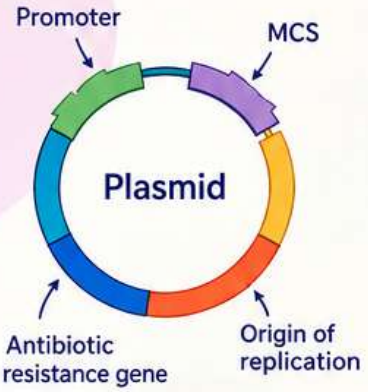
RAMAIAH
College of Arts, Science
& Commerce - Autonomous

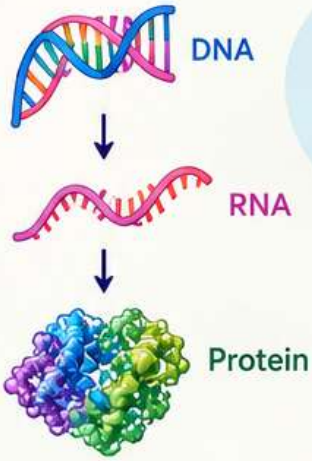
LAB MANUAL

MOLECULAR BIOLOGY AND RDT

(24MIBP401)



II YEAR BSc MICROBIOLOGY



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DEPARTMENT OF MICROBIOLOGY

BSc-MICROBIOLOGY

COURSE-24MIBP401

Molecular Biology and Recombinant DNA Technology

LAB MANUAL

ACADEMIC YEAR 2025-26 ONWARDS

By

Mrs. Soumya S Shanbhag

Dr. Swetha P

Program: BSc

IV Semester-Microbiology

Course: IV Semester MOLECULAR BIOLOGY & RDT

Course Code: 24MIBP301: Microbial Physiology and Genetics		
Sl. No.	**Name of the Experiment**	**Page No.**
1.	Preparation of Phosphate and Tris HCl buffers.	
2.	Estimation of DNA by Diphenylamine (DPA) method.	
3.	Estimation of RNA by Orcinol method.	
4.	Extraction of genomic DNA from bacteria and separation by gel electrophoresis.	
5.	Isolation of plasmid DNA from bacteria and separation by gel electrophoresis.	
6.	Induction and assay of *β*-galactosidase activity in *E. coli.*	
7.	Isolation of antibiotic-resistant mutants using the Replica Plate Method.	
8.	Isolation of antibiotic-resistant mutants using the Gradient Plate Method.	
9.	Demonstration of restriction digestion of DNA.	
10.	Demonstration of *in-vitro* DNA ligation.	
11.	Charts on genetic engineering. i pBR 322 ii pUC 18 and 19 iii SV 40 iv Bacteriophages - Lambda v Gene cloning vi Selection of recombinants by replica plate technique.	

Internal Evaluation Report

Sl. No.	Name of the Experiment	Date	Internal Evaluation			Signature
			Record/ Performance	Viva	Total	
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Signature of the Faculty In-charge **HOD**

INTRODUCTION TO BUFFERS

An aqueous solution that resists any change in pH by adding a small amount of acid or base is called a buffer solution. A buffer solution consists of a weak acid and its conjugate base or a weak base and its conjugate acid.

A buffer solution can resist pH change because of an equilibrium between the acid (HA) and its conjugate base (A^-). The balanced equation for this reaction is:

$$HA \leftrightharpoons H^+ + A^-$$

When a few drops of a strong acid are added to an equilibrium mixture of the weak acid and its conjugate base, the hydrogen ion (H^+) concentration increases very little compared to the volume expected for the quantity of strong acid added. It happens because the equilibrium shifts to the left, following Le Chatelier's principle. If a strong base is added to the equilibrium mixture, the reaction moves to the right to compensate for the lost H^+.

As the buffer's pH changes slightly when a small amount of strong acid or base is added, it is used to prevent any change in the pH of a solution, regardless of solute. Using buffer solutions in various chemical applications keeps the pH at a nearly constant value.

Primarily, buffer solutions are of two types: acidic and basic buffers.

Acidic Buffers

A buffer solution prepared with large quantities of a weak acid and its salt with a strong base is known as an acid buffer. As the name suggests, these buffer solutions have an acidic pH and are used to maintain acidic environments.

Example

A typical example of an acidic buffer is a mixture of an equal concentration of acetic acid (CH_3COOH) and sodium acetate (CH_3COONa) [pH = 4.74].

Basic Buffers

A buffer solution containing relatively large quantities of a weak base and its salt with a strong acid is called a basic buffer. It has a basic pH and is used to maintain basic conditions.

Example

A typical example of a basic buffer solution would be a mixture of ammonium hydroxide (NH_4OH) and ammonium chloride (NH_4Cl) [pH = 9.25].

Mechanism of Buffer Solution

To understand the mechanism of buffer action, let us take an example of an acidic buffer solution of acetic acid (CH_3COOH) and sodium acetate (CH_3COONa).

In the aqueous medium, they dissociate as:

$$CH_3COONa\ (aq) \rightleftharpoons CH_3COO^-\ (aq) + Na^+\ (aq)\ [\text{Completely ionised}]$$

$$CH_3COOH\ (aq) \rightleftharpoons CH_3COO^-\ (aq) + H^+\ (aq)\ [\text{Partially ionised}]$$

When a small quantity of strong acid, like HCl, is added to the buffer solution, the additional hydrogen ions combine with the conjugate base (CH_3COO^-) as follows:

$$H^+\ (aq) + CH_3COO^-\ (aq) \leftrightarrow CH_3COOH\ (aq)$$

Since the extra H^+ ions get consumed, the pH of the resulting solution remains constant. As CH_3COOH is a weak acid and its ions have a strong tendency to form non-ionized CH_3COOH molecules, the reaction nearly goes to completion.

On the contrary, if a strong base like NaOH is added, the additional OH^- ions get neutralized:

$$CH_3COOH\ (aq) + OH^-\ (aq) \rightarrow CH_3COO^-\ (aq) + H_2O\ (l)$$

Also, the added OH^- ion reacts with the H^+ ion to produce water. As a result, the added OH^- ions get removed, and the acid equilibrium shifts to the right to replace the used-up H^+ ions. Thus, the pH changes negligibly.

Uses and Applications of Buffer Solution in Daily Life

Buffer solutions have various uses and applications in everyday life. Some of them are as follows:

- Maintenance of Life: As most of the biochemical processes in our bodies work within a relatively narrow pH range, the body uses different buffers, such as carbonate and bicarbonate buffer, to maintain a constant pH close to 7.4.
- Biochemical Assays: As enzymes act on a particular pH, buffer solutions keep the pH value constant during an enzyme assay.

- Shampoos: Buffers such as citric acid and sodium hydroxide are used in shampoos to balance out the alkalinity that would otherwise burn our scalp. These buffers counteract the alkalinity of the detergents present in the shampoo.
- Baby Lotions: Baby lotions are buffered to a pH of about 6 to hinder the growth of bacteria within the diaper and help prevent diaper rash.
- Brewing Industry: Buffer solutions are added before fermentation begins to make sure the solution is not too acidic and prevent the product from spoiling.
- Textile Industry: Many dyeing processes use buffers to maintain the correct pH for various dyes.
- Laundry Detergents: Many laundry detergents use buffers to prevent their natural ingredients from breaking down.
- Contact Lens: Buffer solutions are used in contact lens solutions to ensure the pH level of the fluid remains compatible with that of the eye.

PREPARATION OF PHOSPHATE BUFFER

Aim: To prepare phosphate buffer of specific pH and molarity of 0.2M

Introduction:

A buffer is defined as a chemical system that resist change in pH upon the addition of small amount of hydrogen or hydroxyl ions.

Buffers are mixtures of a conjugate acid and conjugate base.

An acidic buffer contains weak acid and its salt (acetic acid and sodium acetate. While a basic buffer contains a weak base and its salt.

Depending upon the type of buffers, each conjugate acid base pair has a characteristic pH buffering zone.

Eg. Phosphate buffer has an effective buffering zone in between 5.9 to 7.9 on the other hand ammonium ion and ammonia pair have effective buffering zone between pH 8.3 to 10.3.

Principle:

A **phosphate buffer** is based on the equilibrium between **dihydrogen phosphate ($H_2PO_4^-$)** and **hydrogen phosphate (HPO_4^{2-})** ions. These two forms act as a **conjugate acid–base pair**.

Phosphate buffer maintains a stable pH due to the equilibrium:

$$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$$

According to the **Henderson–Hasselbalch equation**, the pH of a phosphate buffer is controlled by adjusting the **ratio** of the acid form (NaH_2PO_4) to the base form (Na_2HPO_4):

$$pH = pKa + \log([HPO42^-]/[H2PO4^-])$$

The principle of this experiment is to prepare two stock solutions (acidic and basic phosphate) and mix them in appropriate proportions to obtain the desired pH.

Requirements

Glassware (beakers and bottles),

Measuring cylinders,

Tips and pipettes,

pH paper, pH meter,

Magnetic stirrer,

Weighing balance.

Monobasic sodium phosphate (NaH_2PO_4)

Dibasic sodium phosphate (Na_2HPO_4)

Dilute HCl and NaOH solution

Procedure

Prepare phosphate buffer of pH-7.8, pKa-6.8, Molarity-0.2M

According to Henderson-Hasselbalch equation, the pH of the acidic buffer is:

$$pH = pK_a + \log([Salt]/[Acid])$$

Putting the values,

$$pH = 6.8 + \log([salt]/[Acid])$$

$$7.8 = 6.8 + \log([salt]/[Acid])$$

$$\log([Salt]/[Acid]) = 7.8 – 6.8 = 1$$

$$[Salt]/[Acid]=\text{Antilog } 1$$

$$[Salt]/[Acid] = 10^1 = 10$$

$$[Salt] = 10\ [Acid]$$

From the total concentration:

$$[Salt] + [Acid] = 0.2\ M$$

$$10\ [Acid]+ [Acid] = 0.2$$

$$11\ [Acid] = 0.2$$

$$[Acid] = 0.2/11 = 0.018M$$

This is the amount of Monobasic sodium phosphate (NaH_2PO_4)

Now, calculating the amount of Dibasic sodium phosphate (Na_2HPO_4)

[Salt] = 0.2 – 0.018 = 0.182M

Hence:

Monobasic sodium phosphate (NaH_2PO_4): 0.018 Moles

Dibasic sodium phosphate (Na_2HPO_4): 0.182 Moles

*Now mixing of these two ingredients can be done by two ways one using the amount in grams and mixing in specific quantity of distilled water and second is to measure the quantities in millilitre and mix.

Method 1:

Acid: Monobasic sodium phosphate (NaH_2PO_4): 0.018 Moles

Molecular weight of NaH_2PO_4 is 156.01

156.01X0.018 moles= 2.8gms

Salt: Dibasic sodium phosphate (Na_2HPO_4): 0.182 Moles

Molecular weight of Na_2HPO_4 is 141.96

141.96X 0.182= 25.84gms

Therefore, to make phosphate buffer of pH 7.8 and molarity 0.2M, add 2.8gms of NaH_2PO_4 and 25.84gms of Na_2HPO_4 to 1000ml of distilled water.

Method 2:

Acid: Monobasic sodium phosphate (NaH_2PO_4): 0.018 Moles

0.2 moles in 1000ml hence for 0.018 moles we need to take 90ml from 0.2M solution.

Salt: Dibasic sodium phosphate (Na_2HPO_4): 0.182 Moles

0.2 moles in 1000ml hence for 0.182 moles we need to take 910ml from 0.2M solution.

Hence mix 90ml of 0.2M NaH_2PO_4 and 910ml of 0.2M Na_2HPO_4 to make 1000ml of phosphate buffer of pH 7.8 and molarity 0.2M.

Result:

Phosphate buffer is prepared by mixing the appropriate amounts of acid and base (in grams or millilitres)

PREPARATION OF TRIS HCl BUFFER

Aim: Prepare Tris HCl buffer of specific pH and specific molarity.

Introduction:

A buffer is defined as a chemical system that resist change in pH upon the addition of small amount of hydrogen or hydroxyl ions.

Buffers are mixtures of a conjugate acid and conjugate base.

An acidic buffer contains weak acid and its salt (acetic acid and sodium acetate. While a basic buffer contains a weak base and its salt.

Depending upon the type of buffers, each conjugate acid base pair has a characteristic pH buffering zone.

Eg. Phosphate buffer has an effective buffering zone in between 5.9 to 7.9 on the other hand ammonium ion and ammonia pair have effective buffering zone between pH 8.3 to 10.3.

Principle:

Tris (tris(hydroxymethyl)aminomethane) is a weak base with a pKa of ~8.1 at 25°C, making it effective for buffers in the pH 7–9 range.

Tris buffer is based on the equilibrium between **protonated Tris (Tris-H^+)** and **unprotonated Tris (Tris-base)**:

$$\text{Tris–H+} \rightleftharpoons \text{Tris+H+}$$

Using the Henderson–Hasselbalch equation:

$$pH = pKa + \log[\text{Tris}]/[\text{Tris–H+}])$$

The buffer is prepared by dissolving **Tris base** in water and adjusting the pH to the required value using **HCl**.

Requirements:

Glassware (beakers and bottles),

Measuring cylinders,

Tips and pipettes,

pH paper, pH meter,

Magnetic stirrer,

Weighing balance.

Tris HCl

Tris Base

Dilute HCl and NaOH solution

Procedure

Prepare Tris buffer of pH-9.0, pKa-8.1, Molarity-0.05 M

According to Henderson-Hasselbalch equation, the pH of the acidic buffer is:

$$pH = pK_a + \log([Salt]/[Acid])$$

Putting the values,

$$pH = 8.1 + \log([salt]/[Acid])$$

$$9 = 8.1 + \log([salt]/[Acid])$$

$$\log([Salt]/[Acid]) = 9.0 – 8.1 = 0.9$$

$$[Salt]/[Acid]=\text{Antilog } 0.9$$

$$[Salt]/[Acid] = 10^{(0.9)} = 7.94$$

$$[Salt] = 7.94\ [Acid]$$

From the total concentration:

$$[Salt] + [Acid] = 0.05M$$

$$7.94\ [Acid]+ [Acid] = 0.05$$

$$8.94\ [Acid] = 0.05$$

$$[Acid] = 0.05/8.94 = 0.0056M$$

This is the amount of Tris HCl

Now, calculating the amount of Tris Base

[Salt] = 0.05 – 0.0056 = 0.044M

Hence:

Tris HCl: 0.0056 Moles

Tris Base: 0.044 Moles

*Now mixing of these two ingredients can be done by two ways one using the amount in grams and mixing in specific quantity of distilled water and second is to measure the quantities in millilitre and mix.

Method 1:

Acid: Tris HCl: 0.0056 Moles

Molecular weight of Tris HCl is 157.6

157.6X0.0056 moles= 0.88gms

Salt: Tris Base: 0.044 Moles

Molecular weight of Tris Base is 121.14

121.14X 0.044= 5.33gms

Therefore, to make Tris buffer of pH 9.0 and molarity 0.05M, add 0.88gms of Tris HCl and 5.33gms of Tris Base to 1000ml of distilled water.

Method 2:

Acid: Tris HCl: 0.0056 Moles

0.05 moles in 1000ml hence for 0.0056 moles we need to take 112ml from 0.05M solution of Tris HCl

Salt: Tris Base: 0.044 Moles

0.05 moles in 1000ml hence for 0.044 moles we need to take 880ml from 0.05M solution.

Hence mix 112ml of 0.05M Tris HCl and 880ml of 0.05M Tris Base to make 1000ml of Tris buffer of pH 9.0 and molarity 0.05M.

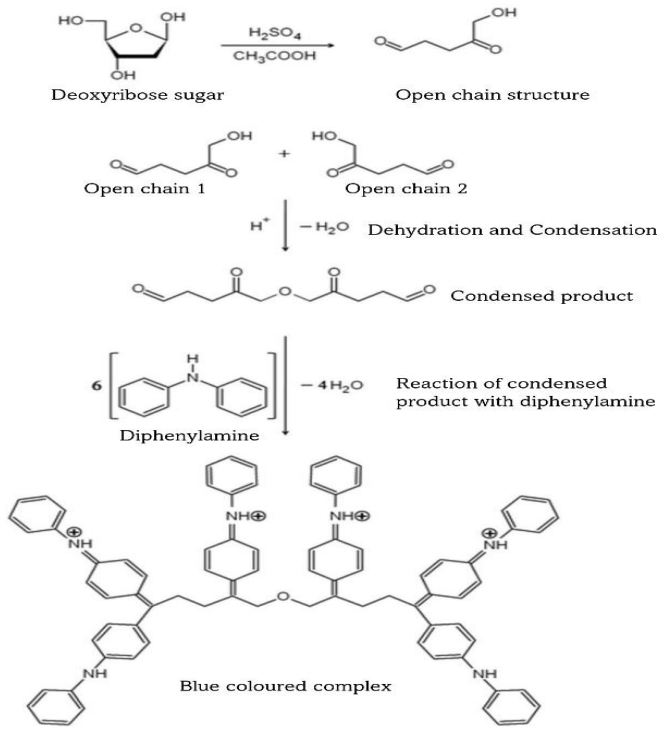
Result: Tris buffer is prepared by mixing Tris HCl and Tris base in appropriate amount.

ESTIMATION OF DNA BY DIPHENYLAMINE METHOD

Aim: To estimate DNA by Diphenylamine method.

Introduction: DNA (Deoxyribonucleic acid) is the hereditary material present in all living organisms, and accurate estimation of its quantity is essential in many biochemical, genetic, and molecular biology studies. One of the classical and reliable methods for DNA estimation is the Diphenylamine (DPA) method, a colorimetric assay based on the reaction between diphenylamine and the deoxyribose present in DNA under acidic conditions. This reaction forms a blue-colored complex, the intensity of which is directly proportional to the concentration of DNA in the sample. By measuring the absorbance of this colored complex at 595 nm, the amount of DNA present can be quantitatively determined. This method is simple, sensitive, and widely used in laboratories for analyzing DNA in various biological samples.

Principle: The assay is a colorimetric procedure for measuring the deoxyribose moiety of DNA. Colour is produced by the reaction of diphenylamine with deoxyribose. The assay is relatively specific, although RNA in high concentrations or sucrose should be absent. In acidic conditions, the ring structure of the deoxyribose in DNA opens and due to the breakage of glycosidic bonds between sugar and purine, depurination occurs. Two open chains of sugar moiety condense to form β-hydroxylevulinic aldehyde, a dehydration product which reacts with diphenylamine to give a blue coloured complex with sharp absorption maximum at 595 nm.



Requirements

1. Test tubes
2. Boiling water bath
3. Pipettes
4. Colorimeter

Preparation of Reagents

1. **DNA stock solution** – 0.05 g of DNA was dissolved in 0.5 ml of 0.1 N NaOH and made up to 100 ml with distilled water.
2. **DPA reagent** – 2 g of DPA was dissolved in 200 ml of glacial acetic acid and 5 ml of conc. H_2SO_4 was added.

Procedure

Preparation of Standard Graph:

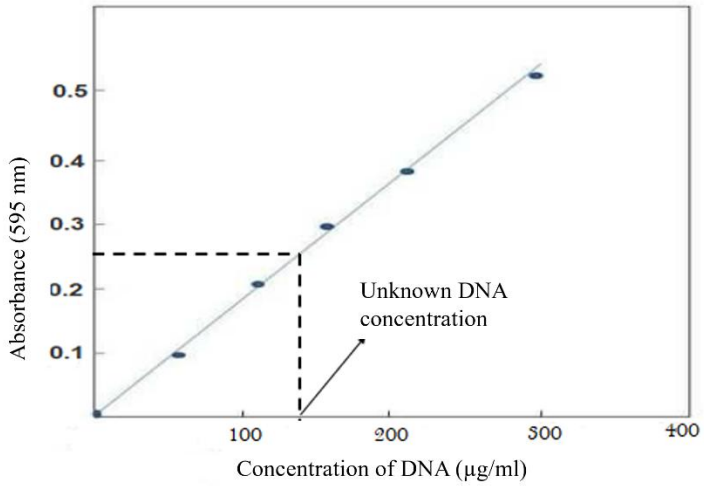
- Different aliquots of standard DNA solution ranging from 0.2 to 1 ml were pipetted out into different test tubes. The volume of each test tube was made up to 1 ml with distilled water.
- 4 ml of DPA reagent was added to all the tubes and boiled in a boiling water bath for 10–15 mins and cooled.
- The absorbance of all the solutions were measured against the blank at 595 nm.
- DNA was isolated from the biological sample cauliflower by SDS method and processed similarly.
- The OD value is measured in the colorimeter.

Stock concentration = 500 µg/ml

Sl. No.	Vol. of stock (ml)	Vol. of of D/W (ml)	Con. Of DNA (µg/ml)	DPA reagent (ml)		Absorbance at 595 nm
1 (Blank)	0	1	0	4	Incubate the tubes in boiling water bath for 10/15 mins	
2	0.2	0.8	100			
3	0.4	0.6	200			
4	0.6	0.4	300			
5	0.8	0.2	400			
6	1.0	0	500			
7	1 ml unknown	0	?			

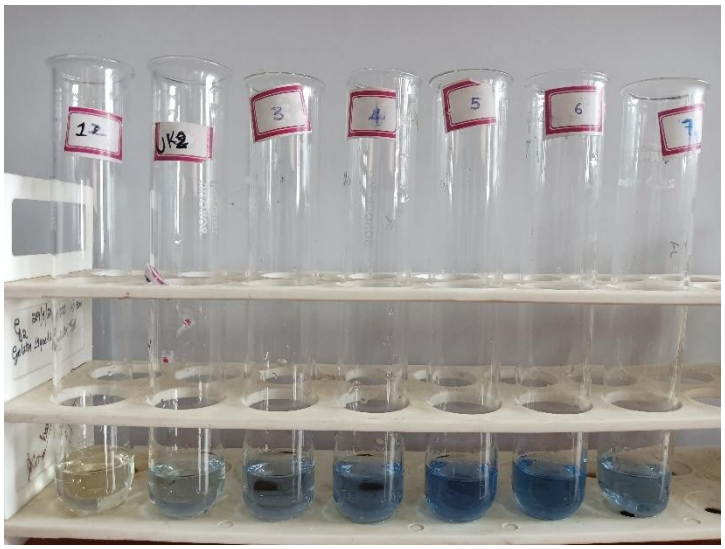
Paste Graph Here

Standard Graph:



Result:

The amount of DNA present in the given sample is found to be _____**µg/ml**.



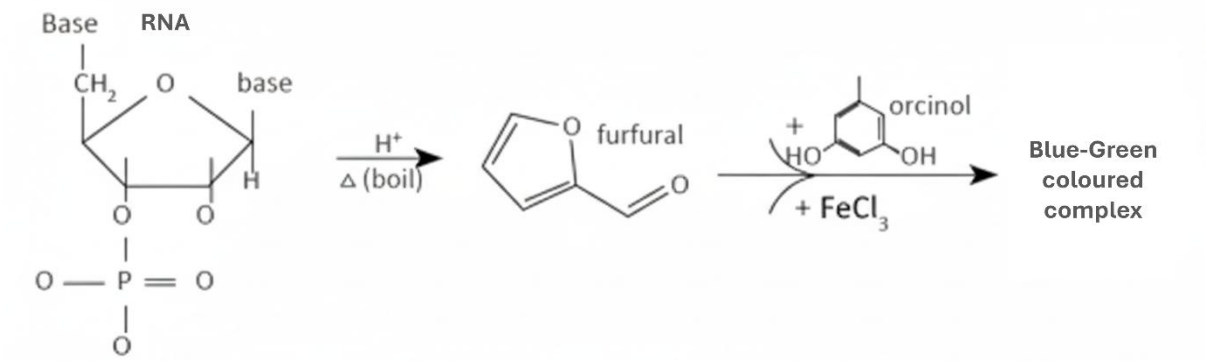
Estimation of DNA by DPA method

ESTIMATION OF RNA BY ORCINOL METHOD

Aim: To estimate RNA by Orcinol method.

Introduction: The Orcinol Method, often referred to as the Bial's reaction, is a widely utilized and sensitive colorimetric assay specifically designed for the quantitative estimation of Ribonucleic Acid (RNA) in biological samples. The core of this technique lies in the chemical distinction between the pentose sugar of RNA (ribose) and the deoxyribose of DNA. Under hot, concentrated acid conditions (hydrochloric acid), the ribose components of RNA are first depurinated and then dehydrated to form furfural. This furfural intermediate subsequently reacts with the key reagent, Orcinol, using ferric chloride (Fe^{3+}) as a catalyst. The successful condensation between furfural and orcinol produces a stable blue-green coloured complex, which exhibits a maximum absorbance at approximately 660–665 nm. By measuring the intensity of this colour using a spectrophotometer, the concentration of RNA in an unknown sample can be accurately determined, as the colour intensity is directly proportional to the amount of ribose sugar present.

Principle: Estimation of RNA using Orcinol is a sensitive colorimetric method for purine bound ribose estimation. In conc. HCl medium, RNA gets depurinated. The resulting ribose phosphates are dephosphorylated and dehydrated to produce the furfural. Furfural then reacts with Orcinol where Fe^{3+} acts as a catalyst. The reaction yields a blue-green coloured condensation product, with an absorption maximum at 660 nm. The colour intensity is measured at 665 nm which is directly proportional to the concentration of RNA. In RNA, only the ribose of purine nucleotide react, so that the value represents half of the total ribose sugar present.



Requirements

Test tubes

Boiling water bath

Colorimeter

Pipettes

Centrifuge

Balance (weighing balance).

REAGENTS REQUIRED:

RNA stock

Orcinol reagent

Alcoholic orcinol

Procedure

(i) Preparation of reagents

RNA stock – 0.05 g of RNA is dissolved in 1 ml of 0.1 N NaOH, and the volume is made up to 100 ml with distilled water in a standard flask.

Orcinol reagent – 0.04 g of orcinol and 0.04 g of $FeCl_3$ in 200 ml of conc. HCl.

Alcoholic orcinol – 0.9 g of orcinol in 15 ml of 95% ethanol.

(2) Preparation of Standard Graph:

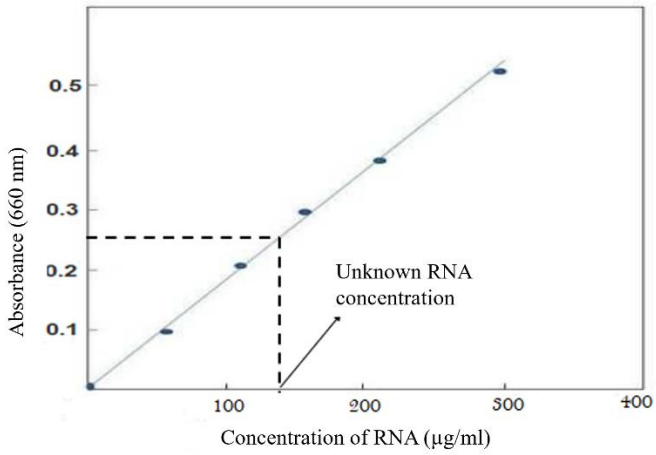
- Different aliquots of standard RNA solution ranging from 0.2 to 1 ml were pipetted out into different test tubes.
- The volume of each test tube was made up to 1 ml with distilled water.
- Six millilitres of orcinol acid was added to all the test tubes including the unknown, and 0.4 ml of alcoholic orcinol was added.
- The tubes were incubated in a boiling water bath for 10–15 minutes and cooled.
- The absorbance of all the solutions was measured against the blank at 665 nm.
- The given unknown RNA sample (from standard) was similarly processed and read.

Stock RNA-500 µg/ml

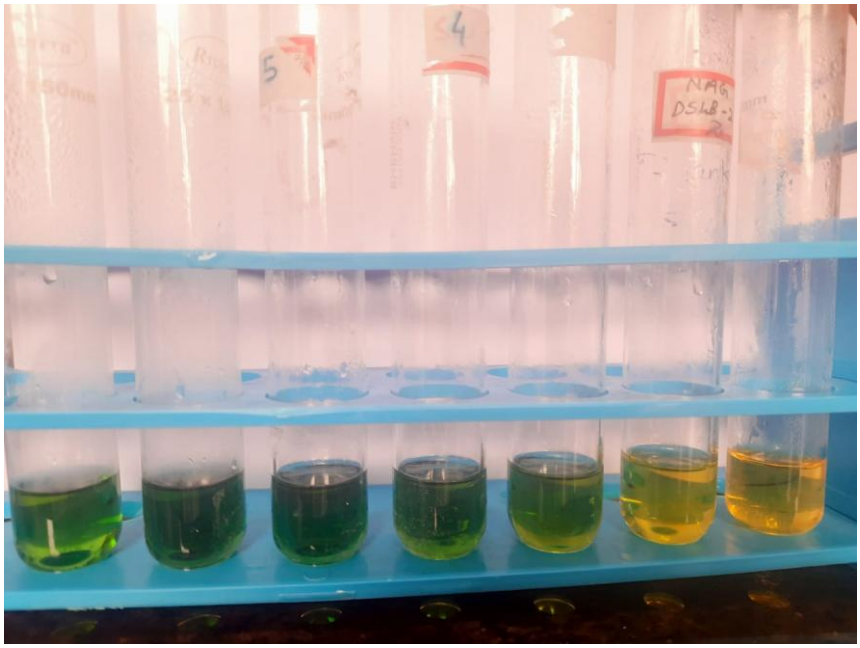
Sl. No.	Vol. of stock (ml)	Vol. of of D/W (ml)	Con. Of RNA (µg/ml)	Volume of orcinol acid (ml)	Volume of 0.6 % alcoholic orcinol (ml)		Absorbance at 660 nm
1 (Blank)	0	1	0	6	0.4	Incubate the tubes in boiling water bath for 10/15 mins	
2	0.2	0.8	100				
3	0.4	0.6	200				
4	0.6	0.4	300				
5	0.8	0.2	400				
6	1.0	0	500				
7	1 ml unknown	0	?				

Paste Graph Here

Standard Graph:



Result: The concentration of given unknown RNA sample was found to be _____µg/ml.



Estimation of RNA by orcinol method

EXTRACTION OF GENOMIC DNA FROM BACTERIA AND SEPERATION BY GEL ELECTROPHORESIS

AIM: To extract genomic DNA from bacteria and separation by gel electrophoresis.

INTRODUCTION: Bacterial genomic DNA extraction is a fundamental technique in molecular biology that serves as a prerequisite for nearly all downstream genetic analyses, such as Polymerase Chain Reaction (PCR), whole-genome sequencing, and gene cloning. The goal is to obtain highly purified, intact DNA that is free from contaminants like proteins, RNA, lipids, and cellular debris. The general method involves a series of chemical and physical steps tailored to overcome the structural challenges presented by the bacterial cell wall and membrane. Agarose gel electrophoresis is method for separation (by size), quantifying, purification of nucleic acids fragments mixture, and analysis of DNA restriction fragments. It is one of the most widely-used techniques in biochemistry and molecular biology. Agarose is a liner polymer composed of alternative residues of D-galactose and 3,6-anhydro-L-galactopyranose joined by α (1→3) and β (1→4) glycosidic linkages. Agarose and acrylamide matrices are used to separate DNA by gel electrophoresis. The choice of gel matrices and gel concentration depends on the size of nuclear acid molecules, as the concentration of the agarose or acrylamide determine the pores size:

w/v % Gel type	Size of DNA fragments (Kb = 1000 bp)
0.5 %	1 kb to 30 kb
0.7 %	800 bp to 12 kb
1.0 %	500 bp to 10 kb
1.2 %	400 bp to 7 kb
1.5 %	200 bp to 3 kb
2.0 %	50 bp to 2 kb

Under physiological conditions, DNA is a negatively charged molecule due to the presence of phosphate groups in the backbone. Therefore, in aqueous media, under the influence of an electrical field, DNA molecules will move through an agarose matrix towards the positively charged anode, at a rate that is inversely proportional to the molecular weight. The electrophoretic migration rate of DNA through agarose gel depends on the following: size of DNA molecules, concentration of agarose gel, voltage applied, conformation of DNA, and the buffer used for electrophoresis.

Successful extraction and visualization ensure that subsequent enzymatic reactions are efficient and reliable, which is critical for accurate research outcomes and clinical diagnostics.

PRINCIPLE: The principle of this bacterial DNA extraction protocol centers on sequential biochemical and physical steps designed to isolate high-purity genomic DNA. It begins with Cell Lysis, where the bacterial cell wall and membrane are disrupted using a lysis buffer containing a detergent like SDS (to dissolve lipids and denature proteins) and EDTA (to inhibit DNA-degrading enzymes, DNases, by chelating Mg^{2+} cofactors). Next, Contaminant Removal is achieved by adding high salt (NaCl) and chloroform, followed by centrifugation. This process selectively precipitates and separates denatured proteins, lipids, and cellular debris into an interface layer, leaving the intact, desired DNA dissolved in the upper aqueous phase. Finally, the DNA is Precipitated by adding cold absolute ethanol, a step where nucleic acids become insoluble and pellet able, allowing for its physical recovery and subsequent washing and rehydration in an appropriate buffer. Nucleic acids are separated by applying an electric field, so these negatively charged molecules will move through an agarose matrix towards the anode, and the biomolecules are separated by size in the agarose gel matrix, where the distance travelled by a DNA molecule is inversely correlated with its size. DNA is visualized in gel electrophoresis by staining the gel with a fluorescent intercalating dye, such as Ethidium Bromide (EtBr), which binds tightly to the DNA fragments, causing them to fluoresce brightly (typically a pale pink or orange color) when illuminated with ultraviolet (UV) light.

REAGENTS REQUIRED:

1. Lysis Buffer

Tris acetate, 40 mM, pH 7.8

Sodium acetate, 20 mM

EDTA, 1 mM

SDS, 1%

2. Chloroform
3. NaCl, 5 M
4. Ethanol, 70%
5. TE Buffer
6. Agarose

7. 1x TAE Buffer
8. EtBr

PROCEDURE:

Extraction of bacterial DNA

1. Grow bacterial colony in 50 ml Nutrient broth.
2. Harvest the fungal/bacterial mat into a muslin cloth and macerate it with a mortar and pestle using liquid nitrogen.
3. Transfer 1.5 ml of culture and spin at 8000 rpm, 4°C, for 10 min.
4. Resuspend the pellet in 200 µl of lysis buffer by vigorous pipetting.
5. Add 66 µl of 5 M NaCl solution to remove most protein and cell debris.
   OR
   Add 80 µl of 4 M NaCl (two phases form on gentle pipetting).
6. Centrifuge at 12000 rpm for 10 min at 4°C.
7. Transfer the clear supernatant into a new vial.
8. Add equal volume of chloroform.
9. Shake the tube gently at least 50 times until a milky solution is formed (Tap bottom of the tube gently).
10. Centrifuge at 12000 rpm for 3 min.
11. Transfer the supernatant (upper aqueous phase) to another vial (Two layers form with debris at the interface).
12. Add equal volume of cold absolute ethanol to precipitate DNA. Incubate at 4°C for at least 30 min or overnight.
13. Centrifuge at 12000 rpm at 4°C for 20 min.
14. Wash DNA with 70% ethanol and centrifuge at 12000 rpm for 20 min.
15. Air-dry DNA for about 15 min and dissolve in 50 µl TE buffer or double-distilled water.
16. Macerate the pellet and centrifuge @ 13000 rpm for 15 min.
17. Expected DNA yield: ~50–150 µg per 10^9 cells.
18. Remove the supernatant carefully and check the concentration using a Photo spectrophotometer.

Pouring a Standard 1% Agarose Gel

1. Measure 1 g of agarose.
2. Mix the agarose powder with 100 mL of 1× TAE buffer in a microwavable flask.
3. Microwave for 1–3 minutes until the agarose is completely dissolved (avoid overboiling).
4. Allow the agarose solution to cool to approximately 50°C (comfortable to touch).
5. Add ethidium bromide to a final concentration of 0.2–0.5 µg/mL.
6. Pour the agarose into a gel tray with the well comb in place.
7. Allow the gel to solidify at 4°C for 10–15 min or at room temperature for 20–30 min.

Loading Samples and Running the Agarose Gel

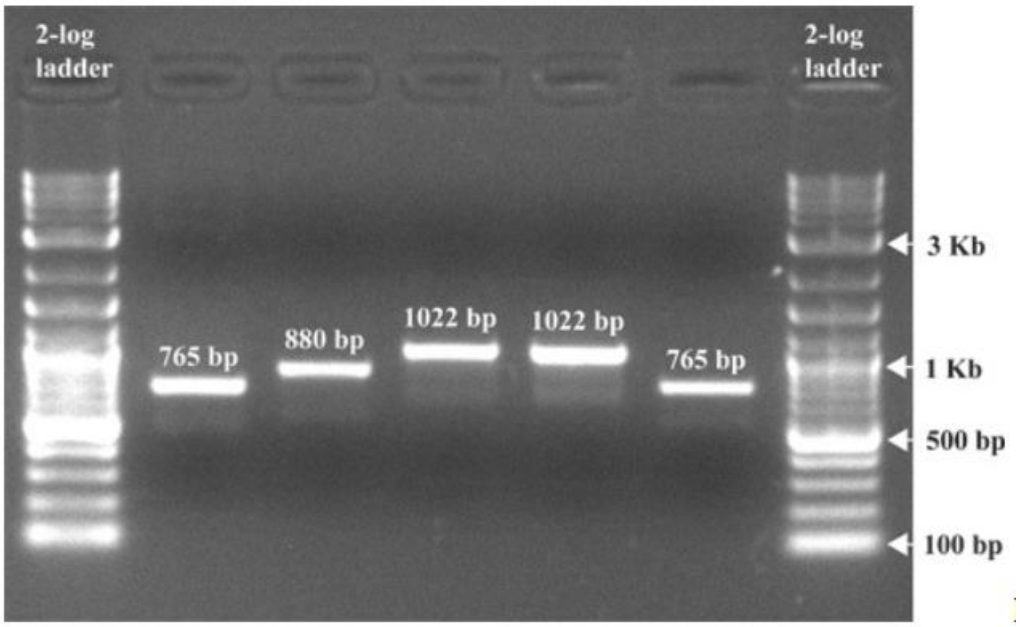
1. Add 5 µL loading buffer per 25 µL DNA sample and mix well.
2. Once the gel has solidified, place it into the gel electrophoresis unit.
3. Fill the gel box with 1× TAE (or TBE) until the gel is fully submerged.
4. Carefully load a molecular weight ladder into the first lane.
5. Load the DNA samples into the remaining wells.
6. Run the gel at 80–150 V until the tracking dye migrates 75–80% of the gel length (approximately 1–1.5 hours).
7. Turn off the power, disconnect the electrodes, and remove the gel from the gel box.
8. Visualize the DNA bands using a UV transilluminator (wear appropriate PPE).

Analyzing the Gel

1. Compare sample DNA bands to the DNA ladder to estimate fragment sizes.

Results:

Distinct DNA bands were observed under UV illumination, indicating successful separation and visualization of DNA fragments by agarose gel electrophoresis.



An image of a gel post electrophoresis. EtBr was added to the gel before electrophoresis to a final concentration of 0.5 µg/ml, followed by separation at 100 V for 1 hour. The gel was exposed to uv light and the picture taken with a gel documentation system (Lee et al., 2012).

ISOLATION OF PLASMID DNA FROM BACTERIA AND SEPERATION BY GEL ELECTROPHORESIS

AIM: To isolate plasmid DNA from bacteria and separation by gel electrophoresis.

INTRODUCTION: Plasmids are extra chromosomal DNA that replicate independently of the bacterial chromosome. They are normally covalently closed, circular, super-coiled molecules. They carry genes encoding functions (such as antibiotic resistance) which may be useful to the cell but are not essential for normal cellular activities. In the recombinant DNA technology plasmid DNA are used as vectors for carrying any foreign DNA. They can replicate with in host cell and possess phenotypic traits by which they can be detected. Genetic engineering makes use of recombinant DNA technology to fuse genes with plasmid vectors and clone them in the host cells. This way large number of isolated genes and their products can be synthesized and used for industrial, therapeutic and agricultural purposes.

PRINCIPLE:

ALKALINE LYSIS

The universal method for plasmid DNA extraction was invented by Bimboim and Doly in 1979. The lysis buffer contains NaOH and SDS. The purpose of which is to completely denature the plasmid and genomic DNA i.e., separate the DNA into single strands. It is critical that this step is performed quickly because too long in the denaturing conditions of this solution, may result in irreversibly denatured plasmid at the end. Next, the sample is neutralized in a potassium acetate solution to renature the plasmid and this is the key to the plasmid separation from the genomic DNA.

ROLE OF DIFFERENT CHEMICALS IN PLASMID DNA

1. Cell Growth and Harvesting

   Bacteria will be grown in a media to achieve sufficient growth. Then cells are pelletted by centrifugation for removal from the growth medium.
2. **Re-suspension**

   Pellet is then suspended in a solution (Soln I) containing Tris, EDTA, Glucose and RNase A. Divalent cations Mg^{2+}, $Ca^{2+}$are essential for DNase activity and the integrity of the bacterial cell wall. EDTA chelates divalent cations in the solution preventing DNase from damaging the plasmid and helps by destabilizing the cell wall. Glucose maintains the

osmotic pressure so that the cells don't burst and RNase A is included to degrade cellular RNA when the cells are lysed.

3. **Lysis**

   The lysis buffer solution-II contains Sodium Hydroxide and detergent SDS. SDS is present to solubilize cell membrane, NaOH helps to break down the cell wall, also it disrupts the hydrogen bonding between dsDNA and converts it to ssDNA [Genomic and plasmid]. This process is called alkaline lysis. SDS also denatures proteins in the cells, which helps with the separation of proteins from the plasmid later in the process. Do not carry out vigorous mixing because genomic DNA will be broken down.

4. **Neutralization**

   Addition of solution-III potassium acetate decreases the alkalinity of the mixture. Under these conditions, the hydrogen bonding between bases of ssDNA can be re–established to form dsDNA. It is easy for small circular plasmid DNA to renature, but it is impossible to properly anneal those huge genomic DNA structures.

   While ssDNA of plasmid can dissolve easily in the solution, the ss genomic DNA, SDS and denatured cellular proteins stick together through hydrophobic interactions to form white precipitate, which can be separated by centrifugation.

5. **Cleaning And Concentration**

   This can be done by Phenol–Chloroform step and precipitation by ethanol.

6. **Phenol – Chloroform**

   This removes all proteins when added. The mixture doesn't mix with water and so, two layers are formed – upper aqueous phase and lower organic phase. DNA is hydrophilic, so it will remain in the upper aqueous layer. On the other hand, proteins which contain polar and non-polar groups (hydrophilic and hydrophobic groups which are retained by folding) will be comfortable to be in organic phase – so they settle in lower organic phase.

7. **Role Of Salt And Ethanol In Precipitation**

   Role of salt and ethanol in precipitation is to neutralize the charges in the sugar-phosphate backbone. Sodium acetate – Na^+ and CH_3COO^- Na^+ will neutralize PO_3^- groups on DNA and make molecules less hydrophilic and less soluble in water.

   Electrostatic attraction between Na^+ and PO_3^- is affected by dielectric constant of the solution. Water has high dielectric constant which makes it difficult for Na^+ and PO_3^- to interact and shield its charge and makes the DNA less hydrophilic and causing it to drop out of the solution.

REAGENTS REQUIRED:

- Alkaline lysis Solution I
  - 50 mM Glucose
  - 10 mM Tris
  - 10 mM EDTA
  - pH of solution – 8

  [NOTE: Sterilize by autoclaving and store at 4°C]
- Alkaline lysis Solution II
  - 0.2 N NaOH
  - 1% SDS

  [NOTE: Prepare fresh and store at room temperature]
- Alkaline lysis Solution III
  - 3 M Potassium
  - 5 M Acetate
  - pH 5.4

  [NOTE: 5M potassium acetate: 60 mL GAA – 11.5 mL and distilled water 38.5 mL.

  Resultant solution is 3M with respect to Potassium and 5M with respect to Acetate. Stored at 4°C]
- Phenol : Chloroform (1:1)
- T.E. Buffer
  - 10 mM Tris
  - 1 mM EDTA
  - pH – 8
- Chilled Ethanol

PROCEDURE:

1.5 mL culture

↓

Centrifuge at 4°C at 10,000 rpm for 10 minutes

↓

Discard the supernatant

↓

Add 100 µL of Alkaline lysis solution I (ice cold). Mix by vortexing the vial once for 10–15 min

↓

Add 200 µL of Alkaline lysis solution II. Mix by inverting 5 times properly

↓

Keep in ice for 5 minutes

↓

Add 200 µL of Alkaline lysis solution III (ice cold), mix properly and gently

↓

Keep in ice for 5 minutes and centrifuge at 4°C, 10,000 rpm for 5–10 minutes

↓

Transfer the supernatant to a new tube and add equal volume of 1:1 Phenol–Chloroform

↓

Mix tubes properly and centrifuge at 4°C for 2 minutes at 10,000 rpm

↓

Transfer the upper aqueous layer to a new tube and add double the volume of chilled ethanol

↓

Keep overnight at 4°C and centrifuge at 10,000 rpm for 5 minutes

↓

Discard the supernatant and dissolve the pellet in 50 µL of TE buffer

↓

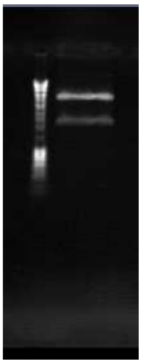
Vortex properly and add gel loading buffer

↓

Detect using Agarose Gel Electrophoresis

RESULT

The plasmid DNA is isolated and separated by Agarose Gel Electrophoresis.



Agarose Gel Electrophoresis Showing Successful Isolation of Plasmid DNA

INDUCTION AND ASSAY OF β-GALACTOSIDASE ACTIVITY IN *E. COLI*

Aim: To Induce *E.coli* for production of β-Galactosidase enzyme and calculate the activity of the enzyme

Introduction: *Escherichia coli* (*E. coli*) can produce the enzyme β-galactosidase which breaks lactose into galactose and glucose. However, the gene for β-galactosidase is normally switched off, except in the presence of lactose/inducer. In this procedure, a sample of *E. coli* is treated with lactose, and then the β-galactosidase activity of this sample and an untreated sample are compared. ONPG (ortho-nitrophenyl-p-D-galactoside) is used as a substrate for the enzyme action which produces galactose and a compound which is yellow in alkaline conditions. The intensity of the yellow colour produced is a qualitative indicator or quantitative measure (with a colorimeter) of the β-galactosidase activity. The procedure indicates that the gene which produces β-galactosidase in *E. coli* is induced or 'switched on' by the presence of inducer**.**

Principle:

The induction and assay of β-galactosidase activity in *E. coli* is based on the regulation of the *lac* operon, where the enzyme β-galactosidase (lacZ product) is normally repressed by the Lac I repressor. When a gratuitous inducer such as IPTG (or the natural inducer allolactose) is added, it binds to Lac I and releases the repression, allowing transcription of the *lac* operon and leading to increased synthesis of β-galactosidase in the cells. The activity of the induced enzyme is then measured using chromogenic substrates such as ONPG, which is cleaved by β-galactosidase to produce o-nitrophenol, a yellow compound whose intensity can be quantified spectrophotometrically at 420 nm. The amount of coloured product formed is directly proportional to the enzyme activity present in the sample. Cells are permeabilized to allow substrate entry, and enzyme activity is usually normalized to cell density to ensure accurate comparison. Thus, the principle relies on inducer-dependent de-repression of the *lac* operon and colorimetric quantification of the enzymatic product.

Reagents:

LB media with Lactose/IPTG

LB media without Lactose/IPTG

Z buffer, per 50 mL:

- O.80g $Na_2HPO_4.7H_2O$ (0.06M)
- 0.28g $NaH_2PO_4.H_2O$ (0.04M)
- 0.5 mL 1M KCl (0.01M)
- 0.05 mL 1M $MgSO_4$ (0.001M)
- 0.135 mL b -mercaptoethanol (BME) (0.05M)
- bring to approximately 40 mL with H_2O, dissolve all the salts
- adjust the pH to 7.0
- use a graduated cylinder to bring the buffer to 50 mL and store at 4^0C.

Note: BME is added to the reaction buffer to stabilize the β-galactosidase enzyme. The important part of BME is a reactive thiol (SH group). Thiols react with oxygen in the air and oxidize (inactivate) over time. Therefore, try not to make much more Z buffer than you will use in a few days. Store the unused portion at 4^0C.

ONPG should be dissolved fresh each day. Dissolve 1.5X as much as you think you will need, because you may have to repeat one or more of the assays *i.e.* for a different amount of time or with a different cell dilution. Dissolve the ONPG to a final concentration of 4mg/mL in 0.1M phosphate buffer pH 7.0.

Phosphate buffer, per 100 mL:

- 1.61g $Na_2HPO_4.7H_2O$ (0.06M)
- 0.55g $NaH_2PO_4.H_2O$ (0.04M)
- pH to 7.0

Procedure:

Day 1: Start overnight cultures in assay medium.

Negative control: cells lacking β-galactosidase, such as LT2;

positive control: cells with high enzyme activity.

Day 2: Dilute cells 1/100 in fresh medium, grow to mid-log.

Prepare solutions: Z buffer, phosphate buffer, ONPG.

Preparation of Cells:

Incubate cultures 20mins on ice to stop growth and wash:

- Pellet at least 2 mL of cells at 4^0C by centrifuging 10mins at 6,000 rpm.
- Pour off the supernatant.
- Resuspend the cell pellet in the same volume of chilled Z buffer.
- Measure the OD_{600} of the resuspended cells (blank against Z buffer)

Dilute cells in Z buffer to 1 mL. For most activities, 0.5 mL cells + 0.5 mL Z buffer will produce a desirable amount of yellow color in 1-2 hours. For higher levels (>500 Miller units), try 0.1 mL cells + 0.9 mL Z buffer.

Permeabilize the diluted cells by adding 100 µl chloroform and 50 µl 0.1% SDS (sodium dodecyl sulfate, sodium laurel sulfate).

Vortex; equilibrate the tubes 5min in a 28^0C water bath.

Assay:

- Start reaction by adding 0.2 mL substrate, *o*-nitrophenyl-β-D-galactoside (ONPG; 4 mg/mL)
  Vortex - Record the time of addition precisely with timer or stopwatch.
- Incubate the cells at 28^0C.
- Stop the reaction after sufficient yellow colour has developed by adding 0.5 mL 1M Na_2CO_3.
- Vortex. - Note time of addition precisely.
- Transfer 1 mL to an Eppendorf tube, spin 5min at maximum to remove debris and chloroform.
- Record the optical density at 420 nm and at 550 nm for each tube.
- Calculate the units of activity

The technique is given by J.H. Miller. In the assay described here, the cells are pelleted and resuspended in assay buffer (Z buffer) to eliminate error because of different carbon sources in the growth medium on the β-galactosidase enzyme activity.

β-Galactosidase can hydrolyze (cleave) β-D-galactosides. This enzyme facilitates growth on carbon sources like lactose by cleaving it into a molecule of glucose and a molecule of galactose which the cells can catabolize and grow on. In the assay described above, the substrate o-nitrophenyl-β-D-galactopyraniside (ONPG) is used in place of lactose. When the β-galactosidase cleaves ONPG, o-nitrophenol is released. This compound has a yellow colour, and absorbs 420 nm light. To measure β-galactosidase activity the accumulation of yellow colour (increase 420 nm absorbance)/minute is monitored.

What is sufficient yellow colour? To get the most accurate measure of activity, the absorbance at 420 nm (A_{420}) should range from 0.6 to 0.9. Readings as low as 0.1 and as high as 1.2 are acceptable. Tubes that have become as yellow as a tube of (unused) LB broth will probably be sufficiently yellow.

If the reading is too low, try the assay again with more cells or longer incubation time. When the element has inserted into a gene that is not expressed much, it will probably take hours to develop enough yellow colour. If your negative control starts to turn yellow (after several or more hours) it means that the substrate is beginning to auto-hydrolyse. The assay can be left overnight. The auto-hydrolysis is then accounted for by subtracting the A_{420} and A_{550} of the negative control from that of the tests before doing any further calculations.

If the reading is too high, try the assay again with fewer cells. Aim to stop the reaction after 15 minutes. For example, if in your first attempt, you added 0.5 mL of cells + 0.5 mL of Z buffer, and it was too yellow after 5 minutes, try adding 0.1 mL cells + 0.9 mL of Z buffer. Watch the tube carefully.

Adding the 1 M Na_2CO_3 stops the reaction by raising the pH of the solution to 11. At this pH the enzyme is not active.

The reading at 420 nm is a combination of absorbance by o-nitrophenol and light scattering by cell debris. Abs_{550} is the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420nm. There is no absorbance from o-nitrophenol at this wavelength. The light scattering at 420 nm is proportional to that at 550 nm:

light scattering at 420 nm = 1.75 x OD_{550}

Use the following equation to calculate units of enzyme activity:

Miller Units = 1000 x [(OD_{420} - 1.75 x OD_{550})] / (T x V x OD_{600})

- OD_{420} and OD_{550} are read from the reaction mixture.
- OD_{600} reflects cell density in the washed cell suspension.
- T = time of the reaction in minutes.
- V = volume of culture used in the assay in ml.

The units give the change in A_{420}/min/mL of cells/OD_{600}

Typical values:

A fully induced lac^+ operon (+IPTG) = 1500 units

An uniduced lac^+ operon (no IPTG) = 1.5-3 units

Procedure in Flow Chart

Take the 2ml of suspension of *E. coli* culture and Centrifuge at 10,000rpm/4^0C/10min

↓

Discard the supernatant and dissolve the pellet in 2ml of Z buffer

↓

Measure the OD at 600nm and then transfer 0.5ml suspension and add 0.5ml Z buffer

↓

Mix well

↓

Add 100µl of chloroform + 50µl of 0.1%SDS

↓

Keep for 5mins at 28^0C water bath

↓

Add 1ml of ONPG substrate (4mg/ml) (note down the time)

↓

Vortex

↓

Incubate at 28^0C for 20mins

↓

Add 0.5ml of 1M Na_2CO_3 (Note down the time)

↓

Mix and centrifuge at 10,000rpm for 5mins

↓

Collect the supernatant

↓

Take OD at 420nm & 550nm.

ISOLATION OF ANTIBIOTIC-RESISTANT MUTANTS USING THE REPLICA PLATE METHOD

Aim: Isolation and identification of mutants by Replica plating technique.

Introduction:

An auxotroph is a bacterial mutant that requires one or more growth factors that the 'wild type' or prototroph can synthesize. A lysine auxotroph, for example, will grow on lysine-supplemented media but not on a medium lacking an adequate supply of lysine because it cannot synthesize this amino acid. Auxotroph's are produced in the laboratory by treating prototrophs with mutagenic agents such as ultraviolet radiation generated by a 15 or 25 W germicidal lamp or nitroguanidine or mitomycin C.

In 1952, Joshua Lederberg and E.M. Lederberg devised a technique called replica plating for detecting and isolating mutant strains of microorganisms.

This technique is used to select auxotrophic mutants. An auxotroph is a mutated prototroph that lacks the ability to synthesize an essential nutrient and therefore must obtain it or a precursor from its surroundings. In other words, it distinguishes itself between mutants and the wild type strains based on their ability to grow in the absence of a particular biosynthetic end product.

Principle:

The principle application of this technique is that it permits the simultaneous transfer of a large number of colonies from one plating medium to another in a single operation by use of a cotton velvet/cloth. One can readily examine thousands of colonies in a short time without having to pick and transfer each one separately.

This exercise deals with the production of auxotrophic mutants by UV irradiation and isolation of lysine auxotroph of E. coli that will grow on lysine-supplemented media but not on a medium lacking an adequate supply of this amino acid because it does not have the ability to synthesize lysine using replica plating technique. By appropriate supplementation of the replica plate, auxotroph's of a specific type (e.g., a single amino acid, vitamin, or nucleic acid precursor) can be identified when used together with a highly specific mutagen such as nitroguanidine. This technique can yield up to 20% auxotroph's or 1 to 2% of a particular mutant type.

In this technique, the mutants are produced by treating a culture with a mutagen. The culture, containing both wild type and auxotroph (i.e., the mutant survivors), is plated on complete medium. After the colonies have developed, the bacteria are transferred by replica plating technique, in which a velvet-covered colony transfer device is used. The sterile velvet/cloth is pressed on the plate surface to pick up bacteria from each colony**.** Then the velvet-covered carrier is pressed on the surface of other plates (one plate with **complete medium** and other plate with **minimal medium** minus lysine) and organisms are transferred to the same position as on the master plate. The plates are incubated for growth of colonies. Observe both the replica plates compared to identify the lys^- auxotrophic mutant colonies.

Requirements:

- 24 hours nutrient broth culture of *Escherichia coli*
- LB broth tube
- Minimal agar plate
- Cotton velvet/screen cloth
- Beaker & 95% alcohol
- Bent glass rod
- Sterile 1mL pipettes
- UV germicidal lamp (15 W)
- Phosphate buffered Saline (PBS)

Preparation of Materials

1. **Preparation of LB broth and agar medium [pH 7.0]**

Constituent	Amount
Tryptone	10.0g
Yeast extract	5.0g
NaCl	5.0g
Distilled water	1000.0 ml

2. Complete Media

Constituent	Amount
Tryptone	10.0g
Yeast extract	5.0g
NaCl	5.0g
Agar	15g
Lysine	0.6g
Distilled water	1000.0 ml
pH	7+/- 0.3

3. Minimal Media

Constituent	Amount
K_2HPO_4	3.0g
Na_2HPO_4	2.0g
NaCl	5.0g
NH_4Cl	2.0g
$MgSO_4$	0.1g
Agar	15g
Glucose	8g
Distilled water	1000.0 ml
pH	7

Weigh and dissolve the above constituents in **distilled water**. Adjust **pH** to 7.0. Pour into 500 ml **Erlenmeyer flasks** and culture. **Autoclave** at 121^0C for15 minutes.

LB broth is a complex medium for rapid growth of *E. coli* and other members of *Enterobacteriaceae*.

LB agar medium: Add 15g of agar per litre of LB broth. Pour 20 to 25 ml into sterile plates.

2. Preparation of Sterile Velvet/Screen Carrier

(i) Cut ordinary cotton velvet/cloth into 15 cm square pieces.

(ii) Wrap them in packets of 10 to 20 in aluminium foil.

(iii) Autoclave them at 121^0C for 10-15 minutes.

(iv) Secure the sterile velvet/cloth on the bottoms of sterile 400ml beakers (beaker acting as a replica block) with rubber bands or metal rings.

Replica Plating: Material Preparation & Assay

Preparation of LB broth and agar medium (Continued from previous page)

- **Autoclave** at 121∘C for 15 minutes. **LB broth** is a complex medium for rapid growth of *E. coli* and other members of *Enterobacteriaceae*.
- **LB agar medium**: Add 15g of agar per litre of LB broth. Pour 20 to 25ml into sterile plates.

2. Preparation of Sterile Velvet/Screen Carrier

(i) Cut ordinary cotton velvet/cloth into 15cm square pieces. (ii) Wrap them in packets of 10 to 20 in aluminium foil. (iii) Autoclave them at 121∘C for 10-15 minutes. (iv) Secure the sterile velvet/cloth on the bottoms of sterile 400ml beakers (**beaker acting as a replica block**) with rubber bands or metal rings.

Assay Procedure

A. Inactivation of *E. coli* culture with UV Radiation

1. Inoculate **LB broth tube** with *E. coli* and incubate it for 16 hours at 37^0C.
2. Dilute the overnight culture 100-fold into fresh LB broth and grow to a cell density of 2×10^8 cells/ml.
3. Wash the bacteria by **dilution** or **centrifugation** and resuspend them in **PBS** to have a cell density of 2×10^8 cells/ml.
4. Plate 0.1ml aliquots of 10−6 dilution in duplicate on solid **LB agar medium**.
5. Incubate plates at 37^0C for 24 to 48 hours.
6. Observe the plates for the development of *E. coli* colonies. Count the number of stable cells per ml prior to **[Induction/Irradiation]**.
7. Irradiate the culture of *E. coli* with UV to get UV-induced mutants of the bacterium as follows:

- Pipet 5 to 6 ml aliquots of bacteria resuspended in **PBS (Phosphate Buffered Saline)**, as prepared in step 3 above, at 2 X 10^8 cells/ml to sterile 100 mm glass Petri dishes.
- Remove the tops of Petri dishes (as glass and plastic are UV opaque) and place these on a **shaker** underneath the **UV light source** at a distance of 30cm, and expose the plates for 15- 20 minutes.

8. Transfer 1 ml of the UV-treated culture to 10ml nutrient broth and incubate overnight to **stationary phase** at 37^0C.
9. Pipet 0.1ml of **undiluted** as well as 10^{-1}- or 10-2-fold dilutions of cultures onto **nutrient agar plates** (i.e., **complete medium**).
10. Spread the organisms over the plate with a **sterile bent glass rod**.
11. Incubate these plates in an **inverted position** at 37^0C for 24 hours.

Observations

- Observe the plates for appearance of colonies (**wild type** and **mutant survivors**) which should be in the range of **100-200 colonies per plate** and allow the colonies to attain 3mm in diameter.

B. Replica Plating techniques for auxotrophic mutants' isolation

12. By holding the **sterile colony carrier handle** in the right hand, carefully lower the **velvet surface** and press on the **master plate surface** having colonies of *E. coli* both wild type and mutant survivors to pick up bacteria from each colony.
13. Inoculate the **minimal agar plate** (i.e., complete medium **minus lysine**) by lightly pressing the velvet onto the medium to get the **replica plate**.
14. Now, without returning the carrier to the master plate, inoculate the **complete medium plate** in the same manner.
15. Incubate both the inoculated plates upside down at 37^0C for 48- 96 hours.

Observations

16. Examine both the **minimal agar** and **complete medium** plates for the appearance of *E. coli* colonies.

17. **Identify those which grew on complete medium but not on the minimal agar plates.**
18. Determine the location of **lysine auxotrophs lys$^-$** on the complete medium replica plates.

Results

19. Differentiate the colonies which grew on the complete medium plates but not on the minimal agar plates.
20. **Complete medium replica plate** will show growth of both **wild type** and **lysine auxotrophs lys$^-$**, UV-induced mutants of *E. coli* (that altered phenotypically).
21. While **minimal agar replica plate** will lack lys$^-$ colonies and have only the **wild type strain**.

Reference

Experiment in Microbiology, Plant Pathology and Biotechnology by K.P. Aneja, 4th edition.

ISOLATION OF ANTIBIOTIC-RESISTANT MUTANTS USING THE GRADIENT PLATE METHOD

Aim: To isolate antibiotic resistant mutants using Gradient Plate Technique.

Introduction: Microorganisms that exhibits a natural, non-mutated inheritance are termed as a wild type or wild strain while a resistant strain refers to an altered version. Mutant strains can show variation in morphology, nutrition, and biochemical characteristics. Genetic control mechanism provides resistance to chemicals, temperature preference and nearly any type of enzyme function.

A mutation is a stable heritable change in the nucleotide sequence of DNA. By manipulating the chemical or physical environment of a bacteria, one can increase the frequency of mutations.

Mutations occur in two ways -

1. Spontaneous Mutation

Arise occasionally in all bacteria and develop in the absence of any added agent.

2. Induced Mutation

Are the results of the bacteria's exposure to a **mutagen**, which is a physical or chemical agent. Genetic and Biochemical investigations in bacteria are often initiated by the isolation of mutants. To detect mutants of a particular organism, one must know the normal or wild characteristic so that altered phenotype can be recognized. Since mutants are generally about one per 10^7 to 10^{11} cells.

An excellent way to determine the ability of organisms to produce mutants that are resistant to antibiotic is to grow them on a gradient plate of a particular antibiotic. The gradient plate consists of two wedges like layers of media: a bottom layer of plain nutrient agar and top layer of antibiotic with nutrient agar. The antibiotic in the top layer, diffuse into the bottom layer producing a gradient of antibiotic concentration from low to high. A gradient plate is made by using Streptomycin in the medium. E. coli, which is normally sensitive to Streptomycin, will be spread over the surface of the plate and incubated for 24 to 72 hours. After incubation colonies will appear on both the gradients. The colonies develop in the high

concentration are resistant to the action of Streptomycin, and are considered as Streptomycin resistant mutants. For isolation of antibiotic resistant of gram-negative enteric bacteria, the antibiotics commonly used are Rifampicin, Streptomycin, and Erythromycin etc.

Principle

The **gradient plate method** consists of two different wedge-like layers of media. A bottom layer of plain nutrient agar and a top layer of nutrient agar with antibiotics. Since antibiotic is present only in the top layer, it tends to diffuse into lower layer producing a **gradient of antibiotic concentration** from low to high.

A gradient plate using **Streptomycin** is described. The sample culture which is normally sensitive to the antibiotic will be spread over the surface of the plate and incubated for 4-7 days. Any colony that develops in the higher concentration zone area will be a **Streptomycin resistant mutant**.

Media Preparation

The media nutrient agar will have a streptomycin concentration of **100 µg/ml**. This concentration is **10 times** the strength used in sensitivity zones in the **Kirby-Bauer test method**.

Materials Required

1. 24-hours old culture of test organism
2. **Nutrient Agar**
3. **Spreader**
4. **Water bath**
5. **Glass marking pencil**
6. **Sterile petri plate**
7. **Nutrient Agar + Streptomycin**

Procedure

I. First Period

Preparation Of Gradient Agar Plate

1. Place a sterile petri plate at an angle by placing either a glass rod or a stick under one side. 2. Aseptically pour a tube of molten nutrient agar on the petri plate. Immediately cover the plate and allow the agar to solidify.
2. Remove the glass rod and place the plate flat on the table.
3. Label the low- and high-level concentrated area on the bottom of the plate.
4. Aseptically pour a tube of Streptomycin containing medium on the surface of gradient agar plate. Place the plate flat ($100 \mu g/ml$) on the plate and allow it to solidify.
5. Label low and high concentrated areas.

Materials Required

1. 24-hours old culture of test organism
2. Nutrient Agar
3. Spreader
4. Water bath
5. Glass marking pencil
6. Sterile petri plate
7. Nutrient Agar + Streptomycin

Procedure

I. First Period

Preparation Of Gradient Agar Plate

1. Place a **sterile petri plate** at an angle by placing either a glass rod or a stick under one side.
2. Aseptically pour a tube of **molten nutrient agar** on the petri plate. Immediately cover the plate and allow the agar to solidify.
3. Remove the **glass rod** and place the plate **flat** on the table.
4. Label the **low**- and high-level **concentrated area** on the bottom of the plate.

5. Aseptically pour a tube of **Streptomycin containing medium** on the surface of gradient agar plate. Place the plate **flat** (100*µg/ml*) on the plate and allow it to solidify.
6. Label **low** and **high concentrated areas**.

Inoculation

Pipette **0.1 ml** of test culture or sample onto the agar surface. Spread the culture evenly on the surface of the agar, with a **cotton swab / glass spreader**.

Incubate the plate inverted at 37^0C for **24-48 hours**.

II. Second Period

1. **Observe** the gradient plate of agar for development of resistant test culture colonies in areas of high concentration of Streptomycin.
2. If growth is observed, prepare suspension of any individual isolated colony grown in high concentration.
3. Prepared suspension is spread on gradient plate of higher concentration of same antibiotic than previous one like 200 µg/ml, 300 µg/ml, 400 µg/ml etc. All the procedure is same as in the first period.

III. Third Period

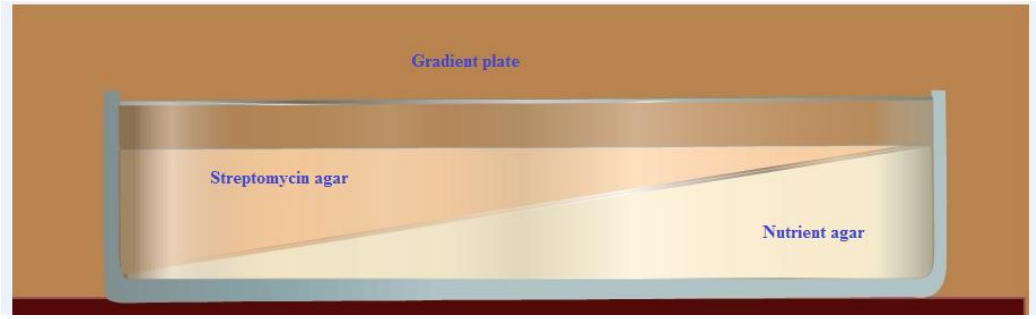
1. **Observe** growth and try with various concentrations to test the ability of resistance against the antibiotic **Streptomycin**.
2. A similar process is followed for resistance of test culture to penicillin antibiotic also.

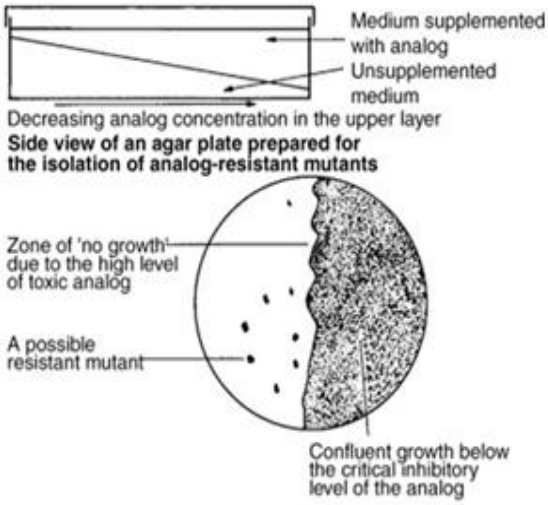
Observation:

Sample (Concentration)	**Streptomycin**	
Colonies	**Low**	**High**
	+++	+

+ indicates less growth

+++ indicates more growth





Result

According to the table, there was growth in low concentration of antibiotic.

In the gradient of 100 µg concentration, a few colonies of culture showed reduced growth.

The bacterial colonies from 100 µg were now exposed to a higher concentration of antibiotic like 500 µg/ml.

DEMONSTRATION OF RESTRICTION DIGESTION OF DNA

Aim: To perform restriction digestion of λ DNA using Eco RI and Hind III enzymes.

Introduction

In 1978, the Nobel prize for medicine was awarded to Warner Arber, Daniel Nathans and Hamilton Smith for their discovery of restriction endonucleases, which led to the development of Recombinant DNA Technology. The restriction enzymes have been discovered in many different bacteria and other multi-cellular organisms. These restriction enzymes are able to scan along a length of DNA looking for a particular sequence of bases that they recognize.

Principle

Restriction digestion involves fragmenting DNA molecule into smaller pieces with special enzyme called restriction endonuclease, commonly known as restriction enzyme, because of this property. The restriction enzymes are also known as molecular scissors. The restriction enzymes are named after the cellular strain from which they are isolated. The Restriction enzymes recognize specific sequence in the double stranded DNA molecules (for ex: G A A T T C) and then cut the DNA to produce fragments called **restriction fragments**. The target site or sequence which the restriction enzyme recognizes is generally from **4-6 base pairs** and arranged in a **palindromic sequence**. Once it is located, the enzyme attaches to the DNA molecule and cut each strand of the **double helix**. The restriction enzyme will continue to do this along the full length of DNA molecule which will then break into fragments. The size of these fragments is measured in **base pairs** or **kilo base pairs** (1000 bases).

Materials Required

1. Lambda (λ) DNA
2. DNA marker
3. Agarose
4. Restriction enzyme: EcoR I
5. Restriction enzyme: Hind III
6. 10x assay buffer for EcoR I
7. 10x assay buffer for Hind III
8. Molecular biology grade water

9. 50x TAE
10. 6x gel loading buffer
11. Polypropylene tubes 0.5ml
12. Measuring cylinder, beaker
13. Ethidium Bromide - 10mg/1ml
14. Electrophoretic apparatus and UV transilluminator

Procedure

1. Place restriction enzymes [EcoRI, HindIII], substrate [λ DNA] and assay buffers on ice.
2. Thaw the assay buffer vials completely.
3. Label fresh vials with the name of enzymes and keep them on ice.
4. Set up the restriction digestion reaction as follows:

Reaction 1: [EcoRI digestion]

Component	**Volume**
Nuclease free water	30 µl
10X Assay buffer	4 µl
DNA	4 µl
Enzyme (EcoRI)	2 µl
Total	**40** µl

Reaction 2: [Hind III digestion]

Component	**Volume**
Nuclease free water	30 µl
10X Assay buffer	4 µl
DNA	4 µl

Enzyme (Hind III)	2 µl
Total	**40** µl

5. **Incubate** for **1 hour at 37∘C** in a water bath incubator.
6. **Stop** the reaction after one hour by adding 3μl of **gel loading dye** to the vial.
7. Prepare **1% Agarose Gel**.
8. Add 10μl of distilled water into a fresh 1.5ml tube.
9. Add 3μl of λ DNA and 2μl of gel loading dye into this vial. Label this as **uncut** λ DNA.
10. **Load** the samples into separate wells in 1% agarose gel in the following order:

    Lane 1 - Marker **500bp Ladder**

    Lane 2 - Uncut **λ DNA (Control)**

    Lane 3 – *EcoR I* digest

    Lane 4 – *Hind III* digest

NOTE: Load 10μl of marker DNA. Load the digested samples completely in lanes 3-4.

Agarose Gel Electrophoresis

1. Preparation of Agarose Gel

To prepare 50ml of **1% Agarose gel**, measure 0.5g agarose in a glass beaker or flask and add 50ml **TAE buffer**. Heat the mixture on a microwave or a burner, swirling the glass flask occasionally until the agarose dissolves completely. (Ensure that the lid of the flask is loose to avoid build-up of pressure.) Allow the solution to cool to about 55-60∘C. Add 0.5μl **Ethidium bromide**, mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about **30 minutes** at room temperature.

2. Loading of DNA Samples

Load 3μl of ready-to-use DNA marker into the well 1. To prepare samples for electrophoresis, add 2μl of 6x gel loading buffer to 10μl of DNA samples. Mix well by pipetting and load the samples into the well.

3. Electrophoresis

Connect the power cord to the electrophoresis power supply according to the conventions: Red - Anode and Black - Cathode. Electrophorese at 100 to 120 V and 90mA until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

Observation:

Eco RI

5' G AATTC 3'

3' CTTAA G 5'

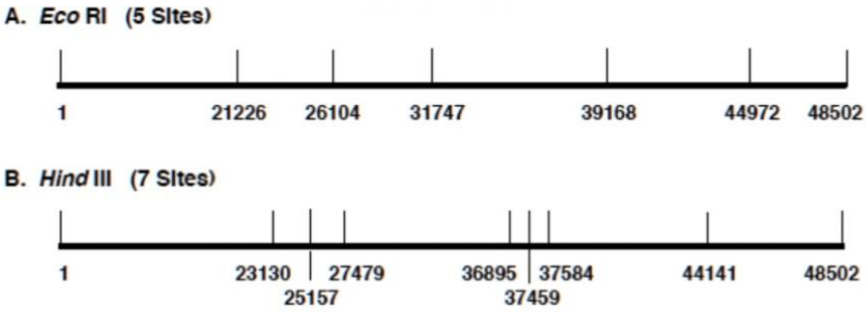
Hind III

5' A AGCTT 3'

3' TTCGA A 5'

Every restriction enzyme has unique target sites for digestion. Lambda DNA has multiple restriction sites for both Eco RI and Hind III which results in several fragments of varying sizes.

Enzymes	Source	Recognition Site	Cut	Fragment Sizes [bp]
EcoRI	Escherichia coli	5' G A A T T C 3' 3' C T T A A G 5'	5' ... G AA T T C ...3' 3' ... C T T A A G ...5'	21226, 7421, 5804, 5643, 4878, 3530
HindIII	Haemophilus influenza	5' A A G C T T 3' 3'T T C G A A 5'	5' ...A A G C T T ... 3' 3' ...T T C G A A ... 5'	23130, 9416, 6557, 4361, 2322, 2027, 564, 125



Result and Interpretation

Restriction digestion patterns of **lambda DNA** obtained upon treatment with **EcoRI** and **HindIII** are marked differently which demonstrates the fact that each **restriction enzyme** recognizes and cleaves only a specific base segment unique to it. The **size of the fragments** can be determined by comparing with that of the **DNA marker** seen on the same gel.

Applications Of Restriction Enzymes

- Construction of **recombinant DNA molecules** and **DNA libraries**.
- **Mapping** the locations of restriction sites.
- **Southern blot hybridization**.

The discovery of restriction enzymes in the late 1960s has had an enormous impact on molecular biology research. Cutting large DNA-molecules with restriction enzymes makes it possible to purify homogenous DNA-fragments of defined lengths that can be subsequently enzymatically manipulated and analysed (Mani et al., 2005a). Cut DNA-fragments can easily be ligated into DNA-molecules with corresponding ends, thereby making recombinant DNA-molecules. Due to the exact specificity of restriction enzymes, and the specific cleavage patterns generated when cut DNA is run on gels, restriction enzymes also enable for mapping of DNA. A common application of this is restriction fragment length polymorphism (RFLP), used for e.g. paternity testing. In RFLP, specific human genomic DNA-areas are cut by several restriction enzymes, and the fragments are subjected to electrophoresis. The generated fragment pattern is unique for a given individual, but shares certain similarities with patterns generated by related individuals. Moreover, restriction enzymes can also be used to detect specific variations/mutations in DNA caused by a single nucleotide change, so called single nucleotide polymorphisms (SNPs) (Dear 2005). The SNP may generate a new cutting site, or it may result in the loss of a cutting site. Therefore, by cutting DNA with restriction enzymes directed at the site of a possible mutation and thereafter subjecting the fragments to electrophoresis, the gain or loss of a band tells whether a SNP is present or not.

DEMONSTRATION OF *IN-VITRO* DNA LIGATION

Aim: To perform ligation of λ (Lambda) Eco RI digest and checking of the ligation reaction through Agarose Gel Electrophoresis.

Introduction

Two DNA molecule ends (either from the same or different molecules) can be joined together through a process called ligation. This process involves the formation of a covalent bond between two DNA fragments (having blunt or overhanging complementary sticky ends) by the help of an enzyme n[1]amed ligase. DNA-ligase forms a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. This process is the key player in constructing recombinant DNA molecules.

Principle

Recombinant DNA is made possible by two important enzymes, Restriction enzymes and DNA ligase. Restriction enzymes cut DNA at a specific location and DNA ligase is used to 'Glue' the fragments of DNA together. The commonly used DNA ligase in nucleic acid research is T4 DNA ligase and E. coli DNA ligase.

E. coli DNA ligase is more specific for cohesive ends that T4 DNA ligase can't, but, E. coli DNA ligase can't be used for cloning purposes. The DNA ligase in the most versatile and commonly used ligase for DNA cloning. The T4 DNA ligase is approximately 60,000 daltons (60 KD) protein produced by T4 bacteriophage. This ATP dependent enzyme covalently joins blunt/compatible cohesive ends as well as nicks in double stranded DNA. A 5' phosphoryl group is required for ligation to a 3' hydroxyl. Generally, cohesive end ligation is carried out at lower temperatures (12^0C to 16^0C) for the maintenance of a good balance between annealing of ends and activity of the enzyme. Blunt end ligation can be carried out at 24^0C as annealing of ends is not a factor due to the lack of cohesive ends. Blunt end ligation is more complex compared to cohesive end ligation.

A typical ligation reaction requires the following components:

a) Two or more fragments of DNA that have either blunt or cohesive ends.

b) A buffer which contains ATP.

c) The DNA ligase.

Materials Required

1. lambda DNA- EcoR I digest
2. 10X Ligase assay buffer
3. T4 DNA ligase
4. Molecular biological grade water
5. Agarose
6. 50X TAE
7. 6X gel loading buffer

Procedure:

a) Thaw the ligase assay buffer and lambda-Eco RI digest vials.

b) Place T4 DNA ligase and a 1.5 mL vial on ice.

c) Set up the reaction mixture as follows:

Component	**Volume**
T4 DNA ligase	1µl
lambda **EcoRI digest**	10 µl
2X ligase assay buffer	10 µl
Total	21 µl

d) Add the above and mix by tapping. Label as ligated sample.

e) Incubate at 16^0C for 2 hours in a preset water bath for ligation.

f) Meanwhile, prepare 1% Agarose gel for electrophoresis.

g) Add 2 µl of Gel loading buffer to the ligated sample at the end of 2 hours.

h) Pipette out 10 µl of lambda EcoRI digest, 2 µl of Gel loading buffer and 12 µl of water into a vial, label it as control.

i) Load the ligated sample and control sample in the wells of the 1% Agarose gel.

j) Electrophorese the samples at 50-100 V for 2 hours and visualize under a UV transilluminator.

Agarose Gel Electrophoresis

a) Preparation

To prepare 50 mL of 1\% Agarose gel, measure 0.5 g agarose. In a glass beaker or flask, add 50 mL 10X TAE buffer. Heat the mixture on a microwave or a burner, by swirling the beaker occasionally, until agarose dissolves completely.

Allow the solution to cool to about 55^0C–60^0C.

Add 0.5 µl Ethidium bromide, 10 ml. Mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature.

NOTE: Ethidium bromide is a powerful mutagen and is very toxic. Appropriate safety precautions should be taken by wearing latex gloves. However, use of nitrile gloves is recommended.

b) Loading the DNA Sample

To prepare the control sample for electrophoresis, take 1 µl of 6X gel loading buffer and 10 µl of lambda-EcoRI digest in a tube, mix well by pipetting & load the sample into the well.

For the ligated sample, add 2 µl of 6X gel loading buffer, to the ligation mix, well by pipetting and load the same into the next well.

c) Electrophoresis

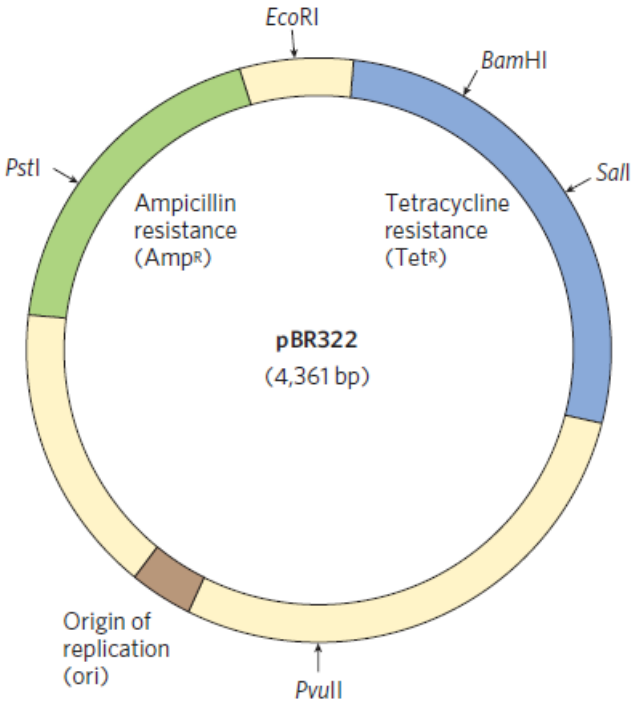
- The power cord to the Electrophoretic power supply is connected according to the conventions. Red for anode & black for cathode. Electrophorese at 100–180V and 90mA, until dye markers have migrated an appropriate distance.

Interpretation Of Results

After running the ligated and un-ligated samples on Agarose gel, one can observe that the seven double stranded fragments formed by digestion of λ DNA with Eco RI are ligated with *T*4 DNA ligase to give a single band.

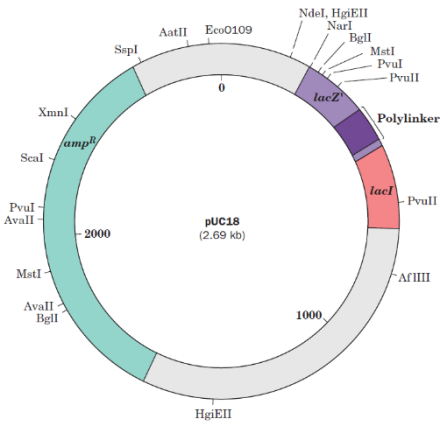
pBR 322

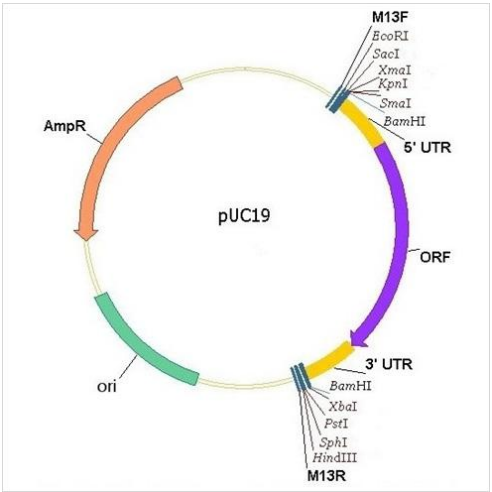
- This plasmid is derived from *E. coli* ColE1
- Prepared by scientists Boliver and Rodriguez.
- This plasmid is modified during reconstruction.
- It is a 4363 base pair long plasmid.
- This plasmid possesses a bacterial origin of replication and restriction sites for different restriction endonuclease enzymes like *Eco*RI, *Hind*III, *Bam*HI, *Sal*I... etc.
- It has two antibiotic resistant genes for Ampicillin and Tetracycline antibiotics.
- It is much smaller than natural plasmid, hence it is easier to handle.
- This plasmid is less susceptible to physical damage.
- It has a simpler restriction map and can be taken up easily by host bacterial cell.



pUC 18 and 19

- pUC 19 is one of a series of plasmid cloning vectors created by Joachim Messing and his coworkers.
- The designation 'pUC' is derived from the classical "p" prefix (denoting plasmid) and the abbreviation "UC" for "University of California". It is a circular double stranded DNA and has 2686 base pairs.
- The artificial plasmid pUC 18 has been genetically engineered to include:
- A gene for antibiotic resistance to Ampicillin
- A gene (and its promoter) for the enzyme β-Galactosidase, Lac Z. The Lac Z contains a Polylinker region, with a series of unique restriction sites found nowhere else in the plasmid.
- Digestion with one of these endonucleases will make a single cut that linearizes the circularized plasmid DNA, and allows it to recombine with a foreign DNA that has been cut with the same endonuclease.
- pUC 19 is one of the most widely used vector molecules. pUC 18 is similar to pUC 19 but the Multiple Cloning Site (MCS) is reversed.





SV-40

The first animal vector was derived from the primate papova virus. Simian Virus-40 was used for cloning in 1979.

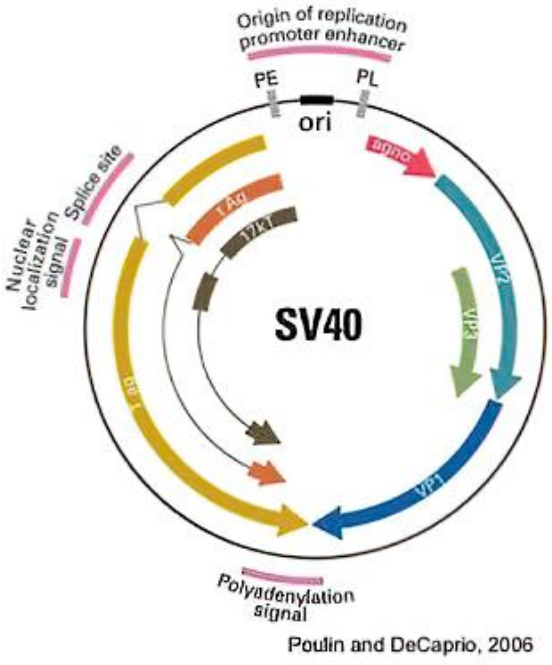
Salient Features

- SV-40 is a sphinoid virus with a circular, double stranded 5243 base pairs (should be 5243 bp).
- The Virus encodes five proteins namely, Small t, Large t, VP1, VP2, VP3.
- It has an origin of replication of 80 bp and is complexed with histones to form chromatin.
- Large t is essential for viral capsule.
- In the lab, it is multiplied in cultured kidney cells of African Green Monkey.
- SV-40 genome has been used to develop mainly the following 3 types of vectors:

  a) Transducing vectors

  b) Plasmid vectors

  c) Transforming vectors



Poulin and DeCaprio, 2006

Bacteriophage Lambda (λ)

General Features

Bacteriophage lambda (λ) is a temperate phage widely used in molecular biology, especially as a cloning vector. Its genome is approximately 48.5 kilobases long and contains about 46 genes.

Structurally, λ phage consists of:

- An icosahedral head that encloses the viral double-stranded DNA.
- A long, flexible tail that facilitates attachment to *Escherichia coli* cells during infection.

The native λ phage contains a specific set of genes that enable its DNA to integrate into the E. coli chromosome, entering the lysogenic cycle.
During vector construction, these non-essential genes can be removed to create space for inserting foreign DNA, making λ phage a valuable tool for gene cloning.

Development of λ as a Cloning Vector

Two major discoveries made bacteriophage λ a promising candidate for use as a cloning vector:

1. **Deletion Tolerance Between J and N Genes**
   It was found that the gene products located between the J and N genes are not essential for the completion of the λ life cycle.
   These non-essential regions can be removed, creating space to insert foreign DNA.
2. **Engineering of Unique Restriction Sites**
   By eliminating unwanted restriction endonuclease recognition sequences from the λ genome, researchers were able to design vectors that contain a single, defined restriction site.
   This allows precise insertion of foreign DNA without unwanted cutting at multiple positions.

Types of λ Vectors

1. **Insertional Vectors**
   - Foreign DNA is inserted into a specific restriction site within the λ genome.

- Only small inserts (typically up to ~5 kb) can be accommodated.

2. **Replacement Vectors**

   - A non-essential region of λ DNA called the stuffer fragment is removed and replaced with foreign DNA.
   - These vectors can carry larger inserts (up to ~20 kb).

Example: λ gt10 Vector

The bacteriophage λ gt10 vector is 43.8 kilobases in size. It contains a strategically placed EcoRI restriction site within the cI gene (which encodes the λ repressor).
Insertion of foreign DNA at this site inactivates the cI gene, preventing lysogeny and ensuring lytic growth—useful for screening recombinants.

λ gt10 can accommodate an insert of up to ~7.6 kb before exceeding the packaging limit for λ DNA.
To create recombinant DNA:

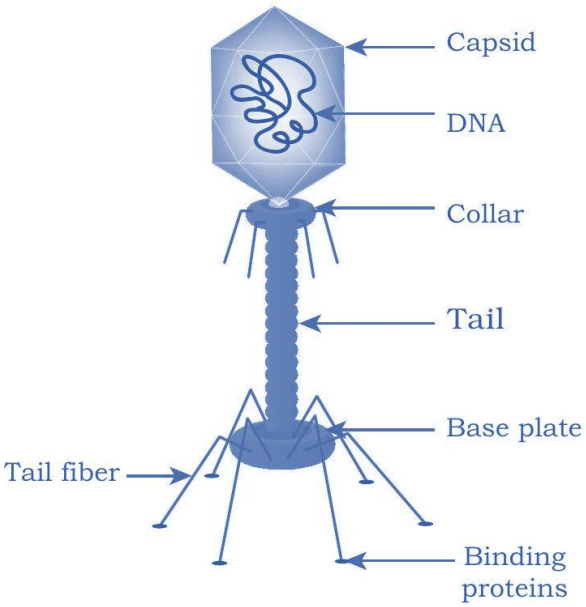
- The vector and the foreign DNA are both cleaved with EcoRI.
- The compatible ends anneal and are sealed with DNA ligase.
- The recombinant DNA is then packaged into λ heads and tails in vitro.

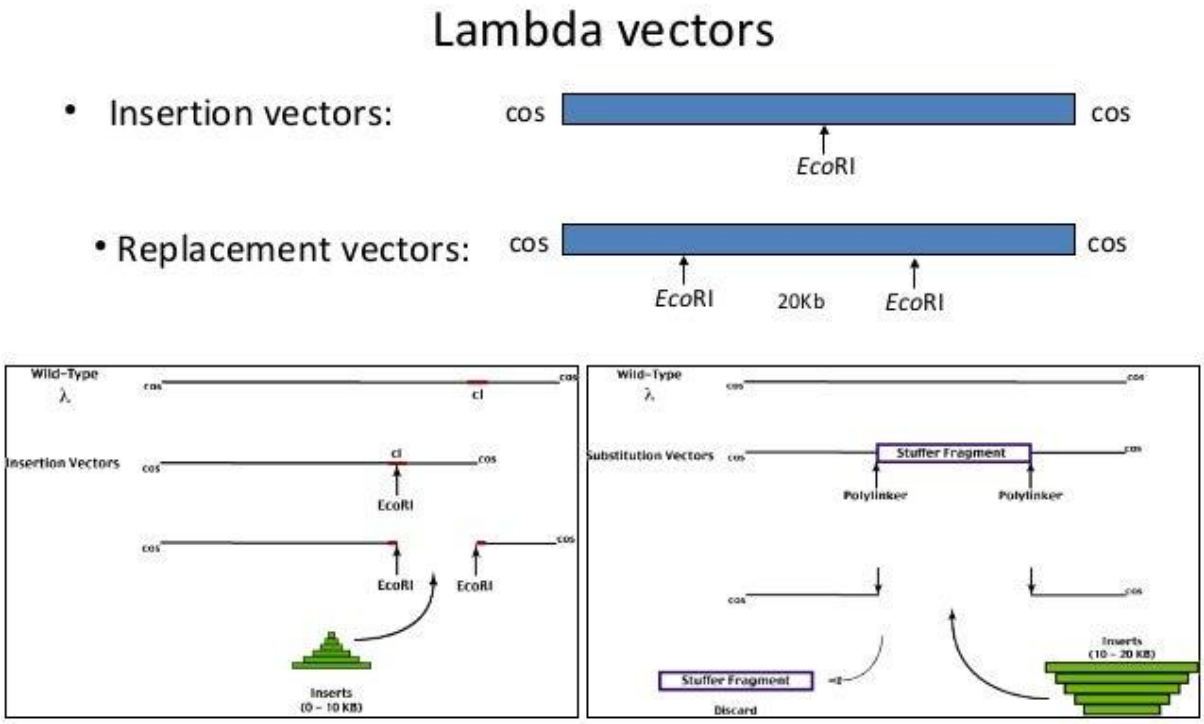
Steps for Using λ gt10

1. **Digest the λ gt10 Vector**
   - Cut λ gt10 DNA with EcoRI.
   - Prepare the double-stranded foreign DNA by also cutting it with EcoRI to create compatible sticky ends.
2. **Ligation**
   - Mix vector and insert.
   - Use DNA ligase to seal the nicks, forming recombinant λ molecules.
3. **In Vitro Packaging**
   - Combine the ligated DNA with a packaging mix (containing λ head and tail precursors) to assemble infectious phage particles.

4. **Infection of *E. coli***

- Infect *E. coli* cells with the packaged recombinant λ particles.
- Allow phage replication and recovery of plaques containing the recombinant phages.





GENE CLONING

Gene Cloning Involves the Following Steps

1. **Isolation of the Desired Gene**

   The gene of interest is isolated using gene isolation techniques that involve treating DNA with a restriction endonuclease enzyme and separating the resulting DNA fragments by gel electrophoresis.

2. **Selection and Cutting of a Suitable Vector**

   A suitable vector, such as the plasmid pUC-18, is selected and cut using the same restriction endonuclease enzyme. The circular plasmid becomes linearized, producing sticky or staggered ends.

3. **Ligation of the Gene into the Vector**

   The gene of interest is ligated into the linearized plasmid using DNA ligase. The staggered ends facilitate proper annealing and ligation. The resulting recombinant molecule is called rDNA, chimeric DNA, or mosaic DNA.

4. **Introduction of rDNA into a Host Cell (Transformation)**

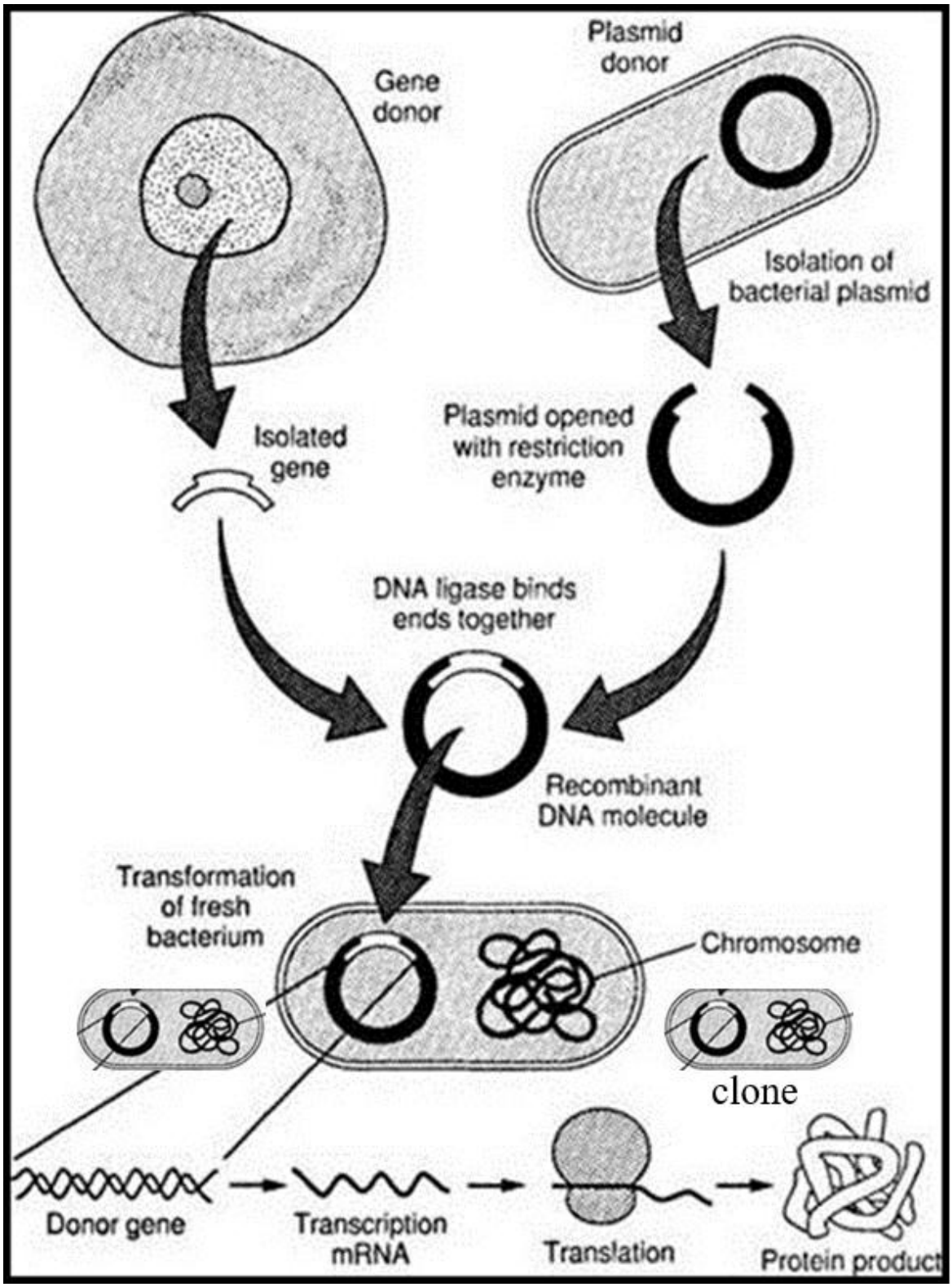
   The rDNA is transferred into a suitable bacterial host cell (e.g., *E. coli*) by artificial transformation. The recombinant plasmid and host cells are suspended in cold $CaCl_2$ solution (maintained at 0°C).

5. **Heat Shock Treatment**

   After incubation on ice, the temperature is suddenly raised to 42°C. The higher temperature destabilizes membrane proteins of the host cell boundary and increases pore size. Due to the concentration gradient, the recombinant DNA enters the bacterial cell.

6. **Selection of Transformed Cells**

   The transformed bacterial cells are cultured on Nutrient Agar plates supplemented with ampicillin. Since the vector (pUC-18) carries an ampicillin-resistance gene, only the bacteria containing the plasmid will survive and form colonies. This process is called Gene Cloning.


Gene donor
Plasmid donor
Isolation of bacterial plasmid
Isolated gene
Plasmid opened with restriction enzyme
DNA ligase binds ends together
Recombinant DNA molecule
Transformation of fresh bacterium
Chromosome
clone
Donor gene
Transcription mRNA
Translation
Protein product

Selection Of Recombinants by Replica Plate Technique

Replica plating is a microbiological technique in which one or more secondary petri-plates containing different solid [Agar-based] selective growth media (lacking nutrients or containing chemical growth inhibitors such as antibiotics) are inoculated with the same colonies of microorganisms from a primary plate [or master disk], reproducing the spatial pattern of colonies.

The technique involves pressing a velvet-covered disk and then immediately inoculating the secondary plate with cells in colonies removed from the original plate by the material. Generally, large numbers of colonies (roughly 30-300) are replica plated, due to the difficulty in streaking each out individually onto a separate dish.

The purpose of replica plating is to be able to compare the master plate and secondary plates, typically to screen for a desired phenotype.

For example, when a colony that was present on the primary plate [master disk], fails to appear on a secondary plate, it shows that the colony was sensitive to a substance on that secondary plate.

Common screenable phenotypes include auxotrophy and antibiotic resistance.

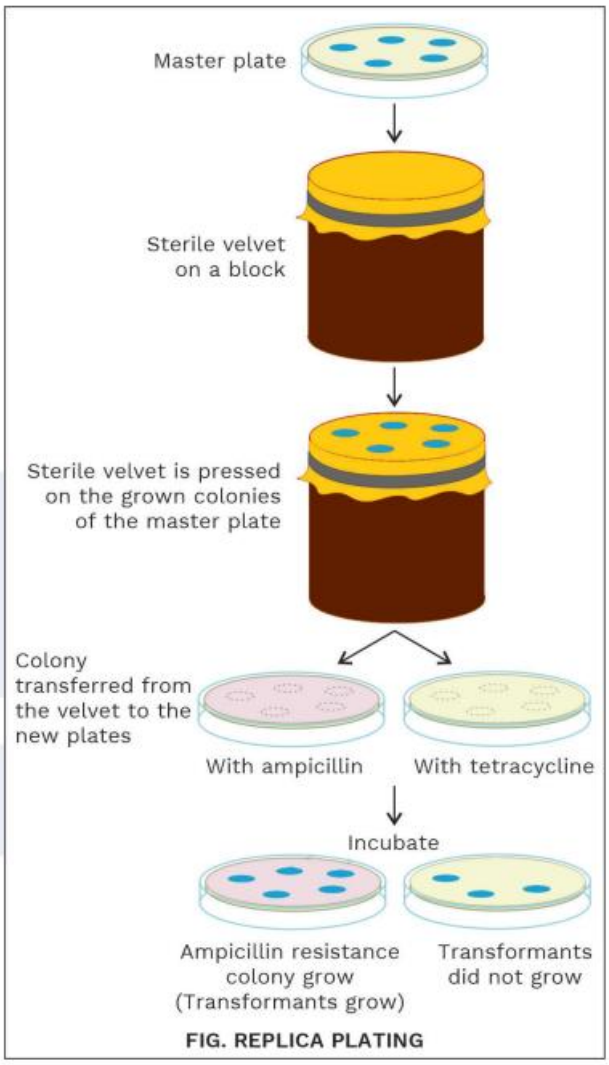


FIG. REPLICA PLATING

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