoresis.

Introduction: -

Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their molecular weight and is an intrinsic part of almost all routine experiments carried out in molecular biology. This technique is used to separate charged molecules. DNA is negatively charged at neutral pH and when electric field is applied across the gel, DNA migrates toward the anode. Migration of DNA through the gel is dependent up on—

1. Molecular size of DNA
2. Agarose concentration
3. Confirmation of DNA
4. Applied current

Agarose gel acts as a molecular sieve through which DNA fragments move on application of electric current. Higher concentration of agarose gives firmer gels i.e. spaces between cross-linked molecules is less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps in movement of larger DNA fragments as the spaces between the cross linked molecules is more. The process of gel electrophoresis is monitered by observing the migration of visible dye through the gel. Two commonly used dyes are Xylene Cyanol and Bromophenol blue that migrate at the same speed as the double stranded DNA of size 5000 bp and 300 bp respectively. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of the run, when the tracking dye reaches towards the anode, run is terminated.

Visualization of DNA: -

Since DNA is not naturally coloured, it will not be visible on the gel. Hence the gel after electrophoresis is stained with a dye specific to DNA. Discrete bands are observed when there is enough DNA material present to bind the dye to make it visible; otherwise the band is not detected. The gel is observed against a light background where in DNA appear as dark coloured bands.

Preparation of 1 % agarose gel: -

1. Prepare 1 X TAE by diluting appropriate amount of 50 X TAE buffer (for one experiment, approximately 200 ml of 1 X TAE is required. Make up 4 ml of 50 X TAE to 200 ml with distilled water.)
2. Weigh 0.5 gm of agarose and add 50 ml of 1 X TAE. This gives 1 % agarose gel.
3. Boil till agarose dissolves completely and clear solution results.
4. Meanwhile place the combs of electrophoresis set such that it is approximately 2 cm away from the cathode.
5. Pour the agarose solution in the central part of the tank when the temperature reaches approximately 60 ^{0} C. Do not generate air bubbles. The thickness of the gel should be around 0.5 to 0.9 cm. Keep the gel undisturbed at room temperature for the agarose to solidify.
6. Pour 1 X TAE buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
7. Gently lift the combs, ensure that wells remain intact.

Electrophoresis: -

1. Connect the power cord to the electrophoretic power supply according to the convention.
   Red : Anode. Black : Cathode
2. Load the samples in the wells in the desired order.
3. Set the voltage to 50 volts and the switch on the power supply.
4. Switch off the power when the tracking dye (Bromophenol blue) from the well reaches 3 / 4 th of the gel. This takes approximately one hour.

Staining procedure to visualize DNA: -

1. Prepare 1 X staining dye by diluting 6 X dye (1 : 6) with distilled water. (Approximately 50 ml of 1 X staining dye is required for one experiment. Therefore make up 8 ml of 6 X dye to 48 ml of distilled water).
2. Carefully transfer the gel (from gel tank) into a tray containing 1 X staining solution. Make sure that gel is completely immersed.
3. For uniform staining, place the tray on a rocker for approximately 1 hour or shake intermittently every 10 to 15 minutes.
4. Pour out the staining dye into a container (the dye can be reused twice). Destain the gel by washing with tap water several times till the DNA is visible as a dark band against a light blue background.

Observation: - Dark bands of DNA are observed.

Result: - Genomic DNA from the *E. coli* is isolated.

Conclusion: - By this method we can observe the genomic DNA of the E. coli.

Expt. No. 4 **Date: -**

Confirmation and Estimation of DNA by diphenylamine

Aim:

To estimate the amount of DNA in a given sample.

Theory:

Disch (1930) found out that DNA gives a blue colour on heating at 100 ^{0} C with a solution of Diphenylamine in glacial acetic acid containing 2.75 % Sulphuric acid. The colour is proportional to the amount of DNA and can be used for its quantitative determination. The deoxyribose sugar in the DNA in presence of acid forms hydroxy levulinialdehyde of purine, which reacts with Diphenylamine to give a blue colour. But only the deoxyribonucleotide of purine reacts.

Requirements:

A) Reagents:

1. Standard DNA 100 microgram / ml
2. Freshly prepared Diphenylamine. It is prepared by dissolving 1 gm of Diphenylamine in 100 ml of glacial acetic acid by adding 2.5 ml of conc. H_2SO_4.
3. Standard saline Citrate solution: 8.77 gm of 0.15 N NaCl dissolved in one liter distilled water and 3.675 gm of 0.015 N Sodium citrate dissolved in one liter distilled water and both were then mixed properly.

B) Apparatus:

1. Spectrophotometer
2. Test tubes
3. Boiling water bath.

Procedure:

1. A reagent blank containing 3 ml standard saline citrate (SSC) was prepared. A set of DNA standards containing 20, 40, 60, 80, 100 microgram / ml was prepared in SSC and each of DNA standard is pipetted out in to a separate tube in 3 ml amount.
2. 5 ml of Diphenylamine reagent is added into each of tube and tubes are shaken thoroughly to mix the reagents.
3. All tubes are then placed in boiling water bath for 15 minutes.
4. Tubes are then cooled. OD at 595 nm wavelengths is measured.

5. Using blank solution spectrophotometer is set at 100 % transmission at 595 nm wavelengths. The OD of colour developed in each tube is measured.
6. Standard graph of OD verses Conc. Of standard DNA is then plotted.
7. From the standard graph and optical density of sample under test, the amount of DNA present in the sample is estimated.

Observation table:

Standard DNA sol. of 100 microgram / ml	**Distilled water**	**Concentration of DNA in microgram / ml**	**Diphenylamine**	**Keep in boiling water bath for 15 minutes**	**OD at 595 nm**
0.6 ml	2.4 ml	20	5 ml	,,	
1.2 ,,	1.8 ,,	40	,,	,,	
1.8 ,,	1.2 ,,	60	,,	,,	
2.4 ,,	0.6	80	,,	,,	
3.0 ,,	Nil	100	,,	,,	
Nil	3 ml	Blank	,,	,,	
Sample 3 ml	Nil	Unknown	,,	,,	

Result:

As shown in the table and graph, concentration of DNA in a given sample is _____ microgram / ml.

Expt. No. 5 **Date**

Measurement of β-galactosidase activity using ONPG.

Aim

To measure β galactosidase activity by using ONPG (Ortho-Nitro Phenyl-Galactopyranoside)

Theory / Approach

Principle:

o-Nitrophenyl-b-D-galactopyranoside (ONPG) $\xrightarrow{\beta\text{-galactosidase}}$ o-Nitrophenol (ONP) + D-Galactose. The appearance of o-nitrophenol is measured at 410 nm by spectrophotemetry.

Unit Definition: One unit causes the formation of one micromole of ONP per minute.

Requirements

1. Phosphate buffer, pH 7.3: 0.1 M (Prepare by mixing 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4 to reach pH 7.3 at 37^oC.)
2. ONPG solution 34 mM (205 mg ONPG/20 ml of Phosphate buffer)
3. Ortho Nitro Phenol (ONP) solution 13.9 gm in 100 ml water to get 1mM
4. E. Coli culture
5. Lactose tube
6. Colorimeter

Procedure

1. For the standard graph of ONP concentration prepare tubes as per protocol and take OD at 410 nm
2. Inoculate the E. coli culture in lactose tube with 0.2 ml ONPG solution and incubate at 37^0 C for 5 minutes.
3. Take OD at 410 nm

Observation table / Protocol

Stock solution in ml	D/W in ml	Conc. of ONP in micromole	OD at 410 nm
0.6	2.4	200	
1.2	1.8	400	
1.8	1.2	600	
2.4	0.6	800	
3.0	Nil	1000	
Test sample 3 ml	Nil		

Result: The β galactosidase units produced by ***E. coli*** are _____

Experiment No. 06 **Date: -**

Selection of recombinant clones on suitable media

Aim: - To select recombinant clones on suitable media..

Theory / Approach: -

When recombinant DNA is constructed and used for transformation of *E. coli* cells, the following types of bacterial cells are obtained: (1) majority of the cells are nontransformed, (2) a proportion of the transformed cells contain unaltered vector, while (3) the remainder cells have recombinant DNA. A mixture of these cells is plated on a suitable medium. In some cases, it may be possible to select for those cells that contain the desired recombinant DNA or the desired gene. But in most cases, the desired recombinant DNA will have to be identified from a collection of a very large number of clones containing different recombinant DNA molecules. A clone containing a recombinant DNA molecule is called ***recombinant clone.***

When the desired gene does not lend itself to direct selection, it is isolated by a two-step indirect selection procedure. The first step of this procedure consists of identification and isolation, following the transformation of host cells, of the small number of cells that contain recombinant DNA from among a very large number of nontransformed cells and those cells that are transformed by the unaltered vector molecules. The next step, therefore, is to identify the clone having the desired DNA insert from among the large number of clones containing recombinant DNA molecules. This is generally achieved by inserting a selectable marker gene into the vector used for producing the recombinant DNAs. **A *marker gene*** or ***reporter gene*** produces a phenotype, which permits either an easy selection or quick identification of the cells in which it is present. A ***Selectable marker*** governs a feature, which enables only such cells that possess it to survive under the selective conditions. For example, genes confering resistance to an antibiotic like ampicillin, are good selectable markers. When a population of bacterial cells is plated on a ampicillin containing medium, only those cells that have the ampicillin resistance gene (amp^r) survive and form colonies.

A good vector has at least two marker genes, of which atleast one is selectable, *e.g. p*BR322 has the genes tet^r -and apm^r (for tetracycline and ampicillin resistance, respectively). The nontransformed bacterial cells are eliminated by plating them on a medium containing the selection agent, *e.g.*, tetracycline or ampicillin in the case of pBR322. All the colonies that develop

on the selective medium are transformed either by the unaltered vector or the recombinant DNA.

Amp^r gene in pBR322 has restriction site for *Pst*I hence it introduces target DNA into Amp^r gene. Thus Amp^r gene gets inactivated and cell loses resistance against ampicillin and do not grow ampicillin containing plate. They are identified by knowing selective position on master plate.

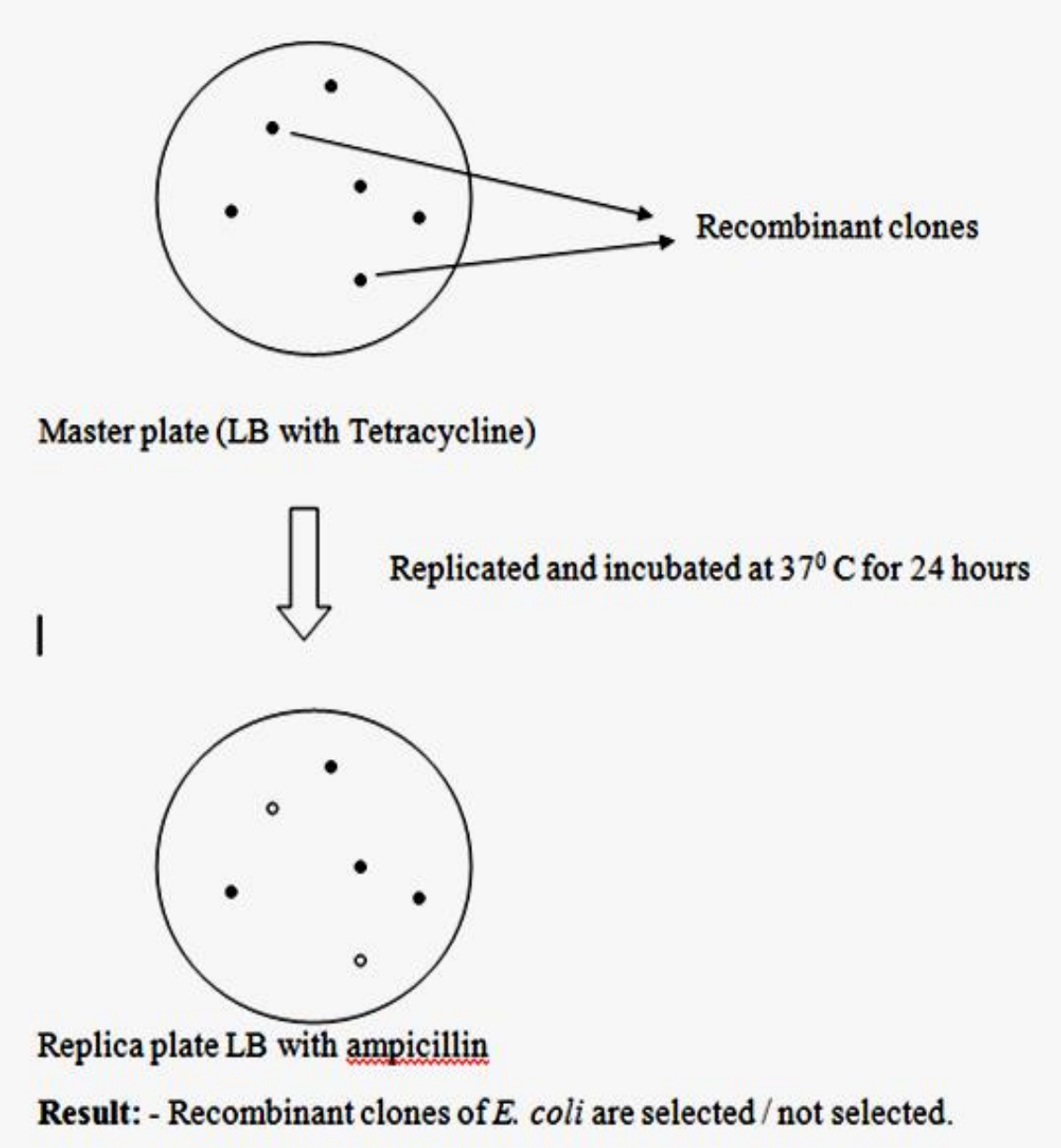
Requirements: -

1. *E. coli* suspension
2. LB plates supplemented with Ampicillin (0.1 %)
3. LB plates supplemented with Tetracycline (0.1 %)
4. pBR322 plasmid
5. Target DNA
6. Restriction Endonuclease *Pst*I (*Pst*I, is a restriction endonuclease from *Providencia stuartii)*
7. Phosphate Buffer of pH 7

Procedure: -

1. 10 ng pBR322 plasmid and 10 ng target DNA are added in a test tube containing buffer and 10 ng Restriction Endonuclease *Pst*I is added in it and allowed to react for 20 minutes.
2. 1 ml *E. coli* suspension is added in it and allowed to stand for 1 hour.
3. 0.1 ml from above step is spread on the LB plate supplemented with tetracycline and incubated at 37^0 C for 24 hours. This is master plate on which E. coli transformed with pBR322 plasmid without target DNA and E. coli transformed with pBR322 plasmid with target DNA introduced into amp^r gene will grow.
4. This plate is replicated on LB plates supplemented with Ampicillin and incubated at 37^0 C for 24 hours. On this plate E. coli transformed with pBR322 plasmid without target DNA only will grow. E. coli transformed with pBR322 plasmid with target DNA introduced into amp^r gene will not grow.
5. The recombinant clones are selected by knowing respective position on master plate.

Observation: - Colonies are appeared / not appeared on the surface of the medium.



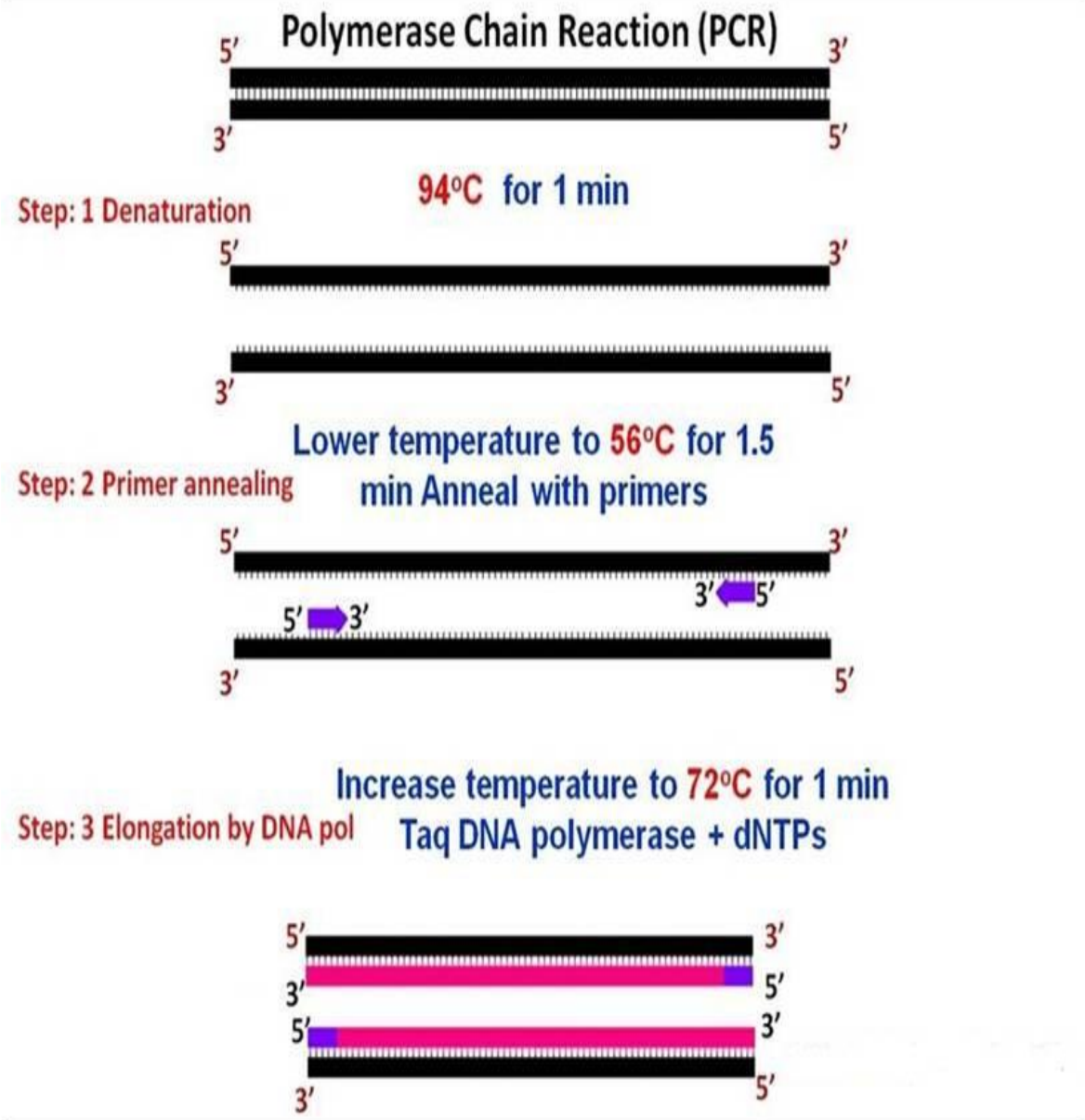
Expt. 7 **Date**

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) technique, developed by Kary Mullis in 1985, is extremely powerful. It generates microgram (µg) quantities of DNA copies (up to billion copies) of the desired DNA (or RNA) segment, present even as a single copy in the initial preparation, in a matter of few hours. The PCR process has been completely automated and compact thermal cyclers are available in the market.

The PCR is carried out *in vitro*. It utilizes the following:

1. a DNA preparation containing the desired segment to be amplified *(target sequence)*,
2. two nucleotide primers (about 20 bases long) specific, *i.e.*, complementary, to the two S'-borders (the sequences present at or beyond the 3'-ends of the two strands) of the desired segment,
3. the four deoxynucleoside triphosphates, *viz.*, TTP (thymidine triphosphate), dCTP (deoxycyctidine triphosphate), dATP (deoxyadenosine triphosphate) and dGTP (deoxyguanosine triphosphate),
4. a heat stable DNA polymerase, *e.g., Taq* (isolated from the bacterium *Thermus acquaticus)*, *Pfu* (from *Pyrococcus furiosus)* and *Vent* (from *Thermococcus litoralis)* polymerases. *Pfu* and *Vent* polymerases are more efficient than the *Taq* polymerase.


Polymerase Chain Reaction (PCR)
5′
3′
3′
5′
94°C for 1 min
Step: 1 Denaturation
5′
3′
3′
5′
Lower temperature to 56°C for 1.5 min Anneal with primers
Step: 2 Primer annealing
5′
3′
3′
5′
5′
3′
3′
5′
Increase temperature to 72°C for 1 min
Taq DNA polymerase + dNTPs
Step: 3 Elongation by DNA pol
5′
3′
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3′
3′
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