# ALAGAPPA UNIVERSITY

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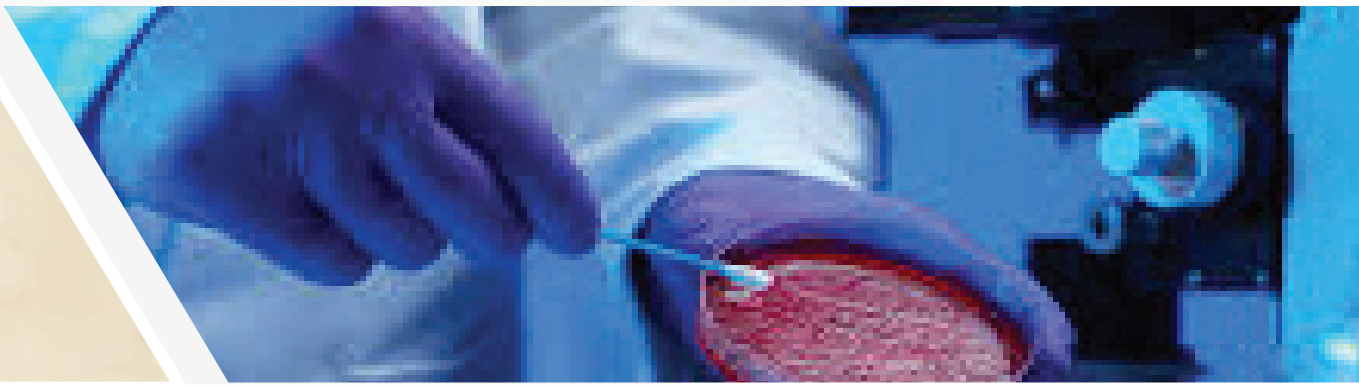
KARAIKUDI – 630 003

DIRECTORATE OF DISTANCE EDUCATION


## M.Sc. [Microbiology]
## 364 24



# LAB II – MICROBIAL GENETICS, MOLECULAR BIOLOGY & rDNA TECHNOLOGY, FOOD & DAIRY MICROBIOLOGY

**II - Semester**

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# LAB II - MICROBIAL GENETICS, MOLECULAR BIOLOGY & rDNA TECHNOLOGY, FOOD & DAIRY MICROBIOLOGY

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# LAB II - MICROBIAL GENETICS, MOLECULAR BIOLOGY & rDNA TECHNOLOGY, FOOD & DAIRY MICROBIOLOGY

## SYLLABI

### MOLECULAR BIOLOGY& r DNA TECHNOLOGY

1. Single colony isolation and checking for genetic markers.
2. Measurement of growth-one step growth curve using a T even phage.
3. Titration of phages (T4).
4. Induction of Lambda Phage.
5. Induced mutagenesis - UV.
6. Isolation of antibiotic resistant mutants.
7. Isolation of auxotrophic mutants.

### MICROBIAL GENETICS

8. Isolation of specialized transducing phage.
9. Bacterial conjugation - transfer of drug resistant factor (Plasmid).
10. Transposon mutagenesis of chromosomal and plasmid DNA.
11. Isolation of plasmid and chromosomal (bacterial) DNA.
12. Quality and quantity checking of DNA by UV Spectrophotometer and Submarine agarose gel electrophoresis.
13. Gene cloning - Preparation of vector and passenger - Ligation - Preparation of competent cells - Transformation of *E.coli* with recombinant plasmids.
14. Selection of recombinants by blue-white selection.
15. PCR amplification - Demo.

### FOOD & DAIRY MICROBIOLOGY

16. Resazurin dye reduction test.
17. Phosphatase test.
18. Litmus milk reactions.
19. Potability analysis of drinking water.
20. Bacterial examination of water (qualitative and quantitative).
21. Membrane filtration technique.

# INTRODUCTION

NOTES

***Microbial Genetics*** is a subject area within microbiology and genetic engineering. Principally, the microbial genetics studies microorganisms for different purposes. The studies of microorganisms involve studies of genotype and expression system. Genotypes are the inherited compositions of an organism. 'Genetic Engineering' is a field of study within microbial genetics, such as the usage of recombinant DNA or rDNA technology and Cloning.

***Molecular Biology*** is a specific branch of biology that studies the molecular basis of biological activity between biomolecules in the various systems of a cell, including the interactions between DNA, RNA, proteins and their biosynthesis, in addition to the regulation of these interactions. Molecular biology, therefore, is the study of molecular foundations of the process of replication, transcription and translation of the genetic material.

***Recombinant DNA*** or ***rDNA***, refers to molecules of DNA from two different species that are inserted into a host organism to produce new genetic combinations that are of great value to science, medicine, agriculture, etc. Since the emphasis of all genetics is the gene, hence the fundamental goal of laboratory geneticists is to isolate, characterize, and manipulate genes. Using this technology, the first drugs of medical biotechnology were produced, namely 'Human Insulin'.

Recombinant DNA technology relates to the usage of three main tools, the Enzymes (Restriction Enzymes, Polymerases, and Ligases), Vectors and Host Organism. The enzymes will help cut (restriction enzymes), synthesize (polymerases), and bind (ligases) DNA. The restriction enzymes play an important role in this technology as they cut at a specific site within the DNA molecule called a restriction site. Usually, the restriction enzyme produce sticky ends in the DNA sequence that helps it bind specifically to the desired gene which will be carried by a 'Vectors'. Vectors are significant parts of the recombinant DNA technology.

***Food Microbiology*** is the study of the microorganisms that inhibit, create, or contaminate food, including the study of microorganisms causing food spoilage, pathogens that may cause disease especially if food is improperly cooked or stored. Specifically those food items that are used to produce fermented foods, such as cheese, yogurt, bread, beer, and wine, and those with other useful roles, such as producing probiotics. Microorganisms are living organisms that are individually too small to see with the naked eye. With regards to the food industry, they can cause spoilage, prevent spoilage through fermentation, or can be the cause of human illness. There are numerous classes of microorganisms, of which bacteria and fungi (yeasts and moulds), the bacterial viruses or bacteriophage are the most common forms.

Food safety is a key emphasis of food microbiology. Numerous agents of disease, pathogens, are readily transmitted via food, including bacteria, and viruses. Microbial toxins are also possible contaminants of food. However, microorganisms and their products can also be used to combat these pathogenic microbes. The bacteriophages, viruses that only infect bacteria, can be used to kill bacterial pathogens.

This book, *Microbial Genetics, Molecular Biology & rDNA Technology, Food & Dairy Microbiology*, deals with the practical aspects of qualitative and quantitative analysis of the techniques used in the laboratory for investigation and analysis.

NOTES

# MOLECULAR BIOLOGY AND RDNA TECHNOLOGY

Molecular biology is a specific branch of biology that studies the molecular basis of biological activity between biomolecules in the various systems of a cell, including the interactions between DNA, RNA, proteins and their biosynthesis, in addition to the regulation of these interactions.

Recombinant DNA or rDNA, refers to molecules of DNA from two different species that are inserted into a host organism to produce new genetic combinations that are of great value to science, medicine, agriculture, etc. Since the emphasis of all genetics is the gene, hence the fundamental goal of laboratory geneticists is to isolate, characterize, and manipulate genes.

## EXPERIMENT 1: SINGLE COLONY ISOLATION AND CHECKING FOR GENETIC MARKERS

### AIM: Single Colony Isolation and Checking for Genetic Markers

### Theory

For many years, **Gene Mapping** was limited to identifying organisms by traditional **Phenotype Markers**. This included genes that encoded easily observable characteristics, such as blood types or seed shapes. The insufficient number of these types of characteristics in several organisms limited the mapping efforts that could be done. This prompted the development of **Gene Markers** which could identify genetic characteristics that are not readily observable in organisms, such as protein variation.

Basically, a '**Genetic Marker**' is a '**Gene**' or '**DNA Sequence**' with a known location on a **Chromosome** that can be used to identify individuals or species.

**Types:** Molecular Genetic Markers can be divided into following two classes:

1. **Biochemical Markers** which detects variation at the Gene Product Level, such as changes in Proteins and Amino Acids.
2. **Molecular Markers** which detects variation at the DNA Level, such as Nucleotide Changes: Deletion, Duplication, Inversion and/or Insertion.

### Some Commonly used Types of Genetic Markers

**RFLP** (Restriction Fragment Length Polymorphism)

**SSLP** (Simple Sequence Length Polymorphism)

**AFLP** (Amplified Fragment Length Polymorphism)

NOTES

**RAPD** (Random Amplification of Polymorphic DNA)

**VNTR** (Variable Number Tandem Repeat)

**SSR** (Simple Sequence Repeat) Microsatellite Polymorphism

**SNP** (Single Nucleotide Polymorphism)

**STR** (Short Tandem Repeat)

**SFP** (Single Feature Polymorphism)

**DArT** (Diversity Arrays Technology)

**RAD Markers** (Restriction site Associated DNA Markers)

**Applications of Genetic Markers**

- Genetic markers can be used to study the relationship between an inherited disease and its genetic cause (for example, a particular mutation of a gene that results in a defective protein). It is known that pieces of DNA that lie near each other on a chromosome tend to be inherited together. This property enables the use of a marker, which can then be used to determine the precise inheritance pattern of the gene that has not yet been exactly localized.
- RFLP analysis was the first DNA profiling technique for genetic fingerprinting and paternity testing. Presence and absence of fragments resulting from changes in recognition sites are used for identification of species or populations.
- By studying the RFLP pattern one can detect the presence of a genetic disease in a certain individual, localization of genes for genetic disorders, determination of risk for disease.
- Some of the methods used to study the Genome or Phylogenetics are RFLP, AFLP, RAPD, and SSR. They can be used to create genetic maps of whatever organism is being studied.
- For identification of bacterial infections in clinical laboratories, the Culture Method is used. Culture is the most sensitive method for identification of bacterial infection in clinical samples, but culture requires at least 16 hours of incubation time. On the other hand additional time is needed to perform the biochemical or immunological tests to identify the bacteria. The 16S rRNA genes of almost all the common bacterial pathogens found in the body fluids have been sequenced.

RFLP analysis is extensively used in molecular biology for detecting variation at the DNA sequence level of various biological samples. RFLP is specific to a single clone/restriction enzyme combination and it occurs when the length of a detected fragment varies between individuals. It is highly locus-specific.

In PCR-RFLP method, only one set of universal primers is used for the amplification of the conserved region of the 16S rRNA gene, in common bacterial

pathogens. PCR products from different individuals are then digested with one or more restriction enzymes and the resulting restriction digestion profiles are compared. Since RFLP patterns of the universal PCR products from different bacterial species are different, this method is expected to identify bacteria.

In this experiment, we are using Universal PCR-RFLP method to check Genetic Markers in Isolated Colony from clinical sample. Procedure/Methods involves following four steps.

**Procedure/Methods**

I. Single Colony Isolation
II. Extraction of Genomic DNA
III. PCR Amplification
IV. Restriction Endonuclease Digestion

**I. Single Colony Isolation**

A transfer of inoculum using the needle is called streaking – a Petri plate so prepared is a streak plate. Streaking is one of the method to obtain pure culture in the form of single isolated colony. On solid medium the bacteria produce a fixed colony of cells that grows to form a visible mass. The separation of single cells from the original inoculum into distinct colonies, such as the **Colony Forming Units (CFU)** which is a fundamental principle for both isolation, characterization and counting of bacteria in cultures.

In this experiment, an amount of bacterial cells from clinical sample of CSF (the Inoculum) is transferred (Inoculated) onto the sterile medium using streak plate method.

**Materials Required**

**Sample:** Cerebrospinal Fluid

**Media:** LB Agar Plate

**Instrument:** Incubator, Laminar Flow

**Other Requirements:** Inoculating Loop, Burner

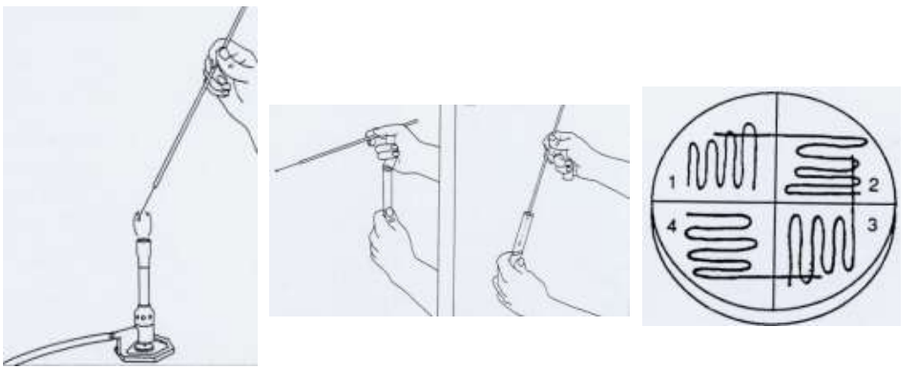
**Procedure**

1. The inoculating loop should be heated by flaming immediately before and after making the transfer. Hold the needle downwards and into the flame, to heat both the needle and lower part of the handle. Refer Figure 1(a).
2. During transfer, hold the tube from which culture has to be taken in the left hand and hold the plug or cap between the fingers of the right hand.

**Caution:** Never lay a plug or cap down! Hold the tube as nearly horizontal as feasible during transfer and do not leave it open longer than necessary. The mouths

of the tube should also be passed through the flame immediately before and after the needle is introduced and removed. Refer Figure 1(b).

3. Transfer a drop of culture at one edge of the agar using the sterile loop. Flame the streaking loop and cool it by jabbing it into another edge of the agar.
4. With the drop of bacteria, streak the culture back and forth from edge to edge in 2 or 3 parallel lines, moving toward you. Be careful not to plunge the loop deep into the agar during streaking. Flame the loop, make another 2 or 3 streaks at about 60°-90° to the first. Make another two repeats of this procedure: flame and streak. Refer Figure 1(c).
5. Keep the streak plate overnight at 37°C.
6. Observe the plate for isolated single colony and extract genomic DNA from single colony as given below.




*(a)* *(b)* *(c)*

***Fig. 1*** *Streaking Method*

## II. Genomic DNA Extraction from Single Colony

1. Pick single colony from the above plate and inoculate in 250 ml flask containing 50 ml LB medium.
2. Keep the flask at 37°C for 24 hours.
3. Extract genomic DNA from overnight grown culture as given in the Experiment 11.
4. Load the isolated Genomic DNA sample on 0.8 % Agarose Gel and check the profile under UV Transilluminator.
5. Quantify the extracted DNA as per Experiment 12.
6. Use extracted DNA for PCR amplification as given below.

## III. PCR Amplification

The conserved fragments of the 16S rRNA genes of the 'Isolated Bacterium' was amplified by the universal PCR method.

**NOTES**

## Materials Required

**Sample:** Genomic DNA

**Chemicals and Reagents:** PCR Buffer, Milli Q Water, Genomic DNA, Forward Primer, Reverse Primer, dNTP Mix, Taq DNA Polymerase

**Instruments:** Thermocycler (PCR Machine)

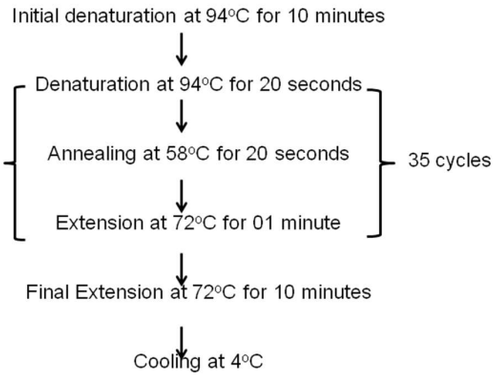
**Other Requirements:** Crushed Ice, PCR Tubes, Micropipettes, Tips

## Steps for PCR

1. Preparation of master mix for PCR is done as follows:

| Sr. No. | Ingredients for PCR | Stock Concentration | Working Concentration | Volume in µl |
|---|---|---|---|---|
| 1 | Milli Q Water | | | 30.5 µl |
| 2 | 10X PCR Buffer (Containing $MgCl_2$) | 10X | 1X | 5 µl |
| 3 | Template DNA | 2.5 µg/ml | 50 ng | 1 µl |
| 4 | Forward Primer | 1 nM | 20 pmol | 1 µl |
| 5 | Reverse Primer | 1 nM | 20 pmol | 1 µl |
| 7 | dNTP Mix | 25 mM | 2.5 mM | 5 µl |
| 8 | Taq DNA Polymerase | 12.5 Units | 2.5 Units | 1.0 µl |
| | Total Volume | | | 50 µl |

2. Tap the PCR tube for 1–2 seconds to mix the contents gently and keep them onto crushed ice until put in thermocycler.
3. Place the tube in the thermocycler block and set the program to get DNA amplification as follows.




4. Ten microliters of PCR product was electrophoresed on 1% agarose gel to determine the size of the product. DNA from some American Type Culture Collection Control and CDC bacteria were examined by the universal PCR.

***Note:*** How to perform agarose gel electrophoresis is discussed in Experiment 12.

**Universal Primers used in PCR**

One pair of primers, designated **Ul** and **U2**, with sequences conserved among all of these bacteria was selected.

**Forward Primer (U1)**

The **sequence** of the **forward primer** is **5'-CCAGCAGCCGCGGTAATACG-3'**, corresponding to **nucleotides 518 to 537 of the *Escherichia coli*** or ***E coli* 16S rRNA Gene.**

**Reverse Primer (U2)**

The **sequence** of the **reverse primer** is **5'-ATC GG(C/T) TACCTTGTTACGACTTC-3'**, corresponding to **nucleotides 1513 to 1491** of the same Gene.

**Observation and Result**

The size of the Universal PCR Product is 996 bp (Lane 2). A nonspecific band of approximately 160 bp was also produced as shown below in Figure 2.

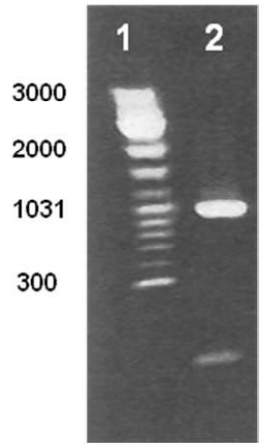



***Fig. 2*** *PCR Products Electrophoresed on 1% Agarose Gel of Universal PCR Products*

Figure 2 illustrates the PCR Products Electrophoresed on 1% Agarose Gel of Universal PCR Products. The Lane 1 is Marker, containing molecular size standards (Base Pairs). Lane 2 is *Escherichia coli.* The size of Universal PCR Product is around 1000 bp. The sizes of the molecular size standards are marked on the left of the gel.

**IV. Restriction Endonuclease Digestion**

The PCR product was digested to determine whether there is a restriction fragment length polymorphism that can be used to identify certain bacteria. For bacterial identification, the Universal PCR Products were digested into several fragments by BsuRI (Hae III) Restriction Enzyme.

## Materials Required

PCR Product, BsuRI (Hae III) Restriction Enzyme, Restriction Enzyme Buffer (R-Buffer), Milli Q Water.

## Steps for Digestion

1. Before starting the experiment, crush ice and place the vials containing PCR Product, Restriction Enzymes and Assay Buffers onto it.
2. Set up the reaction mixture as follows:

| | |
|---|---|
| Milli Q Water | 13.5 µl |
| PCR Product | 8.0 µl |
| BsuRI (Hae III) Restriction Enzyme | 1.0 µl |
| Restriction Enzyme Buffer (R-Buffer) | 2.5 µl |
| Total Volume | 25 µl |

3. After preparing the reaction tubes, mix the components by gentle pipetting and tapping.
4. Incubate the tubes at recommended temperature for 2 hours.
5. Electrophorese Digested DNA on 2% Agarose Gel.

***Note:*** Perform Agarose Gel Electrophoresis as discussed in Experiment 12.

6. Visualize the DNA bands using UV Transilluminator. Compare the restriction profile of unknown sample with that of the reference samples.

## Observation and Result

The RFLP pattern of unknown sample matches with that of reference standard strain of *Streptococcus pyogenes* or *S. pyogenes*. As the restriction enzyme recognizes and digests a particular sequence, any slight change in that results in different restriction profile of a particular sample, as shown in Figure 3.

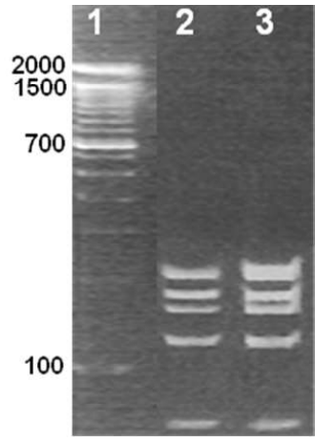



***Fig. 3*** *Hae III Digestion Pattern of Standard Bacteria and CSF Sample Universal PCR Products*

Figure 3 illustrates the Hae III Digestion Pattern of Standard Bacteria and CSF Sample Universal PCR Products. Lane 2 is *Escherichia coli* (CSF sample), Lane 3 is *Escherichia coli* standard sample, while Lane 1 is marker that contained molecular size standards (Base Pairs). The sizes of the molecular size standards are marked on the left of the gel.

**Interpretation**

CSF sample from patient suffering from '**Bacterial Meningitis**' was taken and streaked on LB Agar to get Single Isolated Colony. DNA was extracted from this colony and checked for Genetic Marker using Universal PCR-RFLP method. This method is based on conserved sequence of I6S rRNA Genes of Bacteria. This conserved region could be amplified by PCR, using Universal Primers. Subsequently digestion of this region by restriction enzymes showed unique restriction pattern which matches with a reference ATCC Standard Bacterium taken in the study. Therefore, Universal PCR followed by RFLP, could be considered as a simple, sensitive and rapid method for detection and identification of bacterial pathogens in clinical samples.

This method requires only 1 day to complete. Conventional methods for detection and identification of bacterial pathogens requires at least 2-3 days. The Universal PCR method will provide physicians with results at least 1 day earlier than conventional methods. Although the cost of using the Universal Primer PCR for diagnosis is higher than the conventional methods, the Universal Primer PCR coupled with Restriction Enzyme Analysis can rapidly detect and identify pathogens so that the unnecessary use of broad-spectrum antibiotic therapies can be minimized.

**Precautions**

1. Because of a high concentration of water in the Petri plates, condensation forms during incubation. Moisture is likely to cover the Agar surface resulting in confluent mass of growth rather than well-separated colonies. To avoid this, Petri plates are routinely incubated bottom-side up.
2. The Restriction Enzymes, Assay Buffer are temperature sensitive and should always be placed on ice during the experiment.
3. Ensure that all the components are thoroughly mixed by gentle pipetting after preparing the reaction mixture.
4. Always place the vials containing restriction enzymes on ice as they are temperature sensitive and may lead to degradation of restriction enzyme.
5. Use fresh tip while adding different solution to the tube.
6. While preparing the reaction mixture the enzymes should always be added at last.

# EXPERIMENT 2: MEASUREMENT OF GROWTH - ONE STEP GROWTH CURVE USING A 'T' EVEN PHAGE

NOTES

## AIM: Measurement of Growth - One Step Growth Curve using a 'T' Even Phage

### Theory

Phage infection starts by first attaching to the host known as '**adsorption**' and then injecting its nucleic acid into the cell. Just after the penetration of phage, they start actively transcribing and replicating inside the host. During this period all **Structural Proteins** and **Viral Genomes** are produced in the **cytoplasm** of the host cell and because of this, no free virions are detected in medium. This is known as **Eclipse Period** which can vary from minutes to hours depending on the virus. After maturation of new viral particles they exit by bursting the host cell in case of **Lytic Phages**. Maximum number of phage particles produced per bacterium is known as **Burst Size**. The cycle can then start over infecting new cells, the shape of the growth curve would look step-wise and hence termed as '**One-Step Phage Growth Curve**'.

At the start of the experiment, the plaque count is relatively constant over a time period because each **infected bacterium** will yield only **one plaque**. A rise in **Plaque Forming Units (PFU)** to a plateau level occurs as bacteria are lysed and the newly synthesized phage are released into the medium.

### Materials Required

*Escherichia coli* Culture ($2 \times 10^8$ CFU/ml), T4 Phage Lysate ($4 \times 10^6$ PFU/ml), Soft Agar Tubes, Nutrient Broth (9.9 ml/Tube), Sterile Pipettes, Water Bath maintained at 37°C, 45°C. The CFU refers to Colony Forming Unit.

### Procedure

1. Take 0.9 ml of *Escherichia coli* culture and add 0.1 ml of the Phage Lysate. Mix well and incubate the tube at 37°C Water Bath. Record the exact time.
2. At precisely after 20 minutes of the experiment, remove 0.1 ml of suspension mixture by transferring it to the tube marked $10^{-2}$. Mix well by vortexing.
3. Transfer 0.1 ml from $10^{-2}$ tube to tube marked as $10^4$ tube. Mix meticulously by vortexing and incubate the tubes at 37°C for 10 minutes.
4. After the incubation period, add 0.1 ml from the $10^{-4}$ tube and 0.1 ml of the host culture to a Soft Agar Tube (melted and maintained at 45°C).
5. Quickly mix thoroughly by rolling the tube in the palms of your hands, and pour the Soft Agar onto a base agar plate labeled as '20 Minutes'.

6. Rotate the plate gently so that Soft Agar is spread evenly on the agar plate surface. Allow the soft agar mixture to solidify, incubate the plates at 37°C overnight in inverted position.
7. In similar manner, repeat the above two steps at 25, 30, 35, 40, 45, 50, 55, and so on, till 80 minutes.
8. After 24 hours of incubation count the number of plaques to calculate 'Plaque Forming Units', i.e., PFU/ml for each time point.

## Observation and Results

1. **Multiplicity (Ratio) of Infection:** This is referred as the number of phage available to infect each bacterium. It can be calculated as Number of Phage/ Number of Bacteria in the 'Adsorption Mixture'.
2. **Relative Titers:** First or the average of the first few counts can be taken as the base line for **Eclipse Period**. Divide all other counts by this base line to obtain the Relative Titers. Record your observations in the following table.

| Incubation Time | Number of Plaques Observed on Plate | PFU/ml | Relative Titers |
|---|---|---|---|
| 20 | | | |
| 25 | | | |
| 30 | | | |
| 35 | | | |
| 40 | | | |
| 45 | | | |
| 50 | | | |
| 55 | | | |
| 60 | | | |
| 65 | | | |
| 70 | | | |
| 75 | | | |
| 80 | | | |

3. **Burst Size:** The Maximum Relative Titer is the Burst Size for the Phage.
4. **Plotting the Graph:** Plot a graph between the relative titers on Y-axis over time on X-axis to observe phage 'One Step Growth Curve'.

# EXPERIMENT 3: TITRATION OF PHAGES (T4)

NOTES

## AIM: Titration of Phages (T4)

### Theory

When a suspension of an **infective phage**, viz., **T2**, **T4** phage, is spread over the lawn of susceptible bacterial cells, for example *Escherichia coli*, then the phage attaches the bacterial cell, replicate inside it, and kills it during its Lytic Release. Lysis of the bacteriophage is indicated by the formation of a zone of clearing or plaque within the lawn of bacteria. Each plaque corresponds to a site where a single bacteriophage infected and initiated lytic cycle. The spread of infection by phage to the surrounding bacterial cells results in the lysis in the locality, eventually leading to the development of a plaque. Phage titration or concentration of phages can be determined by double agar overlay method. For this, phage from $10^{-1}$ through $10^{-6}$ fold serial dilutions incubated with bacterial culture in a Soft Agar that forms the upper overlay and the Hard Agar serves as a base layer. Soft Agar permits easy diffusion to nearby uninfected cells by a phage, because the medium used in phage plaque assays has a relatively low percentage of agar and therefore is called soft agar, it permits diffusion of phage to nearby uninfected cells but does not permit new phages to move to remote parts of the plate.

### Materials Required

**Equipment:** Bunsen Burner, Water Bath, Thermometer, 1 ml Sterile Pipettes, Sterile Pasteur Pipettes, Mechanical Pipetting Devices, Test Tube Rack.

**Nutrient Agar:** 15 g Agar, 2.0 g Yeast Extract, 1.0 g Beef Extract, 5.0 g Peptone, 5.0 g NaCl, 1000 ml Dilute $H_2O$.

**Nutrient Soft Agar:** 7 g Agar, 2.0 g Yeast Extract, 1.0 g Beef Extract, 5.0 g Peptone, 5.0 g NaCl, 1000 ml Dilute $H_2O$.

**Saline Solution:** 0.85 % NaCl made in Dilute $H_2O$.

### Procedure

1. Place seven sterile tubes with 4.5 ml of Saline in each tube. Label one tube as 'Saline Control' and the remaining tubes from $10^{-1}$ to $10^{-6}$.
2. Likewise label Nutrient Agar Plates for each dilution.
3. Using a sterile 1 ml pipette, aseptically transfer 0.5 ml of the Bacteriophage suspension to the saline tube marked as $10^{-1}$. Mix the tube well by vortexing.
4. Transfer 0.5 ml from the $10^{-1}$ tube to $10^{-2}$ tube and vortex mix.
5. Similarly continue making serial dilutions for the remaining saline tubes till $10^{-6}$.

6. Dispense melted 3 ml of soft agar in sterile tubes and equilibrate in water bath at 45°C till further use.
7. In a microcentrifuge tube, take 0.3 ml of *Escherichia coli* culture ($OD_{600}$ ~ 0.5) and add 0.1 ml of the $10^{-1}$ phage dilution already made.
8. Mix well and transfer the contents into a soft agar tube. Mix by rolling in between the palms.
9. Quickly, pour the soft agar mix onto the surface of the Nutrient Agar Plate. Rotate the plate gently in circular motion on the surface of the table to evenly distribute the soft agar.
10. Allow the soft agar to solidify.
11. In the same way, plate all the remaining dilutions on accordingly labeled nutrient agar plates.
12. Invert and incubate the plates for 24 hours at 37°C.

## Observation and Results

1. After incubation, count the number of plaques on each plate.
2. Plates having more than 300 plaques should be recorded as TNTC (Too Numerous To Count).
3. Calculate the number of Lytic Phages expressed as Plaque Forming Unit per ml (PFU/ml) using the formula:

$$\textbf{PFU/ml} = \frac{\textbf{Number of Plaques}}{\textbf{Dilution Factor} \times \textbf{Volume of Diluted Virus Added}}$$

Record the 'Results' as follows:

| Dilution of Phage | Number of Plaques | PFU/ml |
|---|---|---|
| $10^{-1}$ | | |
| $10^{-2}$ | | |
| $10^{-3}$ | | |
| $10^{-4}$ | | |
| $10^{-5}$ | | |
| $10^{-6}$ | | |

## Precautions

1. Mix each tube well before and after making the dilutions.
2. Work quickly so that Soft Agar does not solidify before the final plating on Nutrient Agar.
3. Perform the steps aseptically.

# EXPERIMENT 4: INDUCTION OF LAMBDA (λ) PHAGE

NOTES

## AIM: Induction of Lambda (λ) Phage

### Theory

Temperate Bacteriophage can replicate either by a Lytic Growth Cycle eventually lysing the host cell, or by a Lysogenic Cycle in which the Phage DNA is incorporated as a prophage into the Host Cell Genome. The 'Decision' to undergo either a Lytic or Lysogenic Cycle by '**Bacteriophage λ**' in host, is regulated by an intricate regulatory mechanism that is itself influenced by the physiological state of the host cell. Entry into the Lysogenic Cycle is primarily regulated by λ repressor commonly referred to as **CI Protein** which represses the expression of other λ Genes, preventing the prophage from initiating Lytic Growth. The CI protein renders a 'Lysogen Immune' to subsequent infection by other λ particles, not allowing the new phage DNA replicate which is ultimately degraded. Under certain circumstances like host DNA damage or exposure to UV, phage can abandon Lysogeny and initiate Lytic Cycle by killing the host in order to find a new better suited for replication. This process known as induction and can be observed as clear plaques on bacterial plate.

### Materials Required

*Escherichia coli* Culture, Lambda Phage Lysate, Luria Bertani (LB) Broth, LB Agar Plates, 3 µl Soft Agar Tubes, Sterile Pipettes.

### Procedure

1. To obtain Lysogens, add 0.1 ml of actively growing *Escherichia coli* culture to a 3 ml Molten Soft Agar tube and then roll between the palms and immediately pour over a LB Agar Plate (as in case of phage titration). Let the plate stand for 5 minutes to solidify.
2. Put a 5 ml drop of Phage λ Lysate to the surface of the solidified above mentioned Double Agar Plate. Allow the spot to dry for 10-15 minutes, incubate the plate in upright position at 37°C for 24 hours.
3. From a plaque obtained with a turbid center which is due to the unlysed bacterial cells Lysogenized by λ phage, streak the culture with a loop on a fresh LB Agar plate. Incubate the plate at 37°C overnight.
4. Streak the isolated colonies obtained in the grid pattern on a fresh LB Agar plate.
5. Make two double Agar plate as described in Step 1 using non lysogenic *Escherichia coli* culture.
6. Patch each isolated colony by jabbing gently at same position on both the plates. Incubate overnight at 37°C.

7. Expose one plate under a UV Lamp, after removing the lid, for 10-15 seconds to induce phage excision. The second plate unexposed to UV will act as un-induced control.
8. Incubate both the plates at 37°C and record the results for the plaques obtained.

**Result**

| Isolate Number | Plaque Formation (+ or –) After UV Treatment | Plaque Formation (+ or –) After with No UV Treatment |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |
| 7 | | |
| 8 | | |
| 9 | | |
| 10 | | |
| 11 | | |
| 12 | | |

Lysogenic Bacteria can be seen as Turbid Plaque whereas UV Induced Clones would be seen as Clear Plaque due to localized Cell Host Lysis. However, clear plaques if observed on the unexposed plate could be because of spontaneously induced phages.

## EXPERIMENT 5: INDUCED MUTAGENESIS – UV RADIATION

**AIM: To study Induced Mutagenesis by UV Radiation**

**Theory**

Genes are normally copied exactly during Chromosome Duplication. Rarely, however, Changes (Mutations) occur in Genes to give rise to altered forms, most of which function less well than the wild-type Alleles. There is, instead, a strong advantage in there being a small but finite mutation rate, as it provides a constant source of new variability, necessary to allow organisms to adapt to a constantly changing physical and biological environment.

**Mutation** is referred as a heritable change in **DNA Sequence** of the **Genome** of an organism, which may lead to a change in **Phenotype**. An organism whose **Genome** carries **Mutation** is called a **Mutant.**

For a typical bacterium, mutation rates of $10^{-6}$ to $10^{-7}$ per kilo Base Pair are generally observed. The following are the standard classifications of mutation.

### Classification Based on Location of Mutation

Mutations may be classified according to the cell type or chromosomal locations in which they occur.

- **Somatic Mutations:** These mutations are those which are occurring in any cell in the body except Germ Cells. Mutations arising in somatic cells are not transmitted to future generations. **Mutations** in **germ cells** are of greater significance because they may be **transmitted** to **offspring** as **gametes**.
- **Autosomal Mutations**: These are mutations within genes located on the autosomes.
- **X-Linked and Y-Linked Mutations:** These are those mutations which are within the Genes located on the X Chromosome or Y Chromosome, respectively.

### Classification Based on Type of Molecular Change

Geneticists often classify Gene Mutations in terms of the Nucleotide Changes that constitute the Mutation.

- **Point Mutation**: This is caused due to a change in a single base pair, can lead to a single amino acid change in a polypeptide or to no change at all, depending on the particular codon. If a **pyrimidine replaces a pyrimidine** or **a purine replaces a purine**, then a **transition** has occurred. If **a purine replaces a pyrimidine**, or vice versa, then a **trans-version** has occurred. Point mutations are simple changes in single base pairs, the substitution of one base pair for another, or duplication or deletion of single base pairs.
- **Nonsense Mutation:** In it the codon for an amino acid is changed to a stop codon (UAA, UAG, or UGA), and an incomplete polypeptide is made.
- **Missense Mutation:** Mutation in which a single codon is altered so that one amino acid in a protein is replaced with a different amino acid.
- **Silent Mutation:** If the point mutation alters a codon but does not result in a change in the amino acid, hence no effect on the phenotype, at that position in the protein (due to degeneracy of the genetic code), it can be considered a silent mutation.
- **Frame Shift Mutations:** It arises from the insertion or deletion of one or two base pairs within the Coding Region of the Gene. Since the codon consists of precise sequence of triplet codons, the addition or deletion of

fewer than three pairs will cause the reading frame to be shifted for all codons downstream. Frame shift mutations usually are very deleterious and yield mutant phenotypes resulting from the synthesis of non-functional proteins. In addition, frame shift mutation often produces a nonsense or stop codon so that the peptide product is shorter as well as different in sequence.

## Mutations can be Classified as either Spontaneous or Induced Type Based on Occurrences

- **Spontaneous Mutations:** A mutation without a known cause is called Spontaneous Mutations. No specific agents are associated with their occurrence, and they are generally assumed to be accidental. Many arise as a result of normal biological or chemical processes in the organism that alter the structure of Nitrogenous Bases. Often, these mutations occur during the Enzymatic Processes of DNA Replication. These mutations occur at low frequency.
- **Induced Mutations:** These mutations may be the result of a variety of chemical, physical, and biological agents that can increase the mutation rate and are therefore said to Induce Mutations. These agents are called **Mutagens.** These can be either chemical or physical agents. Chemical mutagens induce point mutations, whereas ionizing radiations gives rise to large chromosomal abnormalities.

  An overview of some of the major physical and chemical mutagens with their modes of action is given in Table 1.

***Table 1:*** *Chemical and Physical Mutagens and Their Modes of Action*

| Agent | Action | Result |
|---|---|---|
| **A. Chemical Agents** | | |
| **1. Base Analogs** | | |
| 5-Bromouracil | Incorporated like T; occasional faulty pairing with G | AT to GC and occasionally GC to AT |
| 2-Aminopurine | Incorporated like A; faulty pairing with C | AT to GC and occasionally GC to AT |
| **2. Chemicals Reacting with DNA** | | |
| Nitrous acid ($HNO_2$) | Deaminates A and C | AT to GC and GC to AT |
| Hydroxylamine ($NH_2OH$) | Reacts with C | GC to AT |
| **3. Alkylating Agents** | | |
| Monofunctional (for example, Ethyl Methanesulfonate) | Puts Methyl on G; faulty pairing with T | GC to AT |
| Bifunctional (for example, Mitomycin, Nitrogen Mustards, Nitrosoguanidine) | Cross-Links DNA Strands; faulty region excised by DNase | Both Point Mutations and Deletions |
| **4. Intercalating Dyes** | | |
| Acridines, Ethidium Bromide | Inserts between two Base Pairs | Microinsertions and Microdeletions |
| **B. Physical Agents** | | |
| **Radiation** | | |
| 1. Non Ionizing Radiation (Ultraviolet Rays) | Pyrimidine Dimer Formation | Repair may lead to Error or Deletion |
| 2. Ionizing Radiation (for example, X-Rays) | Free Radical attack on DNA, cause Base Deletion, Single Strand Nicks, Cross-Linking and Chromosomal Breaks | Repair may lead to Error or Deletion |

## Radiations

Radiations can be divided into two main categories: Nonionizing and Ionizing radiation, as shown in Figure 1. Although both kinds of radiation are used to generate mutations, nonionizing radiation, such as UltraViolet (UV) radiation has the widest use.

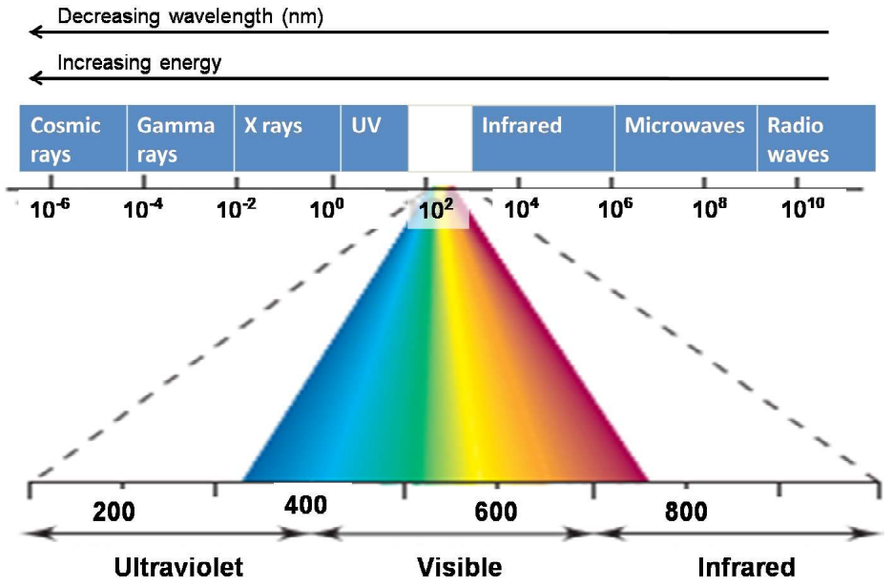



***Fig. 1:*** *Regions of the Electromagnetic Spectrum and their Associated Wavelengths*

Figure 1 illustrates the regions of the electromagnetic spectrum and their associated wavelengths. Ultraviolet radiation consists of wavelengths just shorter than visible light. For any electromagnetic radiation, the shorter the wavelength, the higher the energy. DNA absorbs strongly at 260 nm.

## Ionizing Radiation

Ionizing radiation is a more powerful form of radiation than UV radiation and includes short wavelength rays, such as X-Rays, Cosmic Rays, and Gamma Rays (Refer Figure 1). As a result, they penetrate deeply into tissues, causing ionization of the molecules encountered along the way. Among the potent chemical species formed by ionizing radiation are chemical free radicals, the most important being the hydroxyl radical, **OH•**. Free radicals react with and damage macromolecules in the cell, including DNA. This causes double stranded and single stranded breaks that may lead to rearrangements or large deletions. At low doses of ionizing radiation only a few 'hits' on DNA occur, but at higher doses, multiple hits cause fragmentation of DNA that sometimes cannot be repaired and thus leads to the death of the cell.

In contrast to UV radiation, ionizing radiation penetrates readily through glass and other materials. Therefore, ionizing radiation is used frequently to induce

mutations in animals and plants because its penetrating power makes it possible to reach the gamete producing cells of these organisms. However, because ionizing radiation is more dangerous and is less readily available than UV radiation, it finds less use in microbial genetics.

## Nonionizing Radiations

Ultraviolet (UV) radiation is an electromagnetic radiation with a wavelength from 10 nm to 400 nm (shorter than that of visible light). UV radiation is present in sunlight and also produced by electric arcs, specialized lights, such as mercury lamps. UV radiation is nonionizing in nature. It does not produce ions. The '**Purine**' and '**Pyrimidine**' absorb '**UV Radiation**' most strongly, at a **wavelength** of about **260 nm**. One consequence of which is the **photochemical fusion** of **two pyrimidine** that occupy adjacent positions on the same **polynucleotide chain**. In the case of **two thymine**, the fusion is called a **thymine dimer**, which comprises a **Cyclobutane Ring** generated by covalent bond between **carbon atoms 5** and **6** of **Adjacent Thymine** (Refer Figure 2). In the case of a thymine adjacent to a cytosine, the resulting fusion is a **Thymine – Cytosine** adduct in which the **Thymine** is linked via its carbon atom 6 to the carbon atom 4 of **Cytosine**.

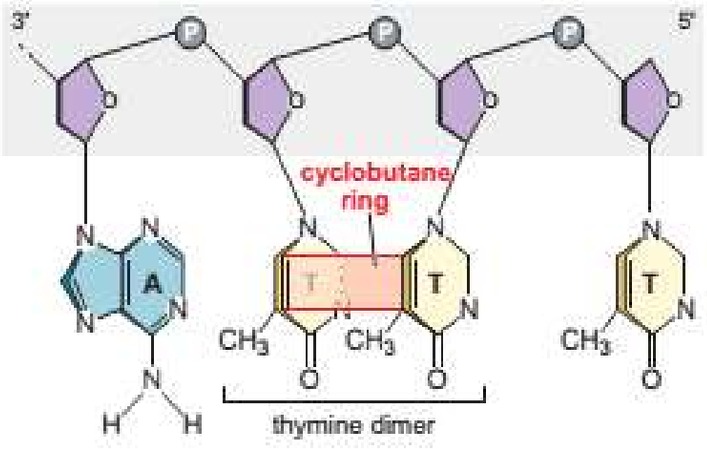



***Fig. 2:*** *Thymine Dimer*

Figure 2 illustrates the 'Thymine Dimer'. The 'Ultraviolet Light' induces the formation of a Cyclobutane Ring between Adjacent Thymine.

The dimers distort the DNA conformation. As a result of the distortion, hydrogen bonding to adenines in the opposing strand is significantly weakened; this structural distortion blocks the growing replication fork. This results either in impeding DNA polymerase or in a greatly increased probability of DNA polymerase misreading the sequence at this point. As a result, errors can be introduced in the base sequence of DNA during replication.

The **UV Radiation source** most commonly used for **mutagenesis** is the **Germicidal Lamp**, which emits UV radiation in the 260 nm region. A dose of

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UV radiation is used that kills about 50–90% of the cell population, and mutants are then selected or screened for among the survivors. If much higher doses of radiation are used, the number of surviving cells is too low. If lower doses are used, damage to DNA is insufficient to generate enough mutations. When used at the correct dose, UV radiation is a very convenient tool for isolating mutants and avoids the need to handle toxic chemicals.

**Materials Required**

**Culture:** *Escherichia coli* Culture

**Media:** Nutrient Agar Plates

**Equipment:** Laminar Flow, UV Lamp

**Glassware and Miscellaneous:** Glass Spreaders, Sterile Tips, Sterile Eppendorf Tubes, Micropipettes, Eppendorf Stand, Distilled Water, Protection Goggles and Gloves, Stopwatch

**Principle**

When **bacteria** are **exposed** to **UV radiations**, they lose the ability to form colonies, generally because of **formation of thymine dimers** which block the activity of **DNA Polymerase**. The loss of viability can be exposed graphically by plotting the fraction of initial population that survives various exposure to radiation against some measure of exposure, i.e., radiation dose per time of exposure. Such a graph is called **Survival Curve.** Samples are removed at intervals from a population of bacteria that is being irradiated. The samples are plated on a Nutrient Agar and the colonies that form are counted. Number of Colonies Forming Units (CFU/ml) is plotted as a function of the dose, assuming that one CFU represents one Bacterium.

Formation of thymine dimmers present the example of **Lethal Mutations**, which prevent the reproducing capability of the organism, and when expressed, results in the death of the microorganism.

**Lethal Dose:** The dose at which 90% of the bacteria are killed is called a **Lethal Dose**. This means that the chance of survival is only 10%.

**Sub-Lethal Dose**: The dose at which 50% of the bacteria are killed is called a **Sub-Lethal Dose**.

**Decimal Reduction Time:** When testing a controlling effect on bacteria in the laboratory, the number of cells surviving the treatment will typically be determined by Plate Counts (Colony Forming Units, CFU/ml). When the CFU number is plotted against the exposure time, the **Decimal Reduction Time**, the '**D Value**' can be determined. D is the time required for a 10-fold reduction in population density at a given treatment. The decimal reduction means that when D is tested at different periods of heating or UV radiation, relationship between D and heat or UV will be an exponentially decreasing relation.

## Procedure

1. Nutrient broth was inoculated with given bacterial culture and incubated at 37°C for 4-5 hours.
2. The Laminar was switched on. Its surface was cleaned with spirit swab and UV sterilized for 5-10 minutes.
3. 100 µl of the culture was pipette (from Step 1) and transferred to 900 µl of distilled water taken in an Eppendorf Tube. Culture mixed by vortexing to ensure that the cells are evenly distributed in the tube. This led to creation of $10^{-1}$ dilution. 100 µl of $10^{-1}$ dilution was pipetted and transferred to 900 µl of distilled water taken in another Eppendorf Tube. This led to creation of $10^{-2}$ dilution. Similarly, dilution up to $10^{-4}$ was made.
4. With a sterile micropipette tip, transfer 100 µl of $10^{-4}$ dilution of the sample onto each of the Agar Plates. Spread immediately the sample evenly on top of the Agar with a sterile glass spreader.
5. All the Nutrient Agar Plates were exposed to UV lamp simultaneously. A stop watch was started. Exactly after 10 seconds, the UV lamp was switched off and one plate was taken out from the Laminar. It was marked as '10 Second Plate' and UV was switched on again.
6. After 20 seconds, it was again switched off and another nutrient agar plate was taken out from the laminar. It was marked as '20 Second Plate'.
7. The same procedure was repeated after every 10 seconds till 100 seconds.
8. The plates were then incubated at 37°C for 24 hours.

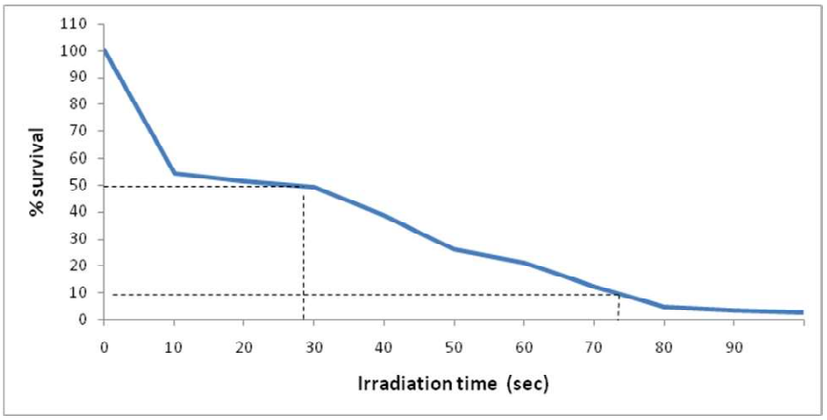
## Observation

The number of CFU observed on plates after UV treatment are recorded as follows:

| Time (in Second) | No. of Colonies | CFU/ml | % Cell Survival |
|---|---|---|---|
| Control (No Exposure) | 580 | $5.8 \times 10^3$ | 100 |
| 10 | 315 | $3.15 \times 10^3$ | 54 |
| 20 | 298 | $2.98 \times 10^3$ | 51 |
| 30 | 287 | $2.87 \times 10^3$ | 49 |
| 40 | 224 | $2.24 \times 10^3$ | 38 |
| 50 | 152 | $1.52 \times 10^3$ | 26 |
| 60 | 122 | $1.22 \times 10^3$ | 21 |
| 70 | 71 | $0.71 \times 10^3$ | 12 |
| 80 | 28 | $0.28 \times 10^3$ | 5 |
| 90 | 21 | $0.21 \times 10^3$ | 4 |
| 100 | 16 | $0.16 \times 10^3$ | 3 |

1. Count the number of colonies on each plate of and plot a graph for % Survival (Y-axis) and Irradiation Time (Time of Exposure of UV Light on X-axis), as shown below.

   **% Survival = Number of UV Exposed Cells/Unexposed Cells × 100**




2. Calculate the CFU/ml and plot a graph between CFU/ml (Y-axis) and Time of Exposure (X-axis) on a semi-log graph paper. Calculate the 'D Value' from the graph plotted.

## Results

The effect of exposing a culture of *Escherichia coli* with UV radiation over increasing periods of time is clearly indicated in Table as well as in graph plotted. Initial number of cells of 5.8 x $10^3$ was reduced to 0.16 x $10^3$ after 100 seconds of radiation.

According to the graph following results are obtained:

1. Lethal Dose (Time for Killing 90% of the Population) is 73 seconds.
2. Sub-Lethal Dose (Time for Killing 90% of the Population) is 29 seconds.
3. The 'D Value' (Time Interval in which Population Reduces by '1 Log Cycle', i.e., 10 Times) as observed from semi-log graph is ……..

## Discussion

In order to study microbial mutants, one must be able to detect them readily, even when they are rare, and then efficiently isolate them from wild-type organisms and other mutants that are not of interest. Microbial geneticists typically increase the likelihood of obtaining mutants by induced mutagenesis to increase the rate of mutation from the usual one mutant per $10^7$ to $10^{11}$ cells to about one per $10^3$ to $10^6$ cells.

In this experiment we induced mutations in *Escherichia coli* bacterial cells using UV radiations. UV radiations produced lethal mutations which prevent the reproducing capability of the organism, and results in the death of the bacterial populations.

The loss of viability, was expressed graphically by plotting the fraction of initial population that survives against various radiation exposure time, assuming that one CFU represents one bacterium.

Initially the curve of an ultraviolet light survival curve is fairly flat because initial damage does not cause killing. As the time of exposure to UV radiation increases, the number of colonies obtained on nutrient agar plates decreases. Longer exposure to UV is very harmful to bacteria since chance of formation of pyrimidine dimmers or mutation in bacteria increases. These linked bases are incapable of base pairing and cause the DNA polymerase to stop during replication. Hence, when bacteria are exposed to UV, they lose the ability to replicate and hence to form colonies on nutrient agar. Hence, UV irradiation can induce lethal mutations, which prevent the reproducing capability of the organism, and when expressed, results in the death of the microorganism.

Note that some cells survived at even 100 seconds of UV radiation. A likely reason for this might be that these bacteria were covered by other cells and received a lower total dose of radiation. Moreover many DNA repair enzyme system are present in the organism.

Some of the **repair mechanisms** involved in **Thymine Dimer Removal** are discussed below.

1. **Photoreactivation Repair/Reversal of UV Damage**: The dimer can be directly repaired by photoreactivation. Photoreactivation directly reverses the formation of pyrimidine dimmers that result from ultraviolet irradiation. In photoreactivation, the **enzyme DNA photolyase,** cleaves **T-T dimmers**, restoring them to the monomeric state. The enzyme is inactive unless exposed to visible light (blue light, 300-600 nm). A folic acid cofactor associated with the enzyme absorbs the light, then the enzyme uses the energy of the absorbed light to break the covalent bonds linking adjacent pyrimidine and restores normal configuration of DNA double helix.
2. **Nucleotide Excision Repair Pathway:** The nucleotide excision repair system works by recognizing distortions to the shape of the double helix, such as those caused by a **thymine dimer**. Repair involves the removal of a short single strand segment (12-13 nucleotide long stretch) including the lesion. Repair is largely accomplished by four proteins: UvrA, UvrB, UvrC, and UvrD. The gap created in the DNA is filled in by DNA polymerase using the undamaged strand as a template and thereby restoring the original nucleotide sequence.
    1. **Post-Replication Repair/ Homologous Recombination Repair:** DNA repair system, called post-replication repair responds after damaged DNA has escaped repair and has failed to be completed replicated. When DNA bearing a thymine dimer, DNA polymerase may stall at the lesion and then skip over it, leaving an un-replicated gap on the newly synthesized strand. To correct the gap, specifically the

'**Rec A Protein**' directs a re-combinational exchange with the corresponding on the undamaged parental strand of the same polarity. When the undamaged segment of the donor strand DNA replaces the gapped segment, a gap is created on the donor strand. The gap can be filled by repair synthesis as replication proceeds. Because a re-combinational event is involved in this type of DNA repair, it is considered to be a form of homologous recombination repair.

**Precautions**

1. All work should be carried out in sterilized condition of the Laminar.
2. The culture of early log phase should be taken (4-5 hours), to get actively growing cells.
3. Pipetting should be done properly while preparing dilutions.
4. Specific distance must be maintained between UV lamp and the platform of exposure, so that level of exposure is taken care off.
5. The glass spreaders used for spreading the plating should not be very hot since it might kill the bacteria.
6. All nutrient agar plates should be exposed to UV radiation simultaneously.
7. Skin should not be exposed to UV radiation.
8. Use the stopwatch to keep track of the time.
9. Use goggles and plastic gloves to protect eyes and skin.
10. Work fast and accurate when you expose the plates to UV light.

## EXPERIMENT 6: ISOLATION OF ANTIBIOTIC RESISTANT MUTANTS

### AIM: Isolation of Antibiotic Resistant Mutants

### Theory

A mutation is an inherited change in base sequence of genome of an organism. Mutations are rare, but represent an important source of genetic variability among living cells. In some cases the changes caused by a mutation enable the mutant cells to survive in a hostile environment. The type of mutation that confers some type of advantage to the mutant organism is referred to as a **selectable mutation**.

An example of a selectable mutation is the development of ***antibiotic resistance***. In an **antibiotic resistant bacterium** the mutation might lead to a change in the permeability of the cell membrane so that access of the antibiotic to the cell is reduced. Alternatively the mutation could result in a change of the cellular target on which the antibiotic normally works, for example the cell wall or the ribosome. These mechanisms are generally different from the mechanisms behind **plasmid encoded resistance**. Plasmid borne genes will often encode enzymes

that are able to degrade or modify the antibiotic or encode efflux pumps that can actively remove the antibiotic from the cell.

The use of **bacterial mutants** continues to be a very powerful tool in **bacterial genetics**. For example, the role of specific genes for important bacterial functions has been addressed through comparing the performance of mutant strains with that of the corresponding wild type. This procedure has been included when identifying the genetic background for production of bioactive metabolites, virulence factors, enzymes, etc. Bacterial mutant strains are also important in test procedures for identifying hazardous chemicals in our environment.

Consequently it is important to have simple methods whereby mutants can be detected and isolated. A selectable mutant can be isolated by direct selection. Direct selection uses selective media supporting growth of the mutant while the parent wild type will be eliminated. Some non-selectable nutritional mutants can be isolated by indirect selection using the replica plating.

## Isolation of a Streptomycin-Resistant Mutant using Gradient Plate Method

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. In brief, a gradient plate is prepared by pouring a lower slanted layer of **Agar Medium** without **Streptomycin**. When this layer has solidified, another layer of agar medium, this time containing streptomycin, is poured on top. The complete plate will contain a concentration gradient of streptomycin. When you inoculate the plates by spreading a liquid culture on top of the plate, **Streptomycin Resistant Colonies** emerge in the region of the plate with a high **Streptomycin Concentration**, as shown below in Figure 1.


***Fig. 1:*** *Streptomycin Agar and Nutrient Agar*

Figure 1 illustrates the light orange (bottom layer) that represents agar without Streptomycin, and the dark orange (top layer) with the Antibiotic included.

## Materials Required

**Culture:** 1 Tube or Flask with an Overnight Culture of *Escherichia coli* Grown in LB Medium.

**Media:** 2 Gradient Plates Prepared from 10 ml LB Agar + 10 ml LB Agar containing 0.03 mg Streptomycin per ml.

**Reagents:** 1% Streptomycin Solution (1 mg/10 ml), a Beaker with 95% Ethanol.

**Glassware and Miscellaneous:** Pipettes and Sterile Tips, Bunsen Burner, Glass Spreader, Incubation Loop.

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**Procedure**

**DAY 1: Preparation of Gradient Plate**

1. Melt two Nutrient Agar Plates maintained at 96°C and cool to 55°C.
2. Pour the contents of one agar tube into a sterile Petri plate. Allow the medium to solidify in a slanting position by placing either a glass rod under one side.
3. After the agar medium is solidified remove the glass rod and place the plate in the horizontal position.
4. Pipette out 0.1 ml of 1% Streptomycin solution into the second tube of the second nutrient agar medium.
5. Rotate the tube between the palms and pour contents to cover the Gradient Layer Agar and allow the medium to solidify on a level table.
6. Label the low and high antibiotic concentration area on the bottom of the plate. The high concentration area is marked with a black dot.

**Inoculation of Culture**

1. Add 0.2 ml of the overnight culture of *Escherichia coli* to the Gradient Plate.
2. Spread the culture over the agar surface of the plate by a sterilized glass spreader.
3. Incubate the plate (bottom up) at 37°C for 48 hours.
4. Observe the plate for appearance of *Escherichia coli* colonies in the area of Low Streptomycin Concentration and High Streptomycin Concentration.

**DAY 3: Confirming Presence of Streptomycin Resistant Colonies of *Escherichia coli***

1. Select a Bacterial Colony from the Middle of the plate and another from the region with High Streptomycin Concentration.
2. With a sterile inoculation loop, streak the two selected colonies, one after the other, from the low concentration to the high concentration region of a new gradient plate.
3. Incubate the plate (bottom up) at 37°C for 48 hours.
4. Observe the growth of streaked colonies towards High Streptomycin Concentration Region.

**Observation**

Two lines of growth of selected isolates were observed on gradient plate. One of them grow at High Streptomycin Concentrations, while another stops in between, as shown in Figure 2.

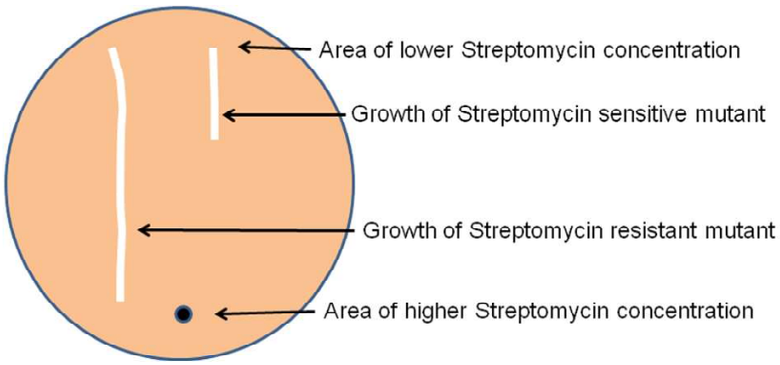


***Fig. 2:*** *Growth of the Bacterial Colony*

Figure 2 illustrates the example of how results from the gradient plate experiment can be presented. The **black dot** indicates the **high Streptomycin concentration area** of the plate.

## Results

Growth of *Escherichia coli* colonies in high streptomycin concentration area indicates the successful isolation of Streptomycin Resistant Mutants.

## Discussion

The gradient plate consists of two sections like layers of media, a bottom layer of plain Nutrient Agar and top layer of Antibiotic with Nutrient Agar. Antibiotic in the top layer, diffuse into the bottom layer producing a gradient of Streptomycin concentration from low to high. *Escherichia coli* is normally sensitive to Streptomycin, but on incubation develops growth lines on both the gradients. One line which goes till black dot is considered as Streptomycin Resistant Mutants, while another one which stops in between is Sensitive Mutant. The experiment permits us to isolate a Streptomycin Resistant Mutant from a Streptomycin Sensitive *Escherichia coli* Culture.

Antibiotic Resistant Mutant can also be isolated by using **Replica Plating** method. Wherein, replica can be made on the plate containing an antibiotic, instead of using nutrient as in isolation of auxotrophic mutant. Only antibiotic resistant colonies will grow on this plate, while antibiotic sensitive (wild type strains) will not grow.

### Precautions

1. All the work must be performed inside the laminar flow to avoid unwanted contamination.
2. Gradient should be made carefully.
3. Mark higher concentration of drug correctly.
4. Be sure that drug gets dissolved in molten Agar before pouring in Petri dish.

# EXPERIMENT 7: ISOLATION OF AUXOTROPHIC MUTANTS

NOTES

## AIM: Isolation of Auxotrophic Mutants

### Theory

**Mutation** is a **heritable change** in the **Nucleotide Sequence** of **DNA**. Mutations may be characterized according to either the kind of **Genotypic Change** that has occurred or their **Phenotypic Consequences**. Mutations can alter the phenotype of a microorganism in several different ways. Morphological mutations change the microorganism's colonial or cellular morphology. Nutritional or biochemical variation may occur in a **gene** that **encodes an enzyme** involved in **Metabolic Pathway** of **Amino Acid Synthesis**. Changes in **gene regulation** occurs when mutation occur in a gene encoding a transcription factor. Lethal mutations prevent the reproducing capability of the organism, and when expressed, it results in the death of the microorganism.

### Principle

Mutations often inactivate a biosynthetic pathway of the microorganism, and frequently make a microorganism unable to grow on a medium lacking an adequate supply of the pathway's end product. A strain bearing such a mutation has a conditional phenotype. It is unable to grow on a medium lacking that molecule, but grow when the molecule is provided. Such mutants are known as **Auxotrophs**.

**Auxotrophs** are mutant for particular nutrient synthesis pathway enzymes. An auxotroph can be grown only on an enriched medium that provides the particular nutrient that the mutant cannot metabolize on its own.

**Prototrophic Organisms** (wild type) is able to grow on a minimal medium. Minimal media contains only salts (to supply needed elements, such as Nitrogen and Phosphorus) and a Carbon source.

### Detection and Isolation of Auxotrophic Mutants by Replica Plating Method

**Replica plating method** can be used for screening large numbers of colonies for the detection of **auxotroph mutant** in a **prototrophic bacterial population**. The replica plating technique was introduced by Joshua and Esther Lederberg in 1952. It distinguishes between mutants and the wild type strain based on their ability to grow in the absence of a particular biosynthetic end product. A replica of the colonies growing on the master plate is made on a velvet surface and transferred to a replica plate containing a minimal medium without nutrient. Subsequently a second replica is made to a minimal medium with nutrient (supplemented medium) to check to transfer efficacy of the experiment. The plates are incubated to allow for colony development, and colonies are scored on all plates. Parental colonies will grow normally on both the media, whereas **auxotrophic mutants** will not

grow on the **medium lacking nutrient**. Thus, the inability of a colony to grow on medium lacking the nutrient signals that it is a mutant. The colony on the master plate corresponding to the vacant spot on the replica plate can then be picked, purified, and characterized, as shown in Figure 1. A mutant with a nutritional requirement for growth is called an **auxotroph**, and the parent from which it was derived is called a **prototroph**. For instance, mutants of *Escherichia coli* with a **His-Phenotype** are **Histidine Auxotrophs**. Figure 1 illustrates the principle of the Replica Plating technique used to isolate a Nutritional Mutant.

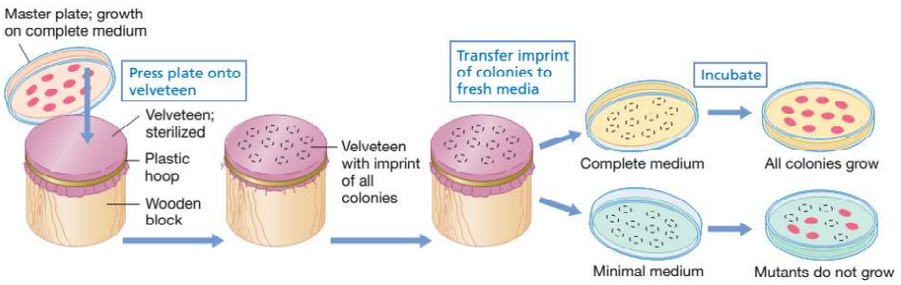


***Fig. 1:*** *Principle of the Replica Plating Technique used to Isolate a Nutritional Mutant*

## Clinical Applications

- This method has been applied in numerous experiments to identify the occurrence of Mutations (Spontaneous Mutants).
- Replica plating technique allows the observation of microbes under a series of growth conditions.
- Detection of Biochemical Mutants (Auxotrophic Mutants).
- Determination of the Spectra of Antibiotic Sensitivity.
- To study Responses to Bacteriophages.

## Materials Required

Culture: One Plate of Nutrient Agar containing 24 Hour Old 20-24 Colonies of *Escherichia coli*.

**Media:** Minimal Medium Plates, One Plate with Minimal Medium Lacking Lysine, One Plates Enriched with Lysine.

**Reagents:** 1% Lysine Solution (1 mg /10 ml of Sterile Water), Beaker with 95% Ethanol.

**Glassware and Miscellaneous:** Sterile Petri Dishes, Bent Glass Rod, Wooden Block, Sterile Velvet Cloth, Ink Marker.

**Equipment:** Incubator.

NOTES

## Procedure

Following are the important steps of the experiment.

### Preparation of Reagents before Starting the Experiment

**Lysine Solution Preparation:** Dissolve 01 mg of Lysine in 10 ml of Sterile Distilled Water to give a final concentration of 1%. Cover it with aluminum foil and store in refrigerator.

**Preparation of One Agar Plates with Minimal Medium Containing Lysine:** Dissolve respective components of complete medium in 60 ml of Sterile Distilled Water. Sterilize by autoclaving and allow the media to cool down to 40°C - 45°C. Add 3 ml of Lysine, to get final concentration of 0.1%, mix properly by rotating between the hands and pour the contents into a Sterile Petri Plate. Allow to solidify.

**Preparation of One Agar Plate with Minimal Media Minus Lysine:** Dissolve respective components of Nutrient Agar in 30 ml of Sterile Distilled Water. Sterilize by autoclaving and allow the media to cool down to 40°C - 45°C and pour the contents into a Sterile Petri Plate. Allow to solidify.

### DAY 1

1. Treat *Escherichia coli* culture with a Mutagen Nitrosoguanidine.
2. Add 200 µl of mutagen treated *Escherichia coli* culture to the surface of the complete medium plate.
3. Using an alcohol dipped and flamed bent glass rod spread the inoculums evenly on the plate.
4. Incubate the plate in an inverted position for 24-48 hours at 37°C.
5. After incubation observe the colonies of *Escherichia coli* on the plate and this plate is considered as the master plate.

### DAY 2

1. Mark the master plate and the two replica plates by the ink marker so that they can all be oriented relative to each other.
2. The sterile velveteen colony carrier was carefully placed on wooden block and gently pressed onto the colonies of the *Escherichia coli* on the master plate. A marking was made on cloth coinciding with that of master plate. Only handle the sheet at the margins to avoid contamination of the areas used for transfer of bacterial colonies.
3. Remove the lid of the master plate and place the Agar Surface on top of the velvet. Gently press the Agar Plate downwards until you can see that the colonies make contact with the velvet.
4. Transfer the replica to the Agar Plate with minimal medium and thereafter to the **minimal medium with Lysine**. When you make the replicas you should gently press the replica plate down towards the velvet until you can see that the colonies on the velvet contact the agar surface.

5. Remember to keep the same orientation of the master plate, the velvet and the replica plates.
6. Incubate both the inoculated plates, in an inverted position for 48-72 hours at 37°C. Master plate is refrigerated.
7. Count the number of colonies on the master and on the two replica plates.
8. Comparing the presence and location of each colony to locate any colony missing on minimal media **without Lysine** but present on minimal media supplemented **with Lysine**.

**Results**

Leucine Auxotroph Mutant was selected in a prototrophic bacterial population by indirect method using the Replica Plating technique. Auxotrophic mutant developed on a complete medium but failed to develop on a minimal medium that lacks Lysine.

**Discussion**

Mutants were generated by treating *Escherichia coli* culture with a Chemical Mutagen, Nitrosoguanidine, which acts by adding Alkyl Groups to the $O^6$ of Guanine and $O^4$ of Thymine, which can lead to transition mutations between GC and AT. Mutagen treated culture was then plated on complete medium. After the colonies have developed, a piece of sterile velveteen was pressed on the plate surface to pick up bacteria from each colony. Then the velvet was pressed to the surface of other nutrient agar medium supplemented with or without a particular nutrient. By comparing the presence of colonies following incubation we can indirectly determine the mutant colonies by their absence in the selective environment. The fibers of velvet act as fine inoculating needles, picking up the bacterial cells from the surface of this master plate.

Although of great utility, replica plating is nevertheless a screening process and it can be laborious to isolate mutants by screening method.

**Precautions**

1. The velvet cloth must be held firmly on the wooden block.
2. Make sure that marking on velvet coincide with that of plate.
3. The pressing of the plate on the velvet should be gentle and the plate must be removed as such without rotating.
4. After the replica plate has been prepared, the velvet must be discarded carefully since it has got Nitrosoguanidine, which is a carcinogen also.
5. Only handle the sheet at the margins to avoid contamination of the areas used for transfer of bacterial colonies.
6. All the work must be performed inside the Laminar flow to avoid unwanted contamination.
7. After the two transfers, the velvet used for the transfer should be soaked in disinfectant.

# MICROBIAL GENETICS

Microbial genetics is a subject area within microbiology and genetic engineering. Principally, the microbial genetics studies microorganisms for different purposes. The microorganisms that are observed are bacteria, and archaea. Some fungi and protozoa are also used as subjects of study for 'Microbiology'. The studies of microorganisms involve studies of genotype and expression system. Genotypes are the inherited compositions of an organism. 'Genetic Engineering' is a field of study within microbial genetics, such as the usage of recombinant DNA or rDNA technology and Cloning. The process involves creating recombinant DNA molecules through manipulating a DNA sequence. Since the discovery of microorganisms by Robert Hooke and Antoni van Leeuwenhoek during the period 1665-1885, they have been used to study many processes and have had applications in various areas of study in genetics. For example, Microorganisms' rapid growth rates and short generation times are used by scientists to study evolution. Antoni van Leeuwenhoek's contribution to the microscopic protozoa and microscopic bacteria yielded to scientific observations and descriptions. Microbial genetics also has applications in being able to study processes and pathways that are similar to those found in humans, such as drug metabolism.

## EXPERIMENT 8: ISOLATION OF SPECIALIZED TRANSDUCING PHAGE

### AIM: Isolation of Specialized Transducing Phage

### Theory

**Transduction** is a method of **genetic recombination** in **bacteria**, in which **DNA** is **transferred** between **bacteria via bacteriophages**. The source cells of the DNA are called '**Donors**' and the cells that receive the DNA are called the '**Recipients**'. In each case, the donor DNA is incorporated into the recipient's cell's DNA by **recombination exchange**. If the exchange involves DNA of the recipient's genome, the recipient's genome and phenotype will change.

### Materials Required

**Equipment:** Conical Flask, Measuring Cylinder, Sterile Micro Centrifuge Tubes, Sterile Tubes (15 and 50 ml), Distilled Water Centrifuge, Incubator, Shaker, Water Bath, Micropipettes, Tips, Sterile Loops and Spreaders, 0.45µ Filters

**Reagents:** Luria Bertani (LB) Broth, LB Agar Plates, Donor and Recipient *Escherichia coli* Strains, P1 Lysate, 50 mM $CaCl_2$, Selective Growth Medium Containing 20 mM Na Citrate (Sodium Citrate), 20% Glucose, 1M $CaCl_2$

**NOTES**

## Procedure

1. Inoculate a 5 ml overnight culture of donor and recipient strain in LB with appropriate antibiotic.
2. Dilute the culture (1:100) into 5 ml LB having 5 mM $CaCl_2$ and 0.2% Glucose.
3. Incubate for 30 minutes at 37°C with shaking or till the culture appears to be slightly cloudy.
4. Add 100 µl of P1 phage stock and grow the culture, shaking at 37°C for 2-4 hours until the culture lyses and appears to be clear.
5. Add 200 µl of chloroform, continue shaking for 10 minutes. This step ensures Complete Cell Lyses and killing of the Bacterial Donor Strain.
6. Centrifuge the culture for 10 minutes at 9200 g, 4°C.
7. Transfer supernatant containing the P1 phages into a fresh tube stored at 4°C till further use.
8. Additionally, the Lysate may be purified using a 0.45 µm filter to remove any residual bacterial cells.
9. For transduction, pellet 1.5 ml of recipient culture by centrifuging at 1500 g for 10 minutes.
10. Re-suspend the cells in 1 ml solution consisting of 10 mM $MgSO_4$ and 5 mM $CaCl_2$.
11. Take 100 µl of cells in microcentrifuge tube, for each transduction experiment.
12. Add 50 µl of P1 Lysate, incubate at 30°C without disturbing/shaking for 30 minutes.
13. Add 1 ml of LB with 10 mM Sodium Citrate, incubate undisturbed at 30°C for 30-40 minutes.
14. Centrifuge for 2 minutes at 8000 rpm (rotations per minute), re-suspend pellet in 100 µl of 1M Sodium Citrate.
15. Plate on selective LB transduction plates containing 5 mM Sodium Citrate.
16. Also, plate P1 Lysate as phage only control.

## Observation

Genetic Loci can be mapped/confirmed by PCR or southern blot from isolated phage DNA or the bacterial DNA after transduction.

# EXPERIMENT 9: BACTERIAL CONJUGATION– TRANSFER OF DRUG RESISTANT FACTOR (PLASMID)

NOTES

**AIM: Bacterial Conjugation, Transfer of Drug Resistant Factor (Plasmid)**

## Theory

**Genetic Variability** is essential for the **evolutionary success** of all organisms. In eukaryotes **crossing over** (exchange of genetic material between homologous chromosomes) and meiosis contribute to this variability while in bacteria, genetic **Recombination** may occur by **Conjugation, Transduction,** and **Transformation**.

- **Conjugation:** The conjugation is a **recombination process** where one bacterial cell **transfers DNA** in one direction to another cell by **direct cell-to-cell** contact. The transferred DNA may be part of or all the **Bacterial Genome**, or it may be an **Extra-Genomic DNA element** called a '**Plasmid**'. A genomic fragment may recombine with the recipient's chromosome after entry.
- **Transformation:** A bacterial cell can also acquire a piece of DNA from the environment and incorporate this DNA into its own chromosome.
- **Transduction:** In addition, certain phages can pick up a piece of DNA from one bacterial cell and inject it into another, where it can be incorporated into the chromosome.

These lateral gene transfer methods not only generate new gene assortments, but are potent evolutionary force that can create diversity within bacterial species. In this experiment, only the process of conjugation is considered.

## Principle

Following are the significant principles for conjugation.

## Discovery of Conjugation

Joshua Lederberg and Edward Tatum, in 1946 discovered a sex like process in bacteria. They were studying **two strains** of ***Escherichia coli*** with different sets of **auxotrophic mutations**. **Strain A** would grow only if the medium were supplemented with **Methionine** and **Biotin** (met-bio-thr+leu+thi+), while **Strain B** would grow only if it were supplemented with **Threonine**, **Leucine**, and **Thiamine** (met+bio+thr-leu-thi-). Cells of Strain A or Strain B cannot grow on an un-supplemented (minimal) medium, because in Strain A and Strain B each carry mutations that cause the inability to synthesize all the constituents needed for cell growth. When Strain A and Strain B are mixed for a few hours and then plated, however, a few colonies appear on the agar plate. These colonies derive from single cells in which an exchange of genetic material has occurred. They are therefore capable of synthesizing all the required constituents of metabolism, become

prototroph. Some of the minimal media were plated only with Strain A bacteria and some only with Strain B bacteria to act as controls, but from these no prototrophs arose (Refer Figure1). Figure 1 illustrates the Lederberg and Tatum's demonstration of Genetic Recombination between Bacterial Cells.

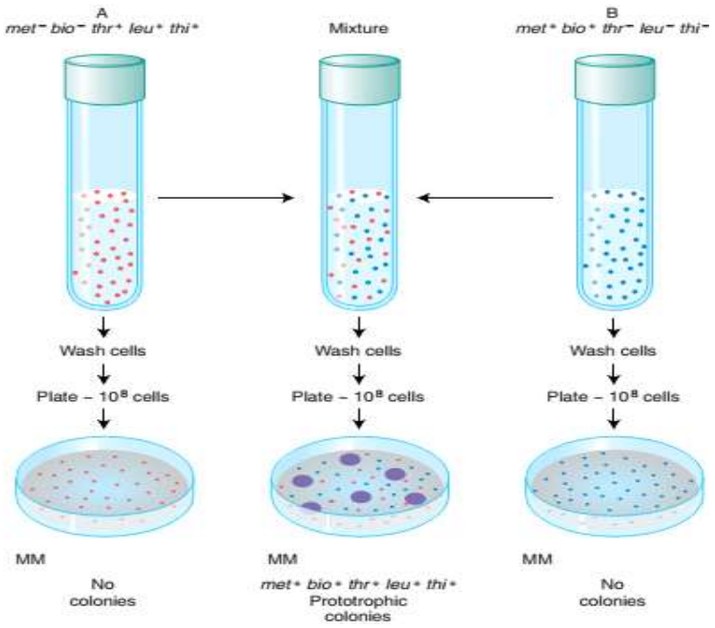


***Fig. 1:*** *Lederberg and Tatum's Demonstration of Genetic Recombination between Bacterial Cells*

## Physical Contact between Bacterial Cells is Required for Genetic Recombination

Lederberg and Tatum did not directly prove that physical contact of the cells was necessary for gene transfer. This evidence was provided by Bernard Davis (1950), who constructed a U tube consisting of two pieces of curved glass tubing fused at the base to form a U shape with a fine glass filter between the halves (Refer Figure 2). The pores of the filter were too small to allow bacteria to pass through but large enough to allow easy passage of any dissolved substances. The U tube was filled with nutrient medium and each side inoculated with a different auxotrophic strain of *Escherichia coli*. During incubation, the medium was pumped back and forth through the filter to ensure medium exchange between the halves. After 4 hours of incubation, the bacteria were plated on minimal medium to see if there were any prototrophic cells, but none were found. Davis discovered that when the two auxotrophic strains were separated from each other by the fine filter, gene transfer could not take place. Therefore direct cell to cell contact was required for the recombination that Lederberg and Tatum had observed. Figure 2 illustrates the U- Tube experiment to show that Genetic Recombination by Conjugation requires Direct Physical Contact between Bacteria.

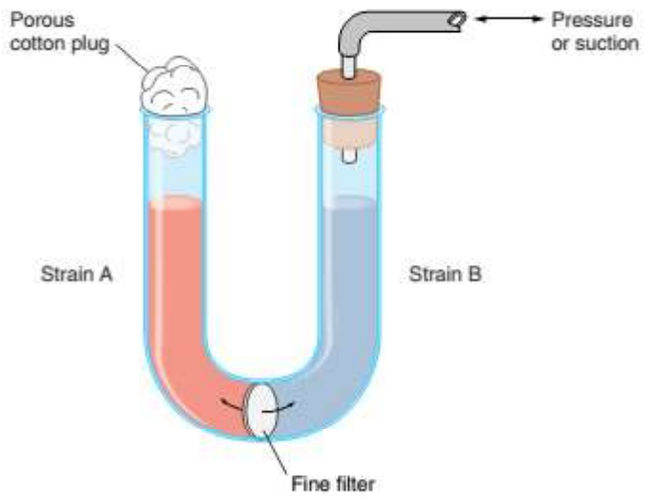


***Fig. 2:*** *U- Tube Experiment for Genetic Recombination by Conjugation*

## Discovery of F Factor

In 1950 William Hayes, Francis Jacob and Elie L. Wollman established that **Conjugating Bacteria** are of **two mating types**. Certain '**Male**' types that carry **Fertility Factor** (designated as **F+**) can **donate** their **DNA** (hence **Donor**) and other '**Female**' types that lack this **Fertility Factor** (designated as **F-**) **receive** the **DNA** (hence **Recipient**) as shown in Figure 3. **F cells** become **F+** when they acquire a small amount of DNA.

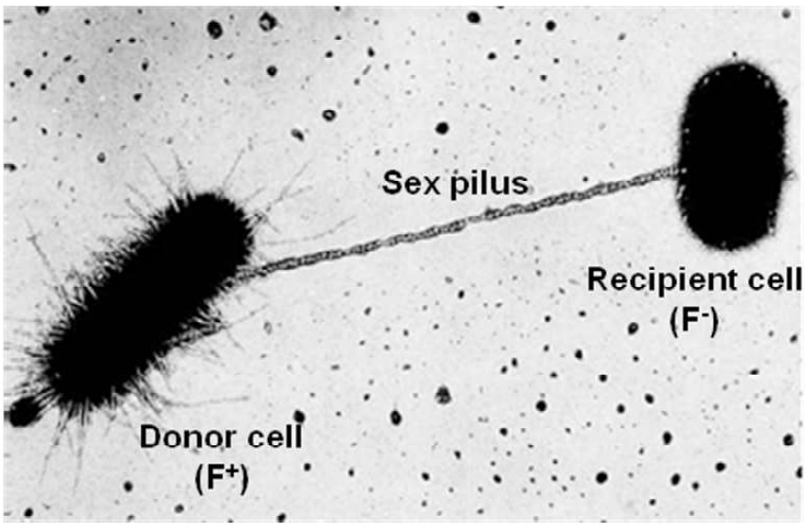


***Fig. 3:*** *Bacteria can Transfer Plasmids (F Factor) Through Conjugation*

Figure 3 illustrates that **Bacteria** can **transfer Plasmids (F Factor)** through **Conjugation**. A donor cell extends one or more projections, '**Pili**' that attach to a recipient cell and pull the two bacteria together.

**Donor's F Factors** are known to be **plasmids** which are the **extra-chromosomal**, **nonessential circular DNA molecule** that can **replicate** in the **cytoplasm** independent of the **host chromosome**. These plasmids are **conjugative plasmids** which carry **genes**, referred to as **tra genes** (transfer genes), which directs the synthesis of **F Pili** or **Sex Pili** (hair like projections) that initiate contact with a recipient and draw it closer.

### DNA Transfer by Bacterial Conjugation

There are following three ways by which DNA gets transferred amongst bacterial population:

1. The presence of the **F Factor Plasmid** enables an *Escherichia coli* cell to act as a **plasmid donor** during **conjugation**.
2. Chromosomal integration of the F Factor creates an **Hfr cell** (High-frequency recombination cell).
3. Hfr cell can transfer **genomic DNA** during **conjugation**.

### Conjugation between F+ and F– Cells

The **transfer** of a copy of the **F Factor Plasmid** from an **F+ Donor Bacterium** to an **F – Recipient** converts the **F – cell** into an **F+ cell**. Plasmid transfer begins at the F factor's origin of transfer.

At the area of contact, a channel or conjugation bridge is formed. Once contact via sex pili has been made, the F Factor (Plasmid) begins replicating by the **rolling circle mechanism**. Transfer always begins at a point on the plasmid called its '**Origin of Transfer**'. The circular plasmid '**Rolls**', and as it turns, it reels out the single stranded copy. This copy passes through the channel to the recipient. When it arrives, enzymes synthesize a complementary strand, and a **double helix** is formed. The **double helix bends** to a **loop** and **reforms** an **F Factor (Plasmid)**, thereby completing the conversion of recipient from **F –** cell to **F+** cell. Meanwhile, back in the donor cell a new strand of DNA forms, to complement the leftover strand of the F Plasmid. The transfer of F Factors involves no activity of the bacterial chromosome; therefore the recipient does not acquire new genes other than those on the F Factor. Figure 4 illustrates the Conjugation and Transfer of an F Plasmid from an F+ Donor to an F – Recipient.

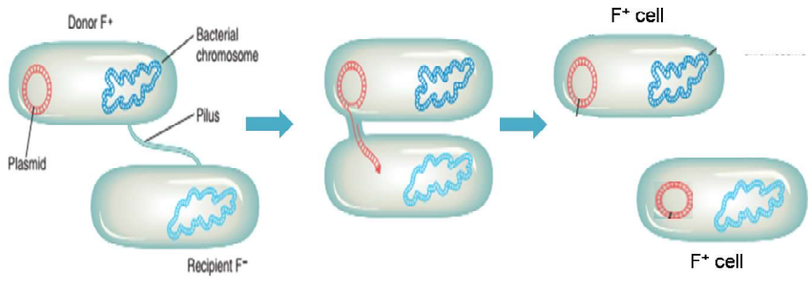


***Fig. 4:*** *Conjugation and Transfer of an F Plasmid from an F+ Donor to an F- Recipient*

## Conversion of an F+ Cell into an Hfr Cell

Here, **F Plasmid integrate** in the **chromosome** of its **bacterial host**, generating an **Hfr** (High frequency of recombination) cell, which is capable of producing a high frequency of recombination in further matings because it can now **transfer genomic DNA** during **conjugation**. Similar to F+ cells this cell can also direct the synthesis of a sex pilus. As the **chromosome** of the **Hfr cell replicates** it may begin to cross the pilus so that **plasmid** and **chromosomal DNA transfers** to the **recipient cell** (Refer Figure 5). Such DNA may recombine with that of its new host, introducing new gene variants. Plasmids encoding antibiotic resistance genes are passed throughout populations of bacteria, and between multiple species of bacteria by conjugation.

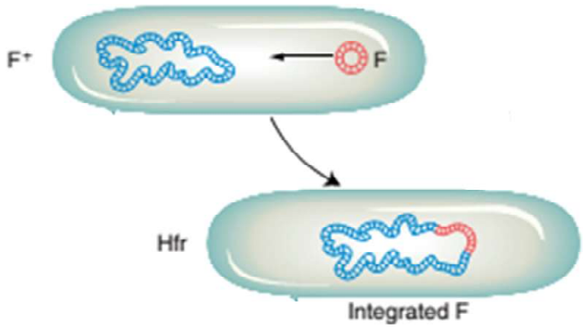


***Fig. 5:*** *Formation of an Hfr Cell*

Figure 5 illustrates the formation of an Hfr cell. Occasionally, the independent F Factor combines with the *Escherichia coli* chromosome, creating an Hfr strain.

## Conjugation between an Hfr Cell and an F⁻ Cell

When an **Hfr Bacterium** is mated to **F⁻ Cell**, the DNA is transferred into the recipient cell. But instead of transferring just the F Factor itself, the Hfr Cell transfers at least part of its **Chromosomal DNA**. Cells rarely remain in contact long enough for the entire bacterial chromosome to be transferred. Transfer begins at the origin of transfer within the integrated F Factor and proceeds in a direction dictated by the orientation of the F Factor within the Chromosome. **Uppercase letters** represent **Alleles** carried by the **Hfr** and the **Lowercase letters** represent **Corresponding Alleles** in the **F⁻ cell** (Refer Figure 6). In the last step, Allele 'A' from the Hfr is recombined into the F⁻ cell's DNA in place of its 'a' Allele.

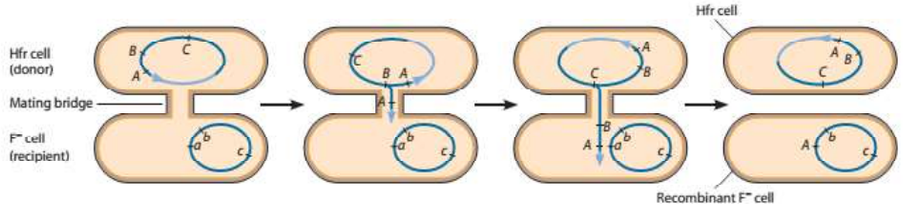


***Fig. 6:*** *Conjugation between an Hfr Cell and an F⁻ Cell*

## Clinical Applications

Conjugation is a major cause of the spread of antibiotic resistance and represents a serious problem in antibiotic therapy of immunosuppressed patients. Several conjugative plasmids are known. One important group is represented by Resistance (R) Plasmids carrying Genes, which confer resistance to antibiotics and/or to heavy metals. Bacteria that carry several resistant genes are called multi-drug-resistant superbugs. The indiscriminate use of antibiotics both within the healthcare profession and the illegal sale of drugs without prescriptions are mainly responsible for the increased spread of antibiotic resistance.

Other conjugative plasmids carry genetic pathways involved in degradation of pesticides while still other plasmids encode restriction/modification systems conveying resistance to attack by bacteriophages.

## Materials Required

**Culture:** Donor Strain A, Recipient Strain B

Store Donor Strain A, Recipient Strain B, Tetracycline Hydrochloride and Streptomycin Sulphate at 2-8°C. Other materials can be stored at room temperature, i.e., 15-25°C.

**Glasswares:** Conical Flask, Measuring Cylinder, Sterile Test Tubes, Petri Plates.

**Reagents and Media:** Streptomycin Sulphate, Tetracycline Hydrochloride, Ethanol, Luria Bertani Broth, Agar Powder, Bacteriological Distilled Water.

**Other Requirements:** Incubator, Shaker, Spectrophotometer, Micropipettes, Tips, Sterile Loops and Spreaders.

## Preparation of Reagents before Starting the Experiment

1. **Tetracycline Solution Preparation:** Dissolve 45 mg of Tetracycline in 1.5 ml of 70% Ethanol, mix by gentle pipetting to give a final concentration of 30 mg/ml. Cover with aluminum foil and store in refrigerator. Use this solution within a month.
2. **Streptomycin Solution Preparation:** Dissolve 150 mg of Streptomycin in 1.5 ml of Sterile Distilled Water to give a final concentration of 100 mg/ml. Cover with aluminum foil and store in the refrigerator. Use this solution within a month.
3. **Preparation of LB (Luria Bertani) Broth (100 ml):** Dissolve 2.5 g of Luria Bertani Broth in 100 ml of Distilled Water and autoclave.
4. **Preparation of LB (Luria Bertani) Agar Plates with Streptomycin (100 ml):** Dissolve 2.5 g of LB Media and 1.5 g of Agar in 100 ml of Sterile Distilled Water. Sterilize by autoclaving and pour on Sterile Petri Plates. Sterilize by autoclaving and allow the Media to cool down to 40-45°C. Add 100 µl of Streptomycin in 100 ml of autoclaved LB Agar Media and pour on Sterile Petri Plates.

**NOTES**

5. **Preparation of LB (Luria Bertani) Agar Plates with Tetracycline (100 ml):** Dissolve 2.5 g of LB Media and 1.5 g of Agar in 100 ml of Sterile Distilled Water. Sterilize by autoclaving and allow the Media to cool down to 40-45°C. Add 100 µl of Tetracycline to 100 ml of autoclaved LB Agar Media and pour on Sterile Petri Plates.
6. **Preparation of LB Agar Plates with Tetracycline + Streptomycin (100 ml):** Dissolve 2.5 g of LB Media and 1.5 g of Agar in 100 ml of Sterile Distilled Water. Sterilize by autoclaving and allow the Media to cool down to 40-45°C. Add 100 µl of Tetracycline and 100 µl of Streptomycin to 100 ml of autoclaved LB Agar Media and pour on Sterile Petri Plates.

**DAY 1**

Using sterile flexi loop, streak a loopful of *Escherichia coli* Donor Strain from the stab onto two LB plates with Tetracycline (30 µg/ml) and *Escherichia coli* Recipient Strain onto two LB plates with Streptomycin (100 µg/ml). Incubate at 37°C for 18-24 hours.

**DAY 2**

Pick up a single colony from Donor and Recipient Strain grown overnight on LB plates and inoculate in 6 ml of LB Broth having respective antibiotics. Incubate the test tubes overnight at 37°C.

**DAY 3**

1. Take 25 ml of LB Broth and add 25 µl of Tetracycline into it and inoculate 1 ml of overnight grown culture into it. Incubate at 37°C in a shaker.
2. Take 25 ml of LB Broth with Streptomycin at a concentration of 100 µg/ml and inoculate 3 ml of overnight grown culture in it. Incubate at 37°C in a shaker.
3. Grow the cultures till O.D. of the donor culture reaches 0.8-0.9 at A600.
4. Add 0.2 ml of each donor and recipient cultures in a sterile test tube labeled as Conjugated Sample. Mix by gentle pipetting and incubate at 37°C for 1-1.5 hours.
5. Take 2 sterile test tubes and label them as Donor and Recipient. Add 0.2 ml of respective cultures to the test tubes and incubate at 37°C for 1-1.5 hours.

   ***Note:*** Do not place the tubes in shaker for conjugation and further incubation period.
6. Add 2 ml of LB Broth into each tube after incubation. Incubate the tubes at 37°C for 1.5 hours.
7. Plate 0.1 ml of each culture on the Antibiotic Plates as indicated in Table 1.
8. Incubate the plates at 37°C overnight.

***Table 1:*** *Samples to be Spread on Respective Plates*

| | LB + Streptomycin | LB + Tetracycline | LB + Streptomycin, Tetracycline |
|---|---|---|---|
| **Donor Strain A** | 0.1 ml | 0.1 ml | 0.1 ml |
| **Recipient Strain B** | 0.1 ml | 0.1 ml | 0.1 ml |
| **Conjugated Sample** | 0.1 ml | 0.1 ml | 0.1 ml |

## Observation and Results

Note down the observations in the following table. Indicate Bacterial Growth with Positive Symbol and Absence of Growth with Negative Symbol.

| | LB + Streptomycin | LB + Tetracycline | LB + Streptomycin, Tetracycline |
|---|---|---|---|
| **Donor Strain A** | – | + | – |
| **Recipient Strain B** | + | – | – |
| **Conjugated Sample** | + | + | + |

## Interpretation

On observing colonies on different plates the following interpretation can be made:

1. Donor Strains will grow only on Tetracycline Plates because they contain Tetracycline Resistance, similarly Recipient Strains will grow only on Streptomycin Plates as they contain Streptomycin Resistance Gene.
2. Donor Strain is SENSITIVE to Streptomycin and Recipient Strain is SENSITIVE to Tetracycline, hence no growth will be seen in these plates.
3. The Conjugated Sample will grow on Tetracycline and Streptomycin Plate. The reason being, transfer of gene has occurred by means of Conjugation.
4. The Donor and Recipient Strain will not grow on Tetracycline + Streptomycin Plate since EACH of the STRAIN is SENSITIVE to ONE ANTIBIOTIC in the Plate.

## Precautions

1. Ensure that the respective antibiotic is added to the LB Media at 40-45°C before and then pour the plates. Adding antibiotics at higher temperature will deactivate its activity.
2. Use plates within 1 month of preparation.
3. Do not place the tubes in shaker for conjugation and further incubation period.
4. All microbiological operations should be done strictly under aseptic conditions.

# EXPERIMENT 10: TRANSPOSON MUTAGENESIS OF CHROMOSOMAL AND PLASMID DNA

NOTES

## AIM: Transposon Mutagenesis of Chromosomal and Plasmid DNA

### Theory

**Transposons** are **DNA elements** which have ability to move from one region to another region in the **genome**. The shifting of transposon element from a heterochromatin region to a transcriptionally active region results in **insertional inactivation**.

**Insertional inactivation** focuses on **suppressing** the **expression** of a **gene** by **disrupting** its **sequence** with an **insertion**. When additional nucleotides are inserted near or into a locus, the locus can suffer a frame shift mutation that could prevent it from being properly expressed into polypeptide chain . Transposon based insertional inactivation is considered for medical research from suppression of antibiotic resistance in bacteria for the treatment of genetic diseases.

Transposon Mutagenesis was first studied by Barbara McClintock in the mid-20th century with corn for which she was awarded Nobel Prize also. She was working on the topic of maize chromosomes.

Due to their simple and intricate design and inherent ability to move DNA sequences, transposons are highly compatible for transducing genetic material, enable them as an ideal genetic too for analysis of gene and protein function.

The study of transposons is well described in the case of *Drosophila* (in which P elements are most commonly used) and in Thale cress (*Arabidopsis thaliana*) and bacteria, such as *Escherichia coli*.

### Structure and Mechanism of Transposition System

In case of **Bacteria**, **Transposition Mutagenesis** is usually done by the help of a **plasmid** from which a **transposon** is **extracted** and **inserted** into the **host chromosome**. This usually requires a set of enzymes including **transposase** to be translated.

Figure 1 describes the components and structure of a two-component gene transfer system based on **Sleeping Beauty Transposon System**. A gene of interest to be mobilized is cloned between the terminal inverted repeats/direct repeats (IR/DR, black arrows) that contain binding sites for the **transposase** (white arrows). The **transposase gene** is **physically separated** from the IR/DRs, and is expressed in cells from a suitable promoter (black arrow). The transposase consists of an N-Terminal DNA-Binding Domain, a Nuclear Localization Signal (NLS) and a catalytic domain characterized by the DDE signature.

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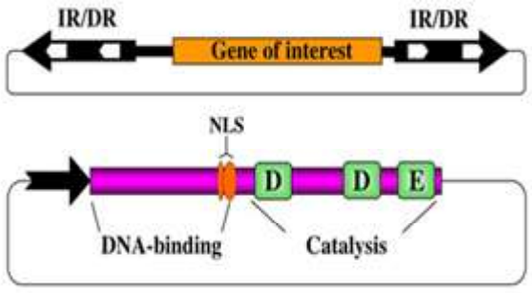



***Fig. 1:*** *Components and Structure of a Two-Component Gene Transfer System based on Sleeping Beauty Transposon System*

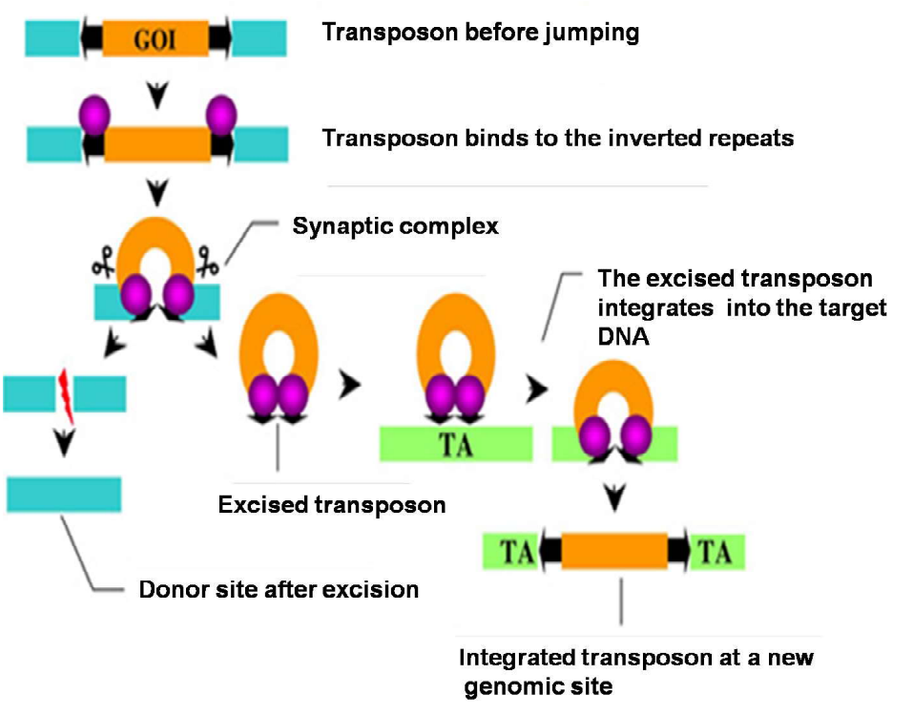



***Fig. 2:*** *Mechanism of Sleeping Beauty Transposition System*

Figure 2 illustrates the mechanism of Sleeping Beauty Transposition System. The transposable element carrying a Gene Of Interest (GOI) is maintained and delivered as part of a DNA vector (left DNA). The **transposase** (small circles) binds to its sites within the transposon inverted repeats (black arrows). Excision takes place in a synaptic complex. Excision separates the transposon from the donor DNA, and the double strand DNA breaks that are generated during this process are repaired by host factors. The excised element integrates into a TA site in the Target DNA (right DNA) that will be duplicated and will be flanking the newly integrated transposon.

## Practical Applications of Transposon Mutagenesis

As a result of the capacity of transposon mutagenesis to incorporate genes into most areas of target chromosomes, there are a number of functions associated with the process.

- Virulence genes in viruses and bacteria can be discovered by disrupting genes and observing for a change in phenotype. This has importance in antibiotic production and disease control. For example, Transposon Mutagenesis identified 13 Pathogenic Loci in the *Mycobacterium tuberculosis* Genome which were not previously associated with disease. This is essential information in understanding the infectious cycle of the bacterium.
- Non-essential genes can be discovered by inducing transposon mutagenesis in an organism. The transformed genes can then be identified by performing PCR on the organism's recovered genome using an ORF-specific primer and a transposon specific primer. Since transposons can incorporate themselves into non-coding regions of DNA, the ORF-specific primer ensures that the transposon interrupted a gene. Because the organism survived after homologous integration, the interrupted gene was clearly non-essential.
- Cancer causing Genes can be identified by genome wide mutagenesis and screening of mutants containing tumours. Based on the mechanism and results of the mutation, cancer causing genes can be identified as Oncogenes or Tumour Suppressor Genes.
- Transposon mutagenesis allows genes to be transferred to a host organism's chromosome, or plasmid interrupting or modifying the function of a gene on the chromosome and causing mutation.

## Materials Required

**Bacterial Strain:** Strain of *Dinoroseobacter shibae* DFL12.

**Media:** Marine Broth (MB), Luria Bertani (LB) Medium, half-concentrated MB or Marine Bouillon (hMB)

**Chemicals and Reagents:** Gentamycin (80 µg/ml), 50 µg/ml of Aminolevulinic Acid

**Instrument:** Shaker, Bottle Flasks

## Procedure

1. The type Strain *Dinoroseobacter shibae* DFL12 was cultured aerobically in Marine Bouillon at 30°C in bottle flasks shaking at 200 rpm in the dark.
2. The Mariner Transposon located on Plasmid pBT20 was used for Transposon Mutagenesis of *Dinoroseobacter shibae* DFL12.

3. For selection of *Dinoroseobacter shibae* Mutants, 80 μg/ml Gentamicin was added after conjugation to half-concentrated Marine Bouillon (hMB).
4. *Escherichia coli* ST18, a mutant of *Escherichia coli* S17, served as the donor strain for the conjugative transfer of plasmid DNA.
5. Luria Bertani (LB) Medium supplemented with 50 μg/ml Aminolevulinic Acid and adjusted to pH 7 was used for its cultivation at 37°C and 200 rpm.
6. For solid medium, Agar was added to a final concentration of 1.5% (wt/vol).

**Selection of Transposon Mutants**

1. For selection of *Dinoroseobacter shibae* transposon mutants with an anaerobic growth deficiency, all clones were cultivated aerobically and anaerobically at 30°C in 96-well plates with hMB supplemented with (80 μg/ml) Gentamicin, respectively.
2. For anaerobic cultivation, 25 mM Nitrate was added. Growth was monitored by measurement of optical density at 595 nm (OD595) in a microtiter plate reader.
3. Strains showing growth deficiencies under anaerobic conditions were isolated for further study.
4. The growth behaviour of the selected *Dinoroseobacter shibae* DFL12 transposon mutants was analyzed aerobically and anaerobically in artificial Sea-Water Medium (SWM) with 16.9 mM Succinate, respectively. For anaerobic cultivation, 25 mM Nitrate was added.
5. The cultivation occurred in 48-well plates at 30°C for 60 hours at 800 rpm in a parallel bioreactor system.
6. Every hour, the OD620, the pH, and the oxygen partial pressure were measured automatically.
7. Transposon integration site was identified.

**Observation and Results**

A total of 4,500 *Dinoroseobacter shibae* transposon mutants were isolated and further screened for growth defects under anaerobic denitrifying conditions. Random integration of the transposon was observed. Of the 1,580 transposon mutants sequenced, 1,134 showed different loci of transposon integration. Taking approximately 12% of the essential genes into account, the saturation of mutagenesis reached 82% of the genome. Out of 53 Mutants, 35 with Transposon integration into Chromosomal Genes and 18 with Transposon integration into Plasmid Genes, showed significantly decrease or even loss of anaerobic growth. For example, plasmid encoded cytochrome 'c' was found to be essential for anaerobic growth in *Dinoroseobacter shibae.*

### Discussion

Transposon mutagenesis is a powerful tool for random mutagenesis of bacterial genomes and insertion of foreign DNA. Overall, new surprising insights into the adaptation of the marine model bacterium *Dinoroseobacter shibae* to oxygen limiting conditions were obtained by using transposons as mutagenic agent. Transposon mutagenesis is much more effective than chemical mutagenesis, with a higher mutation frequency and a lower chance of killing the organism. Other advantages include being able to induce single hit mutations, being able to incorporate selectable markers in strain construction, and being able to recover genes after mutagenesis. Disadvantages include the low frequency of transposition in living systems, and the inaccuracy of most transposition systems.

## EXPERIMENT 11: ISOLATION OF PLASMID AND CHROMOSOMAL (BACTERIAL) DNA

### 1. PLASMID ISOLATION BY ALKALINE LYSIS METHOD

### AIM: Plasmid Isolation by Alkaline Lysis Method

### Theory

**Plasmid DNA** from *Escherichia coli* by **Alkaline Lysis** is based on its different **denaturation properties** as compared to **chromosomal DNA** in cell. The bacterial cells are lysed with Strong Alkali (NaOH) and Detergent (SDS) denaturing chromosomal as well as plasmid DNA. The SDS detergent solubilizes the phospholipids and proteins of the cell membrane resulting in cell lysis and the release of the cells contents. The high concentration of sodium hydroxide denatures the genomic and plasmid DNA, as well as cellular proteins. **Basic pH** of the buffer helps to **denature DNA**, and **EDTA binds divalent cations destabilizing** the membrane and inhibiting **DNA degrading enzymes, DNases**. The cellular DNA becomes linearized and the strands are separated, whereas the plasmid DNA is circular and remains topologically constrained (the two strands, although denatured remain together). Finally, a neutralization buffer of Potassium Acetate is added to neutralize the strong 'Alkaline' conditions. The addition of Potassium Acetate results in a high salt concentration that leads to the formation of a **white precipitate** that consists of **SDS, Lipids** and **Proteins**. In addition, the neutralization of the solution allows the renaturation of DNA. The large chromosomal DNA is captured in the precipitate, whereas the small plasmid DNA remains in solution. The soluble plasmid DNA can then be purified by the Alcohol/ Salt precipitation methods. In addition, released RNA can be degraded by adding RNases.

## Materials and Reagents Required

**Equipment:** 1.5 ml Microcentrifuge Tubes (MCT), Pipettes, MCT Stand

**Sample:** Overnight grown *Escherichia coli* (having Plasmid) Culture under appropriate Antibiotics in Luria–Bertani Broth.

**Media:** Luria–Bertani (LB) Medium 5 gm/L Yeast Extract, 5 gm/L NaCl, 10 gm/L Tryptone.

**TE Buffer:** 10 mM Tris-HCl, pH 8.0, 0.1 mM Ethylenediaminetetraacetic Acid (EDTA), pH 8.0, autoclave and store at room temperature.

**Lysozyme:** 10mM Tris-HCl 20 mg/mL, pH 8.0.

**RNase A:** 10 mg/mL, DNase free.

Ethanol 100% and 70%.

**Plasmid Isolation Solution:** STE (Sucrose/Tris/EDTA) Solution: 8% (w/v) Sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0). Autoclave and store at 4°C.

**Solution I (Resuspension Solution):** 50 mM Glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0). Autoclave and store at 4°C.

**Solution II (Lysis Solution):** Alkaline–SDS Solution: 0.2N NaOH, 1% (w/v) SDS. Prepare fresh and store at room temperature.

**Solution III (Neutralizing Solution):** 3 M Potassium Acetate Solution: 60 mL of 5 M Potassium Acetate, 11.5 mL Glacial Acetic Acid, 28.5 mL Distilled Water. Store at room temperature.

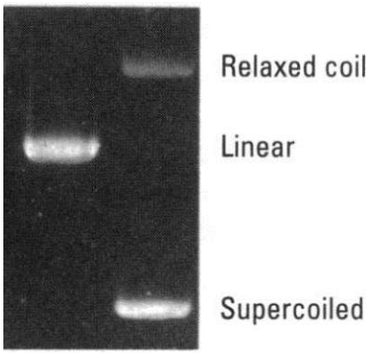
### Procedure

1. Centrifuge 2 mL of culture at 10000 rpm for 1 minute in a microcentrifuge to pellet the bacteria.
2. Decant the supernatant, resuspend the cell pellet in 0.5 mL STE solution. Centrifuge again and remove the supernatant.
3. Resuspend the pellet in 100 μL of Solution I, consisting of two mg/mL of Lysozyme.
4. Add 200 ml of Solution II prepared freshly, invert mix four-five times without vortexing. Incubate the tube on ice for 5 minutes.
5. Add 150 μL of Solution III, invert mix and incubate the tube on ice for 5-10 minutes.
6. Centrifuge at maximum speed for 10 minutes in a microcentrifuge at 4°C.
7. Transfer the supernatant to a fresh tube. Precipitate DNA by adding 2.2-3 volumes of chilled 100% Ethanol, invert mix and incubate the tube for 30 minutes at room temperature or overnight at -20°C.

8. Centrifuge the tube at maximum speed for 10 minutes in a microcentrifuge at 4°C.
9. Decant the supernatant carefully and wash the pellet obtained with 1 mL of 70% Ethanol by centrifuging at maximum speed for 2 minutes.
10. Carefully remove the supernatant as completely as possible and gently dab the tube mouth on the tissue paper to completely drain away the leftover Ethanol droplets.
11. Air-dry the DNA pellet for 10 minutes at room temperature.
12. Resuspend the DNA pellet completely in 50 µL of TE Buffer consisting of 20 µg/mL of RNase A.
13. Analyse the plasmid DNA on agarose electrophoresis gel.

**Observation and Results**

Plasmid DNA can exist in three conformations: Open Circular or Nicked, Linear or Relaxed and Supercoiled Forms. When run on Agarose Gel, Supercoiled DNA will run faster and can be observed at the bottom, Open Circular form or Nicked form would run comparatively slower which can be seen in the middle, and Linear or Relaxed form of plasmid would be seen as the top most band in the Agarose Gel, as shown below.




## 2. ISOLATION OF GENOMIC DNA

**AIM: Isolation of Genomic DNA from *Escherichia coli* DH5 α Cells**

**Theory**

The **cell membranes** are **disrupted** with the help of **Lysozyme** and **SDS** in order to **release the DNA** in the buffer. The **genomic DNA** is separated from **RNA, Protein, Lipid** and other molecules from **Cell Lysate**. Released endogenous DNAase capable of degrading genomic DNA are inactivated DNA by chelating necessary cofactor, $Mg^{2+}$ ions using **EDTA**. Phenol and Chloroform are used to denature and separate Proteins from DNA that form a layer at the interface between the aqueous and the organic phases. Chloroform also stabilizes

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interface between Aqueous Phase and Phenol Organic Layer. DNA is precipitated by Absolute Ethanol or Isopropanol, salts and impurities are removed by 70% Ethanol wash.

**Materials Required**

TES Buffer (50 mM Tris-HCl, 50 mM EDTA, 20% Sucrose pH 8.0).

Lysozyme (10 mg/ml in 0.25 M Tris-HCl pH 8.0)

TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0)

SDS 10%

Phenol (pH 8.0)

Chloroform: Isoamyl Alcohol : 24:1

Ethanol 100%, 70%

RNAse (100 µg/ml)

Microcentrifuge Tubes

**Method A. Nucleic Acid Extraction**

1. Transfer 1.5 ml of *Escherichia coli* culture (grown in LB Medium) to a 1.5 ml Microcentrifuge Tube and centrifuge for 5 minutes at 4500 rpm to pellet the Bacterial Cells, decant the medium. Harvest the cells during late log to early stationary phase for maximum yield.
2. Resuspend the pellet in 300 µl of chilled TES Buffer.
3. Add 30 µl of ice cold Lysozyme and 25 µl of 10 % SDS, invert mix and incubate at Room Temperature (RT) for 15 minutes.
4. Add 4 µ of RNase A, mix gently and keep at 37°C (water bath) for 20 min
5. Add equal volume of Phenol and Chloroform: Isoamyl Alcohol, centrifuge at 10,000 rpm for 5 minutes.
6. Collect aqueous layer in fresh Microcentrifuge Tube.
7. Add 1/10 th volume of Sodium Acetate (pH 5.2) and 2.5 volumes of chilled 100 % Ethanol to the Isolated Aqueous Layer, keep at –20 °C for 15 minutes (or can be left overnight).
8. Centrifuge for 10,000 rpm for 10 minutes at 4°C, decant to obtain the pellet.
9. Wash the pellet twice by adding 1 ml of 70 % Ethanol to the tube. Spin at 10,000 rpm for 10 minutes at 4°C and decant the Ethanol.
10. Air dry the pellet at 37°C to remove the traces of Alcohol.
11. Resuspend the pellet in 50 µl of autoclaved Double Distilled Water or TE Buffer by gently tapping the base of the tube with fingers till the pellet dissolves.

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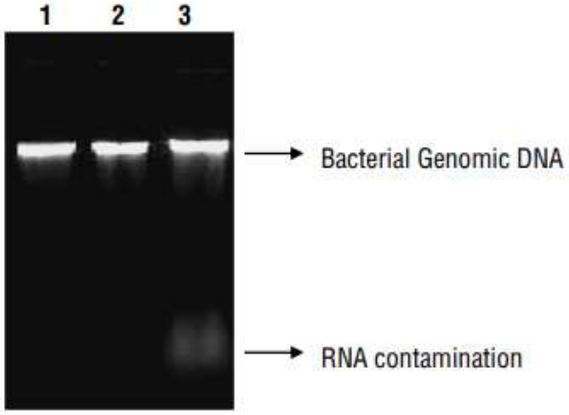
12. Load the Isolated Genomic DNA sample on 0.8 % Agarose Gel and check the profile under UV Transilluminator.
13. Assess the purity of DNA sample by calculating the ratio of absorbance at 260 nm and 280 nm.

**Observation**

DNA smear from high to low molecular weight range are expected on Agarose Gel, although most of DNA if not degraded might be seen accumulated at the well due to their high molecular weight. RNA contamination if present, can be seen as a smear in the lower part of the gel, as shown below.




Ratio of A260/A280 for pure DNA should be 1.8. A ratio of < 1.8 indicates Protein or Phenol Contamination.

**Precautions**

1. Wear gloves while handling Phenol, Chloroform.
2. Use cut/blunt end tips as the Lysate becomes sticky.
3. Nucleases are present on human fingertips also therefore wear gloves throughout to prevent spurious degradation of DNA during the protocol.
4. Perform all centrifugations at 4$^{o}$C.
5. Precipitated pellets might be loosely attached to the side of the tube, so be careful while decanting the Ethanol.
6. Do not use a vortex the tubes as this can lead to DNA shearing.

# EXPERIMENT 12: QUALITY AND QUANTITY CHECKING OF DNA BY UV SPECTROPHOTOMETER AND SUBMARINE AGAROSE GEL ELECTROPHORESIS

**AIM: Quality and Quantity Checking of DNA by UV Spectrophotometer and Submarine Agarose Gel Electrophoresis**

## Theory

After the extraction procedure, the DNA is checked to verify that it is intact and clean of cellular contaminants. Determination of the quality and concentration can be accomplished in two ways: Gel Electrophoresis and UV Spectrophotometry. Strategies for accurately quantifying nucleic acids using these approaches are discussed here.

## 1. QUANTIFICATION USING A UV SPECTROPHOTOMETER

One of the more commonly used practices to **Quantitate DNA** or **RNA** is the use of **Spectrophotometric Analysis** using a **Spectrophotometer**. A spectrophotometer is able to determine the average concentrations of the nucleic acids DNA or RNA present in a mixture, as well as their purity.

**Nucleic Acid Quantification** is commonly performed in a cuvette spectrophotometer. Light absorbed by the nucleic acid in the sample correlates to the concentration of nucleic acid present. Both DNA and RNA absorb light at 260 nm. Nucleic acid samples are also typically measured at 280 nm, which is the absorbance peak for Protein. The ratio of the 260 nm and 280 nm measurements provides a determination of the purity of the nucleic acid, with a ratio near 2 indicating a highly Pure Nucleic Acid sample (Refer Figure 1).

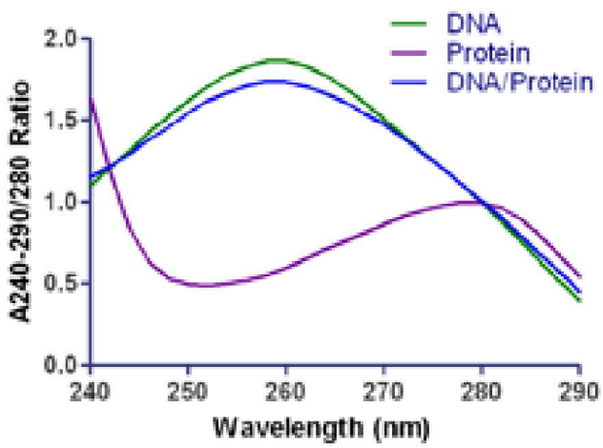



***Fig. 1:*** *Typical Absorbance Spectrum for DNA, RNA and Protein, Indicating the Peak at about 260 nm for DNA and RNA and the Peak at about 280 nm for Protein*

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Figure 1 illustrates the typical absorbance spectrum for DNA, RNA and Protein, indicating the peak at about 260 nm for DNA and RNA and the peak at about 280 nm for Protein.

**Principle**

**Spectrophotometric Analysis** is based on the principles that **Nucleic Acids absorb** the **UltraViolet (UV) light** in a **specific pattern**. In the case of DNA and RNA, a sample is exposed to ultraviolet light at a wavelength of 260 nanometres (nm) and a photo-detector measures the light that passes through the sample. Some of the ultraviolet light will pass through and some will be absorbed by the DNA/RNA. The more light absorbed by the sample, the higher the Nucleic Acid concentration in the sample. The resulting effect is that less light will strike the photo-detector and this will produce a **higher Optical Density (OD)**.

**Beer-Lambert Law**

When using spectrophotometric analysis to determine the concentration of DNA or RNA, the Beer-Lambert law is used to determine unknown concentrations without the need for standard curves. In essence, the Beer-Lambert Law makes it possible to relate the amount of light absorbed to the concentration of the absorbing molecule. Beer-Lambert Law, credited to the separate research of August Beer and Johann Heinrich Lambert in the 19th and 18th centuries, respectively.

The Beer-Lambert equation is shown below:

$$\mathbf{OD = \varepsilon C b}$$

According to this equation, the **Optical Density (OD)** of the sample can be found by the product of the **Extinction Coefficient (ε)** and **Concentration (C)** of the sample and **Path Length (b)** of the measuring vessel. Cuvette-based spectrophotometer has a horizontal light path where the wavelength-specific light is perpendicular to the sample. Most standard cuvettes have fixed optical path lengths of 1 cm. Table 1 illustrates the commonly accepted Extinction Coefficients at known concentrations.

***Table 1:*** *Commonly Accepted Extinction Coefficients at known Concentration*

| Nucleic Acid | Average Extinction Coefficient $(\mu g/mL)^{-1}\ cm^{-1}$ | Concentration (µg/ml) per A260 Unit if OD=1* |
|---|---|---|
| ds DNA | 0.020 | 50 |
| ss DNA | 0.027 | 33 |
| ss RNA | 0.025 | 40 |

* Based on a 1cm Path Length.

## DNA Quality

In research along with determining nucleic acid concentrations, it is also important to ascertain purity before using the sample in downstream applications like Sequencing, Restriction Enzyme Digestions, Ligations, and PCR along with many other applications.

The secondary benefit of using spectrophotometric analysis for nucleic acid quantitation is the ability to determine DNA purity.

Contaminants, such as Protein, RNA, Phenol and Salts associated with the Nucleic Acid Isolation and other Gel Extraction Processes may interfere with subsequent operations, such as cutting with a Restriction Endonuclease. Assessment of purity is therefore important. The most commonly used **assay** is the **A260/A280 Ratio**.

### A260/A280 Ratio

Ratio of absorbance at 260 nm vs 280 nm is commonly used to assess Protein Contamination. Purity of Nucleic Acid Samples is determined by finding the ratio of the Nucleic Acid Measurement at 260 nm and the Protein Measurement at 280 nm, where Protein absorbance peaks. To correct for background turbidity, a measurement at about 320 nm can also be taken, since Protein and Nucleic Acids do not absorb light at this wavelength.

The ratio can be calculated after correcting for Turbidity (Absorbance at 320 nm).

**DNA Purity (A260/A280)**

**= (A260 Reading – A320 Reading) ÷ (A280 Reading – A320 Reading)**

Therefore, taking a spectrum of readings from **230 nm to 320 nm** is most informative.

Table 2 illustrates the sample purity for 260:280 Ratio.

***Table 2:*** *Sample Purity (260:280 Ratio)*

| Protein Contamination or Phenol Contamination | For Pure DNA | RNA Contamination |
|---|---|---|
| Proteins (in particular, the Aromatic Amino Acids) absorb light at 280 nm. Therefore, Protein Contamination will INCREASE the A280 Reading but have little effect on the A260 reading. Thus the A260/A280 Ratio will be….. | | Since RNA also absorb at 260 nm, its presence will lead to higher 260 nm absorbance. This means that if the A260 number is used for calculation of yield, the DNA quantity may be overestimated. |
| Lower than 1.8 | ~1.8 | Greater than 2 |

### A260/A230 Ratio

Strong absorbance around 230 nm can indicate that organic compounds or salts are present in the purified DNA. A ratio of 260 nm to 230 nm can help evaluate the level of **salt carryover** in the Purified DNA. The lower the ratio, the greater the amount of Thiocyanate Salt is present. As a guideline, the A260/A230 is best if greater than 1.5.

However, this is rarely used because common buffers, such as Tris also absorb at 230 nm.

### Preparing Sample for UV Readings

- Water should not be used to dilute the DNA because the pH must be precisely 7.4, and the pH of water can vary. Therefore, TNE Buffer is used when results must be extremely accurate.
- The temperature of the samples is also a variable that must be controlled because as temperature increases, the absorbance reading increases. Therefore, DNA samples and 1X TNE should be at room temperature.

### Working on UV Spectrophotometer

- Before turning the spectrophotometer on, make sure that the sample chamber door is closed. With the sample chamber door closed, turn on the Spectrophotometer and allow it to calibrate and warm up. Leaving the Spectrophotometer open while the machine is warming up can cause damage to the instrument.
- If the protocol is asking for an absorbance or transmittance reading then set the Spectrophotometer accordingly.

### Care While Using Cuvette

- Only matching quartz cuvettes or cuvettes designed exclusively for UV Spectrophotometry should be used as other types of cuvettes do not allow the UV wavelengths to pass through the sample correctly, giving an inaccurate reading. Quartz cuvettes are fragile. Handle them with extra caution and care.
- The amount of buffer is determined by the design of the cuvette. With most quartz cuvettes, 2 ml of Buffer must be added or the light path will be above, rather than through, the buffer.
- In addition, the cuvettes and solutions must be clean and free of particulates that can cause light scattering and give an inaccurate reading. A reading of 1X TNE Buffer only at 325 nm should give an absorbance of 0.01. If it is higher, the cuvette may be dirty or the TNE buffer may have particulates, requiring it to be made fresh and filtered.
- Place the cuvette into the Spectrophotometer correctly, with the arrow on the cuvette top pointing in the direction of the light path.

## Materials Required

**Sample:** DNA Solution

**Reagents:** 1X TNE Buffer

**Glassware and Miscellaneous:** Test Tubes, Test Tube Stand, Micropipettes, Tips, UV-Transparent Cuvettes (depending on the instrument), Quartz Cuvettes

**Instrument:** Spectrophotometer Equipped with a UV Lamp

### 10X TNE Buffer Stock Solution

100 mM Tris-Base (Tris [Hydroxymethyl] Aminomethane)

10 mM EDTA

2.0 M NaCl

Adjust pH to 7.4 using Concentrated HCl and a pH meter. Dilute to 1X Working Concentration using Dilute $H_2O$.

### Procedure

1. Set the absorbance of UV Light at a wavelength of 320, 260, 280, 230 nm to determine DNA Concentration.
2. Dilute the DNA sample by adding 1-2 μL of the DNA sample per ml of 1X TNE Buffer and mix well before inserting the clean cuvette into the Spectrophotometer.
3. Add 2 ml of the Solution (DNA + Buffer) to the cuvette.
4. Place the cuvette into the Spectrophotometer correctly, with the arrow on the cuvette top pointing in the direction of the light path.
5. Take several readings of each sample and average to calculate the concentration and be sure to use the dilution factor.
6. If readings fluctuate greatly from one reading to the next, the DNA concentration is probably too low to give accurate readings with the Spectrophotometer you are using. Try adding more of the DNA sample to the cuvette (10 – 20 μL instead of 1 – 2 μL per mL buffer) and take the readings again.

### Observations and Results

| Sample | Wavelength (nm) | Reading 1 | Reading 2 | Reading 3 | Average |
|---|---|---|---|---|---|
| **Distilled Water** | | | | | |
| **DNA Sample 1** | 260 | 0.64 | 0.65 | 0.67 | 0.65 |
| **DNA Sample 2** | 260 | | | | |
| | | **Ratio 1** | **Ratio 2** | **Ratio 3** | **Average** |
| **DNA Sample 1** | 260/280 | | | | |
| **DNA Sample 2** | 260/280 | | | | |
| | | | | | |

## Calculations

### 1. Quantity of DNA

Determine the concentration of DNA in the original sample using the formula:

**ds DNA Concentration = 50 μg/mL × OD260 × Dilution Factor**

Calculation for Sample 1:

**Concentration = 50 μg/mL × 0.65 × 50**

**= 1.63 mg/mL**

### 2. Quality of DNA

Compare the ratio and comment on quality of DNA

(i) 260/280 = _____

(ii) 260/230 = _____

## Discussion

Comment of concentration and purity of:

1. Sample 1
2. Sample 2

The advantage of a cuvette-based method is that it is simple and precise, while the disadvantages include limited throughput of one-at-a-time sample measurements and the need to dilute samples.

Increasingly, Micro-Plate Spectrophotometers are being used to Quantify Nucleic Acids as well due to increased sample processing. In micro-plate spectrophotometers, the OD Measurement is taken vertically through the samples in the plate, and therefore the Path Length of the samples will vary according to the volume in the well.

The Nano-Drop Spectrophotometer has now become popular because they are very easy to use and only 1-2 μL of sample is used to determine quantity as well quality. It automatically calculates the concentrations and then records them as, ng/μl (1 ng/μl =1 μg/ml).

## Precautions

1. Water should not be used to dilute the DNA because the pH must be precisely 7.4, and the pH of water can vary. Therefore, TNE Buffer is used when results must be extremely accurate.
2. Before turning the Spectrophotometer on, make sure that the sample chamber door is closed. With the sample chamber door closed, turn on the spectrophotometer and allow it to calibrate and warm up. Leaving the spectrophotometer open while the machine is warming up can cause damage to the instrument.

3. Only matching quartz cuvettes or cuvettes designed exclusively for UV Spectrophotometry should be used.
4. With most quartz cuvettes, 2 ml of Buffer must be added or the light path will be above, rather than through, the buffer.
5. In addition, the cuvettes and solutions must be clean and free of particulates that can cause light scattering and give an inaccurate reading.
6. Place the cuvette into the Spectrophotometer correctly, with the arrow on the cuvette top pointing in the direction of the light path.

## 2. QUANTIFICATION USING SUBMARINE AGAROSE GEL ELECTROPHORESIS

### AIM: Quantification using Submarine Agarose Gel Electrophoresis

### Theory

**Agarose Gel Electrophoresis** is the easiest and most popular way of **separating** and **analyzing DNA**. The **Phosphate backbone of DNA molecules** is **negatively** charged therefore **negatively charged DNA** molecules **move from negatively charged electrode (cathode)** towards **positively charged electrode (anode)** when placed in an electric field. Shorter molecules move faster and migrate farther than longer ones because shorter molecules move easily through the small pore size of the gel. This phenomenon is sieving. In order to visualize the DNA in the Agarose Gel, staining with an intercalating dye, such as Ethidium Bromide or SYBR Green is required. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band.

DNA concentration can also be estimated by measuring the UV-Induced Emission of fluorescence from intercalated **Ethidium Bromide**. This method is useful if there is not enough DNA to quantify with a spectrophotometer, or if the DNA solution is contaminated.

In this method, a sample of DNA to be quantified is diluted. It is best to dilute the DNA 1/10 and 1/100 and run both dilutions on the gel with a Molecular Weight Marker (MWM) of known concentration. The DNA concentration is determined by comparing it to one band in the MWM that most resembles it in brightness. Any RNA, Nucleotides and Protein in the sample migrate at different rates compared to the DNA so the band(s) containing the DNA will be distinct.

Concentration and yield can be determined after Gel Electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard. For example, if a 2µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100 ng standard, then the solution concentration is 50 ng/µl (100 ng divided by 2 µl). Standards used for quantitation should be labeled as such and be the same size as the sample DNA being analyzed.

The appearance of the DNA on the gel can also reveal if it is clean and intact. If the DNA is intact, it will appear as a distinct band on the gel. If it is degraded, it will appear as a smear of thousands of small fragments. If the DNA is contaminated with Protein, there will be a bright band of DNA at the bottom of the well and along the migration path from the wells where the slower moving protein trapped DNA. If the well is over loaded with DNA that is too concentrated, the band will have a jagged smear above it.

**Materials Required**

**Chemicals and Reagents:** 1% Agarose, Ethidium Bromide (10 mg/ml), Electrophoresis Buffer (1X TAE Buffer, pH 8.0), Distilled Water, Gel Loading Buffer, DNA Sample, DNA Ladder Appropriately Sized

**Glassware:** Conical Flask, Measuring Cylinder

**Other Requirements:** Microwave/Burner, Adhesive Tapes, Micropipettes, Tips, Gloves

Instruments: Horizontal Gel Electrophoresis Tank (including Gel Casting Tray, Comb, Power Supply), External Power Supply, Transilluminator

**Role of Equipment and Supplies used for Conducting Agarose Gel Electrophoresis**

- An electrophoresis chamber and power supply.
- Gel casting trays, which are available in a variety of sizes and composed of UV-Transparent Plastic.
- Sample combs, around which molten Agarose is poured to form sample wells in the gel.
- Electrophoresis Buffer.
- Loading Buffer, which contains something dense (for example, Glycerol) to allow the sample to 'Fall' into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- Ethidium Bromide, a fluorescent dye used for staining Nucleic Acids.
- Transilluminator (an Ultraviolet Light Box), which is used to visualize Ethidium Bromide-Stained DNA in Gels.
- DNA Ladder, Molecular Weight Size Marker.

**50X Concentrated TAE Electrophoresis Buffer (40 mM Tris-Acetate, 2 mM EDTA)**

Add the following to Diluted $H_2O$ to give a final volume of 1 liter:

1. 242 g Tris-Base
2. 57.1 ml Glacial Acetic Acid
3. 100 ml 0.5 M EDTA (pH 8.0)

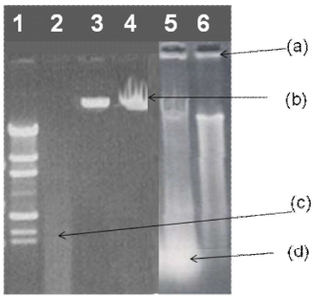
## 1X TAE Electrophoresis Buffer

Dilute 50 x TAE Electrophoresis Buffer with Diluted $H_2O$ to yield 1 liter of 1X TAE Electrophoresis Buffer.

### Procedure

1. Prepare gel tray by sealing the ends with adhesive tape. Place comb in gel tray about 1 inch from one end of the tray and position the comb vertically such that the teeth are about 1-2 mm above the surface of the tray.
2. To prepare 50 ml of 0.8% Agarose Solution, measure 0.4 g Agarose into a glass beaker or flask and add 50 ml 1X TAE Buffer. Heat the mixture on a microwave or hot plate, swirling the glass beaker/flask occasionally, until Agarose is dissolved completely. Ensure that the lid of the flask is loose to avoid buildup of pressure.
3. Allow solution to cool down to about 55-60°C. Add 2.5 µl Ethidium Bromide (to get final Concentrated 0.5 µg/ml from 10 mg/ml stock), mix well and pour the gel solution into the gel tray to a depth of about 5 mm. Allow the gel to solidify for about 30 minutes at room temperature.
4. To start the run, carefully remove the adhesive tape from both the ends of the gel tray, place the tray in electrophoresis chamber, and fill the chamber (just until wells are submerged) with 1X TAE Electrophoresis Buffer and gently remove the comb.
5. Sample of DNA to be quantified is diluted to 1/10 and 1/100.
6. Load 3 µl of the ready to use Molecular Weight Marker (MWM) of known concentration onto Well 3. Load 5 µl of both the dilutions to Well 1 and Well 2.
7. Connect the power cord to the electrophoretic power supply according to the convention, i.e., Red-Anode and Black-Cathode.
8. Electrophorese at 100-120 volts and 90 mA.
9. Switch off the power supply once the tracking dye from the wells reaches 3/4th of the gel which takes approximately 45 minutes.
10. Observe the gel on a UV Transilluminator. Compare fluorescence intensities and estimate DNA concentrations.
11. Comment on the quality of the DNA on following aspects:
    (i) If the DNA is intact, if it is degraded.
    (ii) If the DNA is contaminated with Protein and/or RNA.
    (iii) If the DNA is too concentrated.

## Observations and Results

| A. Quantitative Estimation | B. Qualitative Estimation |
|---|---|
|  |  |



### A. Quantitative Estimation

The band of DNA in Lane 1 is about the same intensity as the MWM band at the arrow. Since, the MWM band designated by the arrow contains 10 ng of DNA, so band in Lane 1 also contains 10 ng of DNA. However, this band is from 1/100 dilution, therefore, the original sample contained 100X as much, i.e., 1 μg.

Since 5 μl were loaded on the gel, therefore concentration is1 μg/ 5 μl. Concentration in the original sample is therefore = 0.2 μg/μl.

### B. Qualitative Estimation

Lane 1 - Molecular Weight Marker DNA.

Lane 2 - Smear (c) indicate Degraded DNA. Smear contains thousands of small fragments.

Lane 3 - Distinct Band indicate that the DNA is intact without any contamination.

Lane 4 - Jagged Smear above band (b) indicate that the well is overloaded that is too concentrated.

Lane 5 - A Bright Band of DNA is seen at the bottom of the well because the slower moving protein get trapped in well (a) indicating that DNA is contaminated with protein. Some DNA is degraded (as smear is also visible). Some DNA is overloaded (jagged smear above band). DNA is also contaminated with RNA (d).

Lane 6 - DNA is contaminated with Protein, some DNA is degraded.

### Discussion

There are Fluorometric and Spectrophotometric methods for Nucleic Acid Quantification. The former is used for applications requiring high sensitivity due to minute available amounts of Nucleic Acid. Nano-Drop Spectrophotometer further reduce this quantity to 1-2 μl of DNA.

**Precaution**

1. Make sure that the Agarose is fully dissolved in the buffer. If it is not dissolved well, again melt it some more time to dissolve completely.
2. Before casting the gel, the tray and comb should wipe with Ethanol.
3. Check that no air bubbles are under or between the teeth of the comb.
4. The gel should be between 3-5 mm thick.
5. Make sure that the gel in the Chamber is immersed in the TAE Buffer.
6. It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel preparation.
7. Electrophoresis apparatus should always be covered to protect against electric shocks. Avoid use of very high voltage which can cause trailing and smearing of DNA bands in the gel, particularly with High-Molecular-Weight DNA.
8. Monitor the temperature of the buffer periodically during the run. If the buffer becomes heated, reduce the voltage. Melting of an Agarose Gel during Electrophoresis is a sign that the voltage is too high, that the buffer may have been incorrectly prepared or has become exhausted during the run.
9. Always wear protective eyewear when observing DNA on a Transilluminator to prevent damage to the eyes from UV Light.
10. Because Ethidium Bromide is a known Mutagen, precautions need to be taken for its proper use and disposal. So wear gloves while handling. All items that were in contact with Ethidium Bromide must be disposed off in the designated as waste container, marked with 'Ethidium Bromide Waste', and to the Gel/Doc Area, including Gels, Tissue Paper used to clean the table, and Nitrile Gloves.

## EXPERIMENT 13: GENE CLONING – PREPARATION OF VECTOR AND PASSENGER – LIGATION – PREPARATION OF COMPETENT CELLS – TRANSFORMATION OF *ESCHERICHIA cOLI* WITH RECOMBINANT PLASMIDS

**AIM: Gene Cloning Preparation of Vector and Passenger, Ligation, Preparation of Competent Cells, Transformation of *Escherichia coli* with Recombinant Plasmids**

**Theory**

**Gene Cloning** is a process through which an **exact copy** of a **particular gene** is made. The basic procedure of molecular cloning involves a series of steps, as discussed below:

- The first step in the production of a **Recombinant Protein** is to select a suitable **Cloning Vector** in which the **desired gene** can be inserted and amplified in a suitable host system.
- Second, the **Cloning Vector** and the **DNA Sequence** containing **desired gene** are first treated with an appropriate **Restriction Endonuclease (RE)**.
- Third, the fragment thus produced is ligated to other DNA molecules that serve as vectors. **Vectors** can **replicate autonomously** (independent of **Host Genome Replication**) in **host cells** and facilitate the manipulation of the newly created **recombinant DNA** molecule.
- Fourth, the **recombinant DNA molecule** is transferred to a **host cell**. Within this cell, the recombinant DNA molecule **replicates**, producing dozens of **identical copies** known as '**Clones**'. As the host cells replicate, the recombinant DNA is passed on to all progeny cells, creating a **population of identical cells**, all carrying the **cloned sequence**.
- Finally, transformants are then selected from non-recombinants either by using selectable markers like **Antibiotic Resistance Genes** (**$Amp^r$/$tet^r$**) or by **Lac Z** method.

Entire cloning procedure is discussed in following steps:

1. Preparation of Vector and Insert DNA
2. Ligation of the Two Linear DNA Fragments
3. Preparation of Competent Cells
4. Transformation of the Ligated Product
5. Screening for the Right Clone

## 1. Preparation of Vector and Insert DNA

The first step in the production of a **Recombinant Protein** is to select a suitable **Cloning Vector** in which the **desired gene** can be inserted and amplified in a suitable **host system**.

The **cloning vector** and the **DNA sequence** containing **desired gene** are first treated with an appropriate **Restriction Endonuclease (RE)**. Restriction Endonucleases or RE recognize a specific **Nucleotide Sequence** on a double-stranded DNA molecule, called a **Restriction Site**, and cleave the DNA at this recognition site. Generally both the Vector and the Foreign DNA are digested with the same restriction enzyme. Foreign DNA has two restriction sites for this particular restriction enzyme while vector has only one restriction site. The restriction digested vector DNA has to be **dephosphorylated** to prevent self-ligation.

**Cloning Vectors:** Several naturally occurring **Plasmids** have been engineered to make different types of **Cloning Vectors**. Cloning vectors are circular DNA molecules in which inserts (desired gene) are maintained and amplified. Vectors should have the following features that make them compatible for a variety of uses in recombinant DNA procedures:

**Size:** Vectors are relatively small molecules; most are only 2.5 - 3 Kb in size.

**Ori:** A vector should contain an **origin of replication (ori)** in order to maintain its autonomous state.

**Marker:** To facilitate selection of cells that contain the vector, a vector should carry at least one genetic marker for which an easy assay exists (for example, **Ampicillin Resistance, Amp$^r$**).

**Cloning Sites:** A vector should contain several different Restriction Endonuclease sites that allow the insertion of a **gene of interest** during **cloning**. Many cloning **vectors** like **pUC19** contain a **synthetic DNA sequence** called a **Polylinker**, which consists of **21 closely-Packed Restriction Endonuclease Recognition Sites** in a small region of DNA.

## 2. Ligation of Insert and Vector

Treatment with **Restriction Endonuclease (RE)** creates blunt or sticky ends at both vector and insert, which can be joined together through a process called **Ligation**, producing a **recombinant DNA molecule**. **T4 DNA Ligase** and ***Escherichia coli* DNA Ligase** commonly used for this process covalently joins the annealed cohesive ends by forming a Phosphodiester bond between **3'-OH** end of one strand and **5'-$PO_4$** of the other end as shown in Figure 1.

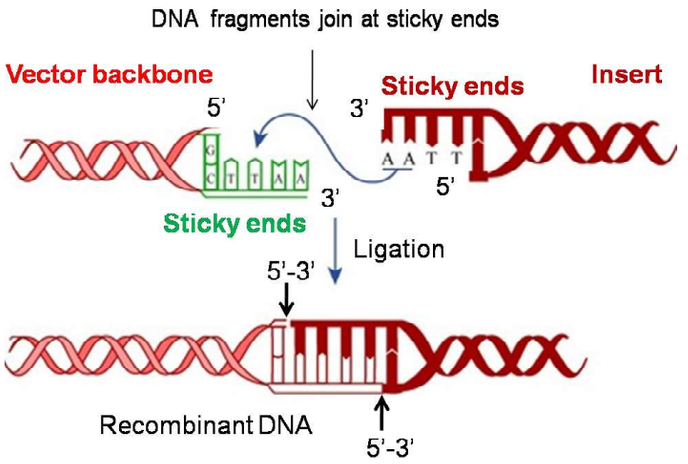



***Fig. 1:*** *Ligase Form a Phosphodiester Bond between 3'-OH and 5'-$PO_4$*

**T4 DNA Ligase** is the most commonly used Ligase for DNA Cloning. It requires ATP for its activity, generally supplied in Ligation Buffer, while the ***Escherichia coli*** enzyme requires **$NAD^+$**. T4 DNA Ligase is approximately 68000 dalton (68 kD) protein produced by **Bacteriophage**.

## Factors Affecting Ligation

The given factors affect ligation.

• **Insert to Vector Ratio**

The ideal ratios for ligating insert to vector for sticky end ligations ranges between 1:1 and 3:1, whereas for blunt ended ligations, the insert to vector ratio should be at least 10:1. If the ratio of plasmid backbone to insert DNA is **too high** then excess '**empty**' mono and polymeric plasmids will be generated. **Too low ratio** results in an excess of linear and circular homo-polymers and hetero-polymers.

## Calculating Insert Amount

**Insert Mass in ng**

**= 6 × [Insert Length in bp / Vector Length in bp] × Vector Mass in ng**

The insert to vector molar ratio can have a significant effect on the outcome of a ligation and subsequent transformation step. Molar ratios can vary from a 1:1 insert to vector molar ratio to 10:1 depending on the basis of sticky or blunt ends. It may be necessary to try several ratios in parallel for best results.

• **DNA Concentration**

Total DNA should not exceed 100 ng for cohesive end ligation and 1.0 µg for blunt end ligations.

• **Ligase Concentration**

The higher the ligase concentration, the faster the rate of ligation. Blunt-end ligation is much less efficient than sticky end ligation, so a higher concentration of ligase is used in blunt-end ligations. High DNA ligase concentration may be used in conjunction with PEG for a faster ligation. Generally, 0.1 units for cohesive end ligations; and 1 unit for blunt end ligations is recommended. While preparing the reaction mixture the ligase should always be added at the last.

• **Temperature**

T4 DNA Ligase has the unique ability to join sticky and blunt ended fragments. However, it is easier to ligate molecules with complementary sticky ends than blunt ends due to complementary base pairing between the ends. Cohesive end ligation is carried out at 12°C to 16°C to maintain a good balance between annealing of ends and activity of the Enzymes. If reaction is set at higher temperatures annealing of the ends become difficult, while lower temperatures diminishes the Ligase Activity. All the T4 DNA Ligase is inactivated by heating at 65°C for 10 minutes. Lack of cohesive termini makes blunt end ligation more complex and significantly slower. Since annealing of ends is not a factor, the reaction is done at 24°C. However, 10 - 100 times more enzyme is required to achieve similar ligation efficiency as that of cohesive end ligation.

**• Buffer Composition**

The ionic strength of the buffer used can affect the ligation. The kinds of cations present can also influence the ligation reaction, for example, excess amount of $Na^+$ can cause the DNA to become more rigid and increase the likelihood of intermolecular ligation. At high concentration of monovalent cation (>200 mM) ligation can also be almost completely inhibited. The standard buffer used for ligation is designed to minimize ionic effect. T4 DNA Ligase requires ATP for its activity, therefore, buffer ATP should be supplied in ligation buffer.

### 3. Preparation of Competent Cells

Competence is the ability of a cell to take up extracellular DNA from its environment. Uptake of DNA requires the recipient cells to be in a specialized physiological state called competent state. Competent cells possess altered cell walls which enhance foreign DNA transfer inside the cell. It distinguished into Natural Competence, and Induced or Artificial Competence.

**• Natural Competence**

Some types of bacteria are naturally transformable, which means they can take up DNA from their environment without requiring special treatment. Natural competence was first discovered by Frederich Griffith in 1928. A genetically specified ability of bacteria is thought to occur under natural conditions as well as in the laboratory. Once the DNA has been brought into the cell's cytoplasm, it may be degraded by the nuclease enzymes or if it is very similar to the cells own DNA, the DNA repairing enzymes may recombine it with the chromosome.

**• Induced or Artificial Competence**

Natural ability of a cell to take up cell free DNA present in extracellular environment is low and only 1% cells are capable to take DNA. Most cells cannot take up DNA efficiently unless they have been exposed to special chemical or electrical treatments to make them competent. The standard method for making the bacteria permeable to DNA involves treatment with Calcium Ions. In $CaCl_2$ method, the competency can be obtained by creating pores in bacterial cells by suspending them in a solution containing high concentration of Calcium. DNA can then forced into the host cell by heat shock treatment at 42°C for the process of transformation.

### 4. Transformation of the Ligated (Recombinant) Product

An appropriate host, such as *Escherichia coli*, is selected and then transformed with the recombinant molecule thus created. **Transformation** is a process of **Bacterial Gene Exchange**, which was discovered by Frederick Griffith in 1928. During the process, **genes are transferred** from **one Bacterium to another** as '**naked**' **DNA solution**.

Successfully transformed bacteria will carry either Recombinant or Non-Recombinant Plasmid DNA. Multiplication of the Plasmid DNA occurs within

each transformed cell. A single bacterial cell placed on a Solid Surface (Agar Plate) containing nutrients can multiply to form a visible colony made of millions of identical cells. As the host cell divides, the plasmid vectors are passed on to progeny, where they continue to replicate.

Since DNA is a very **Hydrophobic Molecule**, it will not normally pass through a **Bacterial Cell Membrane**. In order to uptake the Foreign DNA, the bacterial cells must first be made competent as discussed above.

There are two forms of **transformation**: **Natural** and **Artificial**.

### Natural Transformation

In nature, some bacteria, perhaps after death and Cell Lysis, release their DNA (double-stranded) segment into the environment. Other bacteria can then encounter the double-stranded DNA fragment, which gets attached to the cell wall of the bacteria. However, only one strand passes through the cell wall and into the cell's cytoplasm, other get degraded. If there is sufficient sequence similarity, the foreign DNA undergoes homologous recombination with the recipient chromosome. The genome of the recipient cell has now been modified to contain DNA with genetic characteristics of the donor cell. Naturally occurring transformations are of great interest medically because they may serve as a vehicle for genetic exchange among pathogenic organisms. Interestingly, it appears that a larger percentage of pathogenic bacteria, such as ***Streptococcus pneumoniae, Neisseria gonorrhoeae,*** and ***Haemophilus influenzae***, is capable of natural transformation than the nonpathogenic bacteria. This raises the intriguing possibility that the exchange of genetic material allows pathogenic cells to acquire the ability to evade a host's bodily defenses. Transformation works best when the donor and recipient cells are very closely related.

### Artificial Transformation

Not all bacteria are naturally transformable, however, methods have been developed to produce competency in various types of cells and transform those cells artificially.

- **Calcium Chloride Treatment:** This mode of transformation is easy to perform and requires minimum number of equipment. In this method cells are incubated in a **Concentrated Calcium Salt Solution** to make their membranes leaky. The permeable '**competent**' cells are then mixed with **DNA** (**ligation mix**) to allow entry of the DNA into the bacterial cell, the Calcium ions in ice-cold condition. As a result small pores are formed on the cell membrane, which makes it permeable. The plasmid DNA may adhere to the surface of the cell and uptake is mediated by a pulsed **heat shock** at 42°C for 90 seconds. Heat shock is known to create transient pores from which DNA can enter the cell. A rapid chilling step on ice ensures the closure of the pores.

- **Electroporation:** Another membrane-altering method is **Electroporation**. In this method, cells are suspended in a DNA solution and subjected to high-voltage electric impulses that destabilize the cell membrane, resulting in increased permeability and enabling DNA to pass into the cells. Several hundred volts across plasma membrane is applied in this process. The deliver process is ~10 times more efficient than Calcium Chloride mediated transformation. Electroporation is performed with the help of **Electroporator**, which can create very high electric field in a specialized cuvette. The transformants are then selected from non-recombinants either by using selectable markers like **Antibiotic resistance genes (Amp$^r$/tet$^r$)** or by **Lac Z** method of selection.

Figure 2 illustrates the process of Bacterial Transformation.

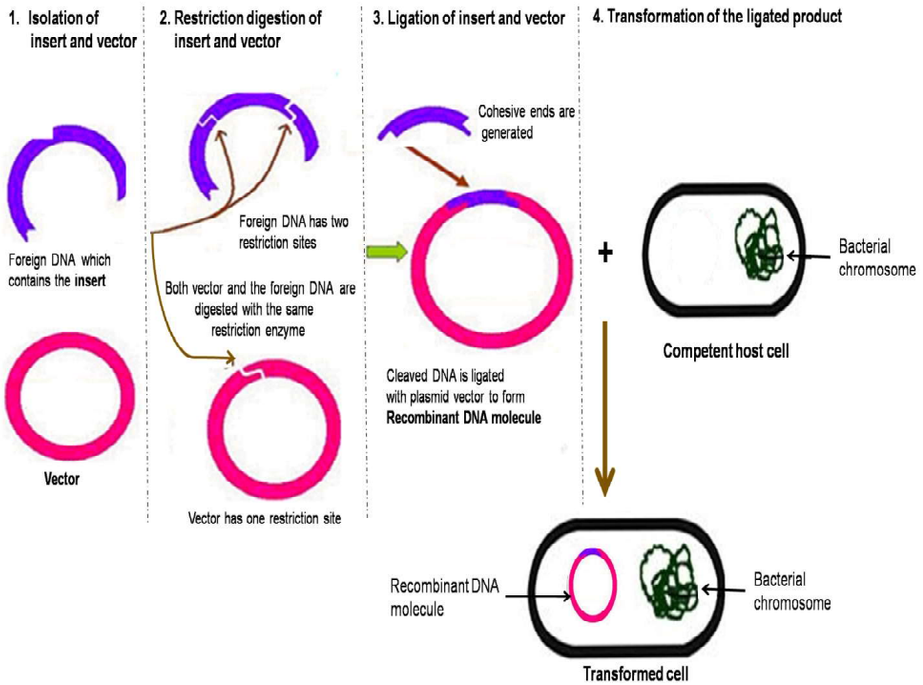



***Fig. 2:*** *The Process of Bacterial Transformation*

## Materials Required

**Culture:** *Escherichia coli* Host with Plasmid pUC19.

**Chemicals and Reagents:** Ampicillin, Luria Bertani (LB) Broth, Luria Bertani Agar, *Escherichia coli* Host, Vector, Insert (Lambda DNA –Hind III Digest), Positive Control Plasmid, Negative Control Plasmid, 10 X Ligase Assay Buffer, T4 DNA Ligase, X-Gal, IPTG, 1M Calcium Chloride (Sterile).

**Glassware:** Conical Flask, Measuring Cylinder, Beaker.

NOTES

**Instrument:** Water Bath (42°C), 37°C Incubator, 37°C Shaking Incubator, Centrifuge.

**Other Requirements:** Micropipettes, Tips, 50 ml Centrifuge Tubes, Crushed Ice, Sterile Double Distilled Water, Sterile Loop and Spreader, Inoculation Loop.

**Preparation of Reagents**

1. **0.1M Calcium Chloride (Sterile) (200 ml):** To prepare 200 ml of 0.1M Calcium Chloride, take 20 ml of 1M Calcium Chloride and add 180 ml of Sterile Distilled Water. Store at 2-8°C.
2. **Ampicillin**: Dissolve 30 mg of Ampicillin Powder in 600 µl of Sterile Double Distilled Water to give a Concentration of 50 mg/ml.
3. **X-Gal:** Make a 2% (wt/vl or w/v) Stock Solution by Dissolving X-Gal in Dimethylformamide at a Concentration of 20 mg/ml Solution. The X-Gal Tube should be wrapped with aluminum foil in order to prevent the damage caused by light and store at -20°C.
4. **IPTG:** Make a 20% (w/v, 0.8 M) Solution of IPTG by Dissolving 2 g of IPTG with 8 ml of Distilled $H_2O$. Prepare the aliquots and store them at -20°C.
5. **LB (Luria Bertani) Broth (55 ml):** Dissolve 1.5 g of LB Media in 60 ml Distilled Water Sterilize by autoclaving.
6. **LB (Luria Bertani) Agar Plates (20 ml):** Dissolve 0.8 g of LB Agar in 20 ml of Sterile Distilled Water. Sterilize by autoclaving and pour on Sterile Petri Plate.
7. **LB (Luria Bertani) Agar Plates containing Ampicillin, X-Gal and IPTG (100 ml):** Dissolve 4.0 g of LB Agar Media in 100 ml of Distilled Water. Sterilize by autoclaving and allow the Media to cool down to 40-45°C. Add 100 µl of Ampicillin, 200 µl of X-Gal and of 100 µl IPTG to 100 ml of autoclaved LB Agar Media, mix well and pour on Sterile Petri Plates.

**Procedure**

The following procedures are involved:

**A. Revive the Culture**

1. Pick up a loopful of culture and streak onto LB Agar Plate.
2. Incubate overnight at 37°C.

**B. Ligation**

1. For setting up of a Ligation Mix, T4 DNA Ligase and 10X Ligase Buffers are temperature sensitive and should always be placed and thawed/defrosted on ice. T4 DNA Ligase is very unstable on ice (use as fast as possible and keep at -20°C).

2. Set up the Ligation Reaction as follows:

| | | |
|---|---|---|
| Deionized Water | | 2 µl |
| Vector DNA * | 1µ µg | 3 µl |
| Insert DNA * | 1 µg | 3 µl |
| 10X Ligase Buffer | 1X | 1 µl |
| T4 DNA Ligase** | 1 Unit | 1 µl |
| Total Reaction Volume | | 10 µl |

**Ligase Buffer Contains:** 250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) Polyethyene Glycol (PEG)-8000. Remember that the buffer contains ATP so repeated freeze, thaw cycles can degrade the ATP thereby decreasing the efficiency of Ligation.

*Total DNA should not exceed 100 ng for cohesive end ligation and 1.0 µg for blunt end ligations.

**0.1 Units for cohesive end ligations, and 1 Unit for blunt end ligations.

3. While preparing the reaction mixture the Ligase should always be added at the last.
4. After preparing the reaction tube, mix the components by gentle pipetting and tapping.
5. Incubate the tubes at 16°C Water Bath, overnight.

   **Note:** Different incubation can be performed. For blunt end ligations it is better to incubate at 16°C overnight. For cohesive end ligations, one hour at RT (Room Temperature) is more than enough.
6. Although not necessary, DNA Ligase can be inactivated at 65°C for 10 minutes.
7. After incubation, the Ligated Mixture is taken for doing Transformation.

## C. Preparation of Competent Cells

1. Pick a Single Bacterial Colony from the Revived Culture Plate.
2. Transfer the Colony into Sterile 50 ml LB Broth in a 500 ml Flask. Incubate the Culture at 37°C shaker at 250 rpm for 2-3 hours till the O.D. reaches ~ 0.6.
3. Transfer the above culture into a pre-chilled 50 ml Polypropylene Tube.
4. Allow the culture to cool down to 4°C by storing on ice for 10 minutes.
5. Recover the cells by centrifuge at 4000 rpm for 10 minutes at 4°C.
6. Decant the medium completely. No traces of medium should be left.
7. Resuspend the cell pellet in 30 ml pre-chilled Sterile 0.1 M Calcium Chloride Solution.
8. Incubate on ice for 30 minutes.

9. Centrifuge at 5000 rpm for 10 minutes at 4°C.
10. Decant the Calcium Chloride Solution completely. No traces of solution should be left.
11. Resuspend the pellet in 2 ml pre-chilled Sterile 0.1M Calcium Chloride Solution.
12. This Cell Suspension contains Competent Cells and can be used for Transformation.

## D. Transformation of Cells

1. Take 3 Eppendorf Tubes and label them as:
   (i) **Control:** 200 µl of the Competent Cell Suspension only.
   (ii) **Plasmid:** 200 µl of the Competent Cell Suspension + Plasmid (without Insert).
   (iii) **Ligation Mixture:** 200 µl of the Competent Cell Suspension + Ligation Mixture (containing both Plasmid and Insert).
2. Mix all three tubes and incubate all the 3 tubes on ice for 30 minutes.
3. Transfer them to a Preheated Water Bath set at a temperature of 42°C for 2 minutes (heat shock).
4. Rapidly transfer the tubes on ice. Allow the cells to chill for 5 minutes.
5. Add 800 µl of LB Broth to all the tubes. Incubate the tubes for 1 hour at 37°C to allow the Bacteria to RECOVER and to express the Antibiotic Resistance Marker Encoded by the Plasmid.
6. Spread 200 µl of cell culture from each tube on LB Agar Plate containing Ampicillin, X-Gal, and IPTG using a sterile spreader until the liquid has been absorbed in by the medium.
7. Store at room temperature till the plates are dry.
8. Incubate the plates overnight at 37°C.
9. Observe the plates for Transformed Colony.

### Observation and Results

Figure 3 illustrates the non-transformant and transformant Bacterial Growth on plates.

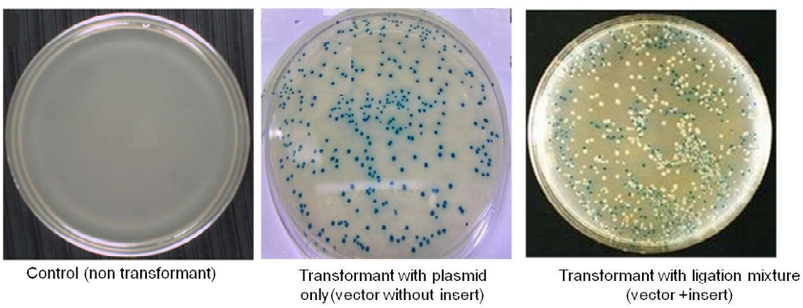



***Fig. 3:*** *Non-Transformant and Transformant Bacterial Growth on Plates*

- After incubation, observe the Bacterial Growth on plates and look for the transformants and non-transformants on the plate.
- No growth observed on Plating Competent Cells Untransformed with Plasmid (Control).
- Growth observed on plating with Ligation Mixture.

Write your observations in Table as provided below and interpret the results.

**Observation Table for Bacterial Growth**

| Control (Only Competent Cells) | Competent Cells + Plasmid (Vector Only) | Competent Cells + Ligation Mixture (Vector+ Insert) |
|---|---|---|
| No Growth | Blue Colonies | White Colonies |

**Interpretation**

In the process of Transformation, the Competent Cells are incubated with DNA in ice. Then it is placed in a Water Bath at 42ºC and further plunging them in ice. This process will take up the DNA into the Bacterial Cell. Then it is plated in an Agar Plate containing appropriate Antibiotic. The presence of an Antibiotic Marker on the Plasmid allows for rapid screening of successful transformants. Those cells that GROW in presence of Ampicillin are TRANSFORMED CELLS. In contrast, NO GROWTH is observed on Plating Competent Cells UNTRANSFORMED with PLASMID as the host ***Escherichia coli*** cell is SENSITIVE to AMPICILLIN.

Blue –White Selection can be used to determine which plasmids carry an inserted fragment of DNA and which do not. The Transformants turn BLUE on X-Gal and IPTG containing plates due to the production of Active β-Galactosidase. X-Gal is the Chromogenic Substrate of β-Galactosidase and IPTG acts as the inducer for the expression of this enzyme. Ligation Mixture has Insert Cloned in the N-Terminal Coding Sequence for β-Galactosidase Gene. As a result enzymatically Active β-Galactosidase is not produced and the Transformants are WHITE in colour.

**Note:** See Blue –White Screening in detail in next Experiment 14.

**Precautions**

1. The revival step is necessary both to allow the Plasmid Establishment and to allow expression of the Resistance Genes.
2. It is essential that the cells used are in a Rapid Growth Phase when harvested. Do not let them approach stationary phase.
3. While making the plates add accurate amount of Ampicillin.
4. Add Ampicillin to the Media after it cools down to 40-45°C to avoid inactivation of Ampicillin.

5. Always thaw the Ligase Buffer and the enzyme on ice.
6. Make sure the buffer is completely melted and dissolved.
7. T4 DNA Ligase is very sensitive to shear, so spinning your Ligation Mix or vortexing it to mix it can affect your yields. Instead try mixing with the pipette tip or slowly resuspending the solution.
8. Try to maintain temperature during Ligation.
9. Always keep the Competent Cells on ice and make sure that the centrifuge machine is cold throughout the experiment.
10. Competent Cells can be stored at 4°C once competent. Holding cells in $CaCl_2$ at 4°C will, in fact, increase transformation efficiency although this declines with more than 24 hours storage. Long periods of storage can be achieved by freezing the Competent Cells.
11. Heat shock treatment should be very specific with respect to temperature and time.
12. Incubate the plates at 37°C for minimum 18 hours to avoid few or no transformants.
13. Make sure that the entire procedure is performed aseptically to avoid contamination on plates.

## EXPERIMENT 14: SELECTION OF RECOMBINANTS BY BLUE –WHITE SELECTION

**AIM: Selection of Recombinants by Blue –White Selection**

**Theory**

**Gene Cloning** is a process through which an **exact copy** of a **particular gene** is made. The **basic procedure** of **molecular cloning** involves a series of steps, as discussed below.

- The first step in the production of a **Recombinant Protein** is to select a suitable **Cloning Vector** in which the **desired gene** can be inserted and amplified in a suitable host system.
- Second, the **cloning vector** and the **DNA sequence** containing desired gene are first treated with an appropriate **Restriction Endonuclease (RE)**. Restriction Endonucleases or RE recognizes a specific **Nucleotide Sequence** on a double-stranded DNA molecule, called a **Restriction Site**, and cleave the DNA at this recognition site. Generally both the Vector and the Foreign DNA are digested with the same restriction enzyme. The **restriction digested vector DNA** has to be **dephosphorylated** to prevent self-ligation.

- Third, the fragment thus produced is ligated to other DNA molecules that serve as vectors. Vectors can replicate autonomously (independent of Host Genome Replication) in host cells and facilitate the manipulation of the newly created recombinant DNA molecule.
- Fourth, the recombinant DNA molecule is transferred to a host cell. Within this cell, the **recombinant DNA** molecule **replicates**, producing dozens of **identical copies** known as '**Clones**'. As the host cells replicate, the recombinant DNA is passed on to all Progeny Cells, creating a population of identical cells, all carrying the **cloned sequence**.
- Finally, transformants are then selected from non-recombinants either by using selectable markers like **Antibiotic resistance genes** (**Amp$^r$/tet$^r$**) or by **Lac Z** method of selection.

Figure 1 illustrates the process of Bacterial Transformation.

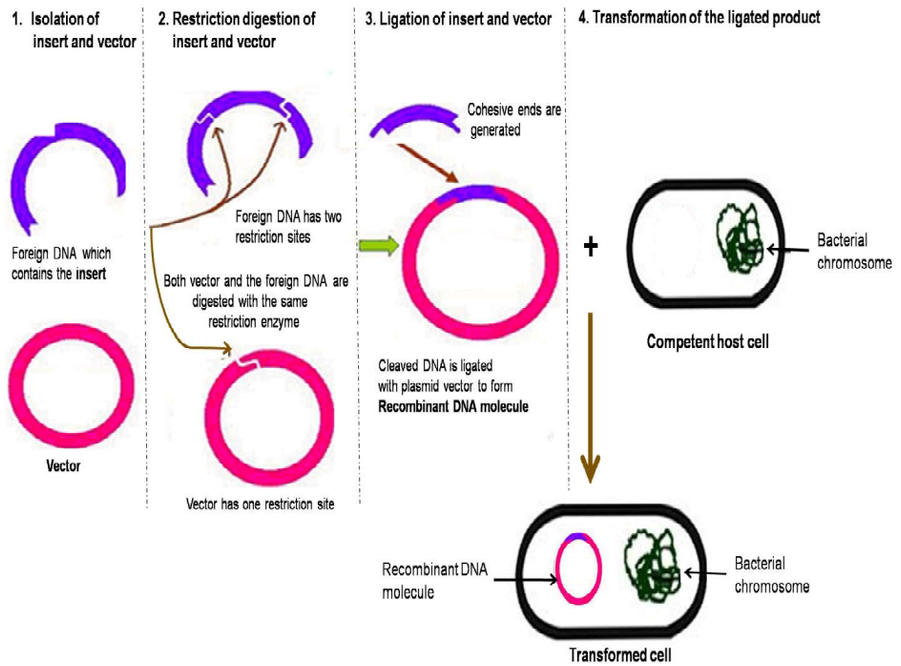



***Fig. 1:*** *Bacterial Transformation Process*

## Blue–White Screening Method or Lac Z Method of Detection

The last step during cloning was the screening for the right clone. **Screening** means to **detect** the **right clone** among a population of colonies. Screening procedure depends upon the vector used for cloning. In this experiment we are using **pUC19** as **Plasmid Vector**.

***Escherichia coli*** **host strain** used for **cloning** purpose has a **deletion** at the **Amino terminal** of the **Lac Z Gene**, therefore it carry a defective Lac Z Gene which produces only **ω-Peptide** of the functional enzyme **β-Galactosidase**.

Cloning vector (*Escherichia coli* Plasmid pUC19) carries Lac Z Gene is capable of producing only the **α-Peptide** of the **β-Galactosidase Gene**. So, when vector is transformed into *Escherichia coli* Competent Cells, the truncated products from both complement each other and as a result enzymatically active β-Galactosidase is produced. This is called **α-Complementation**.

**α-Peptide + ω-Peptide → Functional β-Galactosidase Gene**

**(Vector Coded) (Host Coded)**

In addition, this **Vector (pUC19)** also carries sites for different restriction enzymes (Multiple Coning Site, MCS) within the N-Terminal Coding Sequence for Lac Z Gene. If an insert is cloned within the MCS of pUC19, Lac Z gene within vector gets disrupted and no longer code for α-Peptide. So, when recombinant DNA molecule (carrying Insert into Vector) is transformed inside *Escherichia coli* Competent Cell, only ω-Peptide is produced by defective Lac Z gene present in *Escherichia coli* Chromosome. Thus there is no α-Peptide present to complement and hence no full β-Galactosidase Gene produced.

The transformants turn BLUE on X-Gal and IPTG containing plates due to the production of Active β-Galactosidase. X-Gal is the Chromogenic Substrate of β-Galactosidase and IPTG acts as the inducer for the expression of this enzyme. Ligation Mixture has Insert Cloned in the N-Terminal Coding Sequence for β-Galactosidase Gene. As a result enzymatically Active β-Galactosidase is not produced and the recombinants (Vector containing Insert) are WHITE in colour.

Figure 2 illustrates the Blue–White screening method.

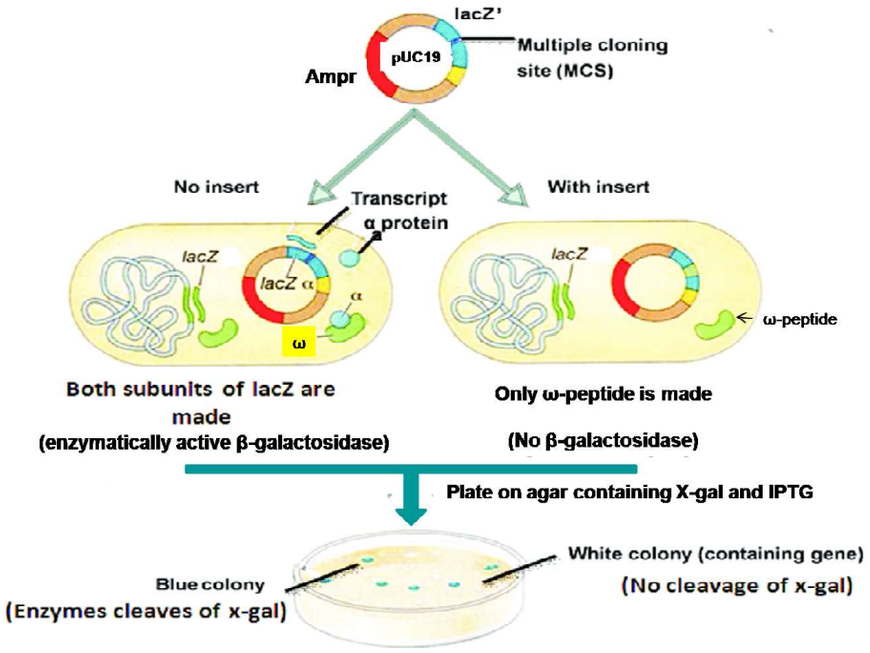



***Fig. 2:*** *Blue–White Screening Method*

## Materials Required

**Culture:** *Escherichia coli* Host with Plasmid pUC19.

**Chemicals and Reagents:** Ampicillin, Luria Bertani (LB) Broth, Luria Bertani Agar, *Escherichia coli* Host, Vector, Insert (Lambda DNA –Hind III Digest), Positive Control Plasmid, Negative Control Plasmid, 10X Ligase Assay Buffer, T4 DNA Ligase, X-Gal, IPTG, 1M Calcium Chloride (Sterile).

**Glassware:** Conical Flask, Measuring Cylinder, Beaker.

**Instrument:** Water Bath (42°C), 37°C Incubator, 37°C Shaking Incubator, Centrifuge.

**Other Requirements:** Micropipettes, Tips, 50 ml Centrifuge Tubes, Crushed Ice, Sterile Double Distilled Water, Sterile Loop and Spreader, Inoculation Loop.

## Preparation of Reagents

1. **0.1M Calcium Chloride (Sterile) (200 ml):** To prepare 200 ml of 0.1M Calcium Chloride, take 20 ml of 1M Calcium Chloride and add 180 ml of Sterile Distilled Water. Store at 2-8°C.
2. **Ampicillin**: Dissolve 30 mg of Ampicillin Powder in 600 µl of Sterile Double Distilled Water to give a Concentration of 50 mg/ml.
3. **X-Gal:** Make a 2% (w/v) Stock Solution by dissolving X-Gal in Dimethylformamide at a Concentration of 20 mg/ml solution. The X-Gal Tube should be wrapped with Aluminum foil in order to prevent the damage caused by light and store at –20°C.
4. **IPTG:** Make a 20% (w/v, 0.8 M) Solution of IPTG by dissolving 2 g of IPTG with 8 ml of Distilled $H_2O$. Prepare the aliquots and store them at -20°C.
5. **LB (Luria Bertani) Broth (55 ml):** Dissolve 1.5 g of LB Media in 60 ml Distilled Water Sterilize by autoclaving.
6. **LB (Luria Bertani) Agar Plates (20 ml):** Dissolve 0.8 g of LB Agar in 20 ml of Sterile Distilled Water. Sterilize by autoclaving and pour on Sterile Petri Plate.
7. **LB (Luria Bertani) Agar Plates containing Ampicillin, X-Gal and IPTG (100 ml):** Dissolve 4.0 g of LB Agar Media in 100 ml of Distilled Water. Sterilize by autoclaving and allow the Media to cool down to 40-45°C. Add 100 µl of Ampicillin, 200 µl of X-Gal and of 100 µl IPTG to 100 ml of autoclaved LB Agar Media, mix well and pour on Sterile Petri Plates.

## Procedure

Following are the important procedure steps.

## A. Ligation

1. For setting up of a Ligation Mix, T4 DNA Ligase and 10X Ligase Buffers are temperature sensitive and should always be placed and thawed on ice.
2. Set up the Ligation Reaction as follows:

| | |
|---|---|
| Molecular Biology Grade Water | 2 µl |
| Vector DNA | 3 µl |
| Insert DNA | 3 µl |
| 10X Ligase Buffer | 1 µl |
| T4 DNA Ligase | 1 µl |
| **Total Reaction Volume** | **10 µl** |

3. While preparing the Reaction Mixture the Ligase should always be added at the last.
4. After preparing the reaction tube, mix the components by gentle pipetting and tapping.
5. Incubate the tubes at 16°C Water Bath for 3 hours.
6. After incubation, the Ligated Mixture is taken for doing Transformation.

## B. Transformation of Cells

Use Competent Cells made in earlier experiment.

1. Take 2 Eppendorf Tubes. In one add 200 µl of the Competent Cell Suspension only (control). In another add 200 µl of the Competent Cell Suspension + 10 µl Ligation Mixture. Ligation Mixture contains both Plasmid and Insert (Recombinant DNA Molecule).
2. Mix and incubate both the tubes on ice for 30 minutes.
3. Transfer them to a Preheated Water Bath set at a temperature of 42°C for 2 minutes (heat shock).
4. Rapidly transfer the tubes on ice. Allow the cells to chill for 5 minutes.
5. Add 800 µl of LB Broth to all the tubes. Incubate the tubes for 1 hour at 37ºC to allow the Bacteria to recover and to express the Antibiotic Resistance Marker encoded by the Plasmid.
6. Spread 200 µl of Cell Culture from each tube on LB Agar Plate containing Ampicillin, X-Gal, IPTG using a sterile spreader until the liquid has been absorbed in by the medium.
7. Store at room temperature till the plates are dry.
8. Incubate the plates overnight at 37°C.
9. Observe the plates for transformed colony.

## Observation and Results

- After incubation observe the Bacterial Growth on plates and look for the Transformants and Non-Transformants on the plate.
- No growth observed on plating Competent Cells Untransformed with Plasmid (Control).
- Blue and White Colonies observed on plating with Ligation Mixture.

Figure 3 illustrates the Blue–White Colonies on transformant plate.

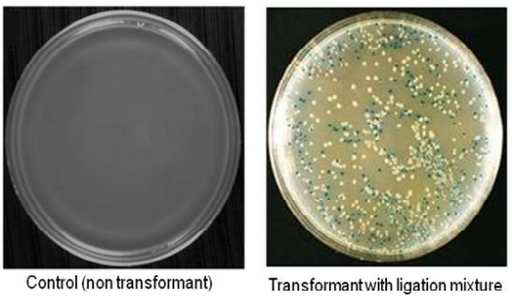



***Fig. 3:*** *Blue–White Colonies on Transformant Plate*

## Interpretation

***Escherichia coli*** **host strain** used for **cloning purpose** has a **deletion** at the **Amino** terminal of the **Lac Z Gene**, therefore it carry a defective Lac Z Gene which produces only ω-Peptide of the functional enzyme β-Galactosidase.

The ***Escherichia coli*** **Plasmid pUC19 (Vector)** has following three characteristics:

1. It has **Ampicillin Resistance Marker** that enables only transformed cells to grow on LB – Ampicillin plates. In contrast, no growth is observed on Plating Competent Cells untransformed with Plasmid as the Host *Escherichia coli* cell is SENSITIVE to AMPICILLIN.
2. It carries the N-Terminal Coding Sequence for the α–peptide of the β-Galactosidase gene. So, when vector is transformed into *Escherichia coli* competent cells, the truncated products from both complement each other and as a result enzymatically active β-Galactosidase is produced. This is called **α-Complementation**.
3. It also carries sites for different Restriction Enzymes (Multiple Coning Site, MCS) within the N-Terminal Coding Sequence for Lac Z Gene. If an insert is cloned within the MCS of pUC19, Lac Z Gene within vector gets disrupted and no longer code for α-Peptide. So, when recombinant DNA molecule (carrying insert into vector) is transformed inside *Escherichia coli* competent cell, only ω-Peptide is produced by defective Lac Z Gene present in *Escherichia coli* Chromosome. Thus there is no α–Peptide present to complement and hence no full β-Galactosidase gene produced.

This process of direct selection of recombinants is called **Insertional-Inactivation**.

**Media** used has **X–Gal** (**5-bromo-4-chloro-3-indoyl-β-D-galactoside**) and an inducer called **IPTG** (**Isopropyl β-D-Thiogalactopyronoside**). X-Gal is a Chromogenic Substrate of β-Galactosidase and IPTG acts as the inducer for the expression of this enzyme.

Based on the above information, the results obtained can be interpreted as follows:

| No Growth | Blue Colonies | White Colonies |
|---|---|---|
| Control (Only Competent Cells) | Competent Cells + Plasmid (Vector Only) | Competent Cells + Ligation Mixture (Vector+ Insert) |
| Non-Transformant | Non-Recombinant (Transformant without Insert) | Recombinants (Transformant with Insert) |
| No Growth is observed on plating Competent Cells Untransformed with Plasmid as the Host *Escherichia coli* cell is SENSITIVE to AMPICILLIN. | Vector is not defective and able to produce α–Peptide. So, when Vector is Transformed into *Escherichia coli* Competent Cells, enzymatically Active β-Galactosidase is produced. X-Gal is a Lactose Analog which is broken down by to a product that is coloured BLUE and therefore COLONIES are seen BLUE. | Vector is defective and not able to produce α–Peptide. As a result enzymatically Active β-Galactosidase is not produced. Therefore colonies that carry Recombinant (Vector containing Insert) molecule will remain WHITE and the recombinants are WHITE in colour. |

## Precautions

1. The revival step is necessary both to allow the Plasmid Establishment and to allow expression of the Resistance Genes.
2. It is essential that the cells used are in a Rapid Growth Phase when harvested. Do not let them approach stationary phase.
3. While making the plates add accurate amount of Ampicillin.
4. Add Ampicillin to the Media after it cools down to 40-45°C to avoid inactivation of Ampicillin.
5. Always thaw the Ligase Buffer and the enzyme on ice.
6. Make sure the buffer is completely melted and dissolved.
7. T4 DNA Ligase is very sensitive to shear, so spinning your Ligation mix or vortexing it to mix it can affect your yields. Instead try mixing with the pipette tip or slowly resuspending the solution. Try to maintain temperature during Ligation.
8. Always keep the competent cells on ice and make sure the centrifuge machine is cold throughout the experiment.
9. Competent Cells can be stored at 4°C once competent. Holding cells in $CaCl_2$ at 4°C will, in fact, increase transformation efficiency although this

declines with more than 24 hours storage. Long periods of storage can be achieved by freezing the competent cells.

10. Heat shock treatment should be very specific with respect to temperature and time.
11. Incubate the plates at 37°C for minimum 18 hours to avoid few or no transformants.
12. Make sure that the entire procedure is performed aseptically to avoid contamination on plates.

## EXPERIMENT 15: PCR AMPLIFICATION – DEMO

**AIM: Polymerase Chain Reaction – Demo**

**Theory**

**Polymerase Chain Reaction (PCR)**, a technique used to **amplify** or **copy** a **specific DNA target** from a mixture of DNA molecules. It was developed by Karry Mullis in 1983. In 1993, he got Nobel Prize in Chemistry for his work on PCR. In **PCR**, **a short region** of **a DNA molecule**, a single gene for instance, is **copied many times** by a **DNA polymerase enzyme** which results in the selective amplification of a chosen region of a DNA molecule. Any region of any DNA molecule can be chosen, so long as the sequences at the borders of the region are known because in order to carry out a PCR, two short **Oligonucleotides** must **Hybridize** to the **DNA molecule**, one to each strand of the double helix, so these **Oligonucleotides**, which act as primers for the DNA synthesis reactions, delimit the region that will be amplified.

A basic PCR requires the following components.

**1. Template DNA**

DNA template contains the region to be amplified. Generally ng of Plasmid DNA or mg of Genomic DNA is used. Higher amount inhibits or result is non-specific amplification.

**2. A Pair of Synthetic Oligonucleotides to Prime DNA Synthesis**

**PCR reaction** needs **two primers**; a **forward** and a **reverse** that must correspond with the sequences flanking the **Target Region** on the template molecule. Each primer must, of course, be complementary (not identical) to the **3' ends** of each of the sense and anti-sense strand of the DNA in order for hybridization to occur, and the **3' ends** of the **hybridized primers** should point toward one another. Primers are designed such that at the end they do not have more than two bases complementary to each other, as this results in **Primer-Dimer Formation**.

**G + C** content is 40 to 60%. TM of both the primers is usually the same.

In practice **17-30 mer primers** are used. If the primers are **too short** then they might **hybridize** to non-target sites and give undesired amplification products. The length of the primer influences the rate at which it hybridizes to the template DNA, **long primers** hybridizing at a slower rate. The efficiency of the PCR, measured by the number of amplified molecules produced during the experiment, is therefore reduced if the primers are too long, as complete hybridization to the template molecules cannot occur in the time allowed during the reaction cycle.

### 3. A Thermostable DNA Polymerase

**Amplification** is usually carried out in the presence of heat stabled **DNA Polymerase**, such as **Taq Polymerase**, an enzyme originally isolated from the **Bacterium *Thermus aquaticus***. This organism lives in hot springs. These enzymes are thermostable, meaning that they are resistant to denaturation by heat treatment. For most amplification reactions 1-2 Units of enzyme are recommended as higher enzyme concentration leads to non-specific amplification.

Table 1 illustrates the sources of DNA Polymerases with Proofreading 3' $\rightarrow$ 5' Exonuclease Activity.

***Table 1:*** *Sources of DNA Polymerases with Proofreading 3' $\rightarrow$ 5' Exonuclease Activity*

| DNA Polymerase | Source |
|---|---|
| TMA | *Thermotoga maritima* |
| VENT | *Pyrococcus sp.* |
| TLI | *Thermococcus litoralis* |
| PFU | *Pyrococcus furiosis* |
| PWO | *Purococcus woesei* |

### 4. Deoxynucleoside Triphosphates (dNTPs)

Deoxynucleoside Triphosphates (dATP, dCTP, dGTPand dTTP), are the building blocks from which the DNA Polymerase synthesizes a new DNA strand.

### 5. Buffer to Maintain pH

Buffer solution provides a suitable chemical environment for optimal activity and stability of DNA Polymerase. In some buffer solutions, $MgCl_2$ is mixed while in some it has to be added in the reaction mixture.

10X PCR Assay Buffer /DNA Polymerase Buffer contains:

100 mM Tris-Cl (pH 9.0)

500 mM KCl

15 mM $MgCl_2$

0.1% w/v Gelatin

## 6. Divalent Cations

**Bivalent Magnesium/Manganese ions**, are essential cofactors required for the maximum **Taq Polymerase** activity and influences the efficiency of primer to template annealing.

### Principle

As the name implies, it is a **chain reaction**, one DNA molecule is used to produce two copies, then four, then eight, and so forth. This continuous doubling is accomplished by specific proteins known as **Polymerases, Enzymes** that are able to string together individual **DNA building blocks** to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e., the **nucleotides** consisting of the four bases - **Adenine (A), Thymine (T), Cytosine (C)** and **Guanine (G)**. They also need a small fragment of DNA, known as the **primer**, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. Figure1 illustrates the Amplification cycle.

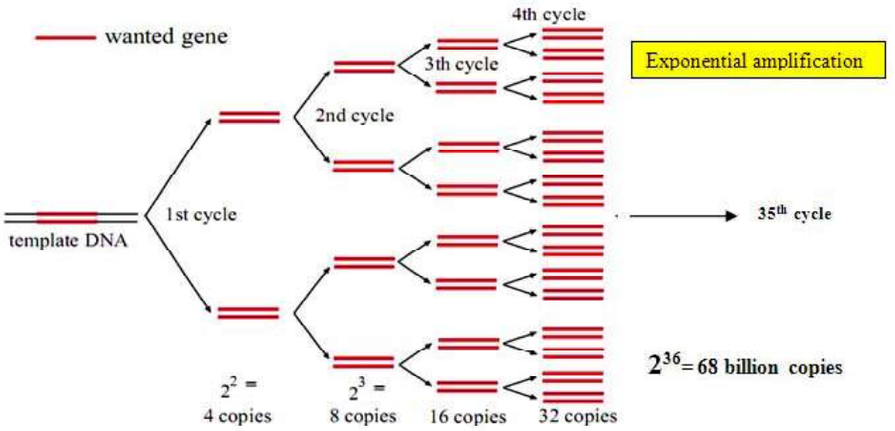



***Fig. 1:*** *Amplification Cycle*

The purpose of a PCR is to amplify a specific DNA or RNA fragment. PCR comprises of following three steps:

### 1. DENATURATION

It includes the following steps.

- **Initialization Step: Double stranded DNA templates** denature at a temperature that is determined in part by their **G+C content**. The higher the proportion of G+C, the higher the temperature required to separate the strands of template DNA. The longer the DNA molecules, the greater the time required at the chosen denaturation temperature to separate the two strands completely. If the temperature for denaturation is too low or if the

time is too short at **AT** rich regions of the template DNA will be denatured. When the temperature is reduced later in the PCR cycle, the template DNA will reanneal into fully native condition. In PCRs catalyzed by **Taq Polymerase**, denaturation is carried out at 94-95°C, which is the highest temperature that the enzyme can endure for 30 or more cycles without sustaining excessive damage. In the first cycle of PCR, denaturation is sometimes carried out for 5 minutes to increase the probability that long molecules of template DNA are fully denatured. In this step the **Hydrogen bonds** that hold together the **two Polynucleotides** of the **double helix** are broken, so the target DNA becomes denatured into single stranded molecule.

- **Denaturation Step:** However this extended period of denaturation temperature is unnecessary for linear DNA molecules as it may be deleterious sometimes. **Denaturation** for 20-30/45 seconds at 94-95°C/98°C is routinely used to amplify linear DNA molecules. As a result the template DNA denatures due to disruption of the **Hydrogen Bonds** between complementary bases of the DNA strands, yielding single strands of DNA.

  Complete denaturation of temperature DNA at the start of the PCR reaction is of key importance. Incomplete denaturation results in insufficient utilization of the temperature in the first amplification cycle and in a poor yield of the PCR product. Initial denaturation step is performed only once at the beginning of the reaction.

## 2. ANNEALING OF PRIMERS TO TEMPLATE DNA

The **annealing temperature** is the important one because, again, this can affect the specificity of the reaction. If the annealing temperature is **too high**, **no** or **little hybridization** takes place; instead the primers and templates remain dissociated. However, if the temperature is **too low**, **mismatched hybrids**—ones in which not all the correct base pairs have formed—are stable. So, the temperature must be low enough to enable hybridization between primer and template, but high enough to prevent mismatched hybrids from forming. Typically the annealing temperature is 3-5°C below the TM (Melting Temperature) at which the **Oligonucleotide primers** dissociate from their templates. This temperature can be estimated by determining the Melting Temperature or (TM) of the **Primer–Template Hybrid**, which can be calculated from following the simple formula:

**TM = (4 x [G+C]) + (2x [A+T]) C**

In which **[G+C]** is the number of **G** and **C nucleotides** in the **primer sequence**, and **[A+T]** is the number of **A** and **T nucleotides**. If non-specific products are obtained in addition to the expected product, it is best to optimize the annealing temperature by performing a series of trial PCRs by increasing the temperature by 1-2°C.

Stable **DNA-DNA Hydrogen Bonds** are only formed when the **primer sequence** very closely matches the template sequence. The polymerase binds to the **Primer-Template Hybrid** and begins DNA synthesis.

## 3. EXTENSION/ELONGATION OF OLIGONUCLEOTIDE PRIMERS

In this step, the **temperature depends** on the **DNA Polymerase** used in PCR. For example, **Taq Polymerase** has its optimum activity at 72–78°C. Commonly a temperature of 68-72°C is used with this enzyme. The DNA Polymerase synthesizes a new DNA strand complementary to the DNA template strand by incorporating **dNTPs** that are complementary to the template in 5' to 3' direction, condensing the **5'-Phosphate group** of the dNTPs with the **3'-Hydroxyl group** at the end of the nascent (extending) DNA strand. The extension time depends both upon the DNA Polymerase used and on the length of the DNA fragment to be amplified. The DNA Polymerase will **Polymerize** a **1000 Bases/Minute** at its optimum temperature. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

**Final Elongation:** This single step is performed at a temperature of 70–74°C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. It allows completion of all amplified products.

**Final Hold:** This step may be employed for short-term storage of the reaction mixture at 4°C for an indefinite time.

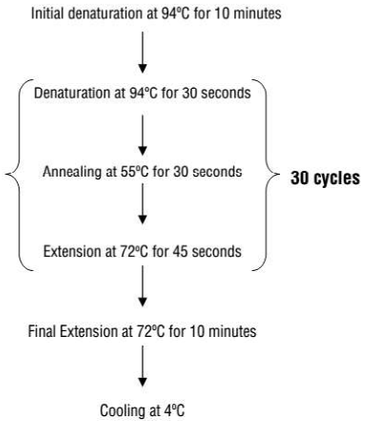
### Number of Cycles

**Denaturation**, **Annealing** and **Extension** steps are repeated 25-30 times in an automated thermocycler that can heat and cool the reaction mixture in tubes within a very short time. This results in exponential accumulation of specific DNA fragments, ends of which are defined by **5' ends** of the **primers**. The doubling of the number of DNA strands corresponding to the target sequences allows us to **estimate** the **Amplification** associated with each cycle using the formula:

**Amplification = $2^n$,** where **n = No. of Cycles.**

### PCR Amplification Cycle

Carry out the amplification in a thermocycler for 30 cycles using the following reaction conditions.



## Applications

PCR can amplify a desired DNA sequence of any region, hundreds of millions of times in matter of hours, a task that would require days with RDT.

It is especially valuable because the reaction is highly specific, easily automated and very sensitive. It has thus revolutionized fields like:

### 1. Clinical Medicine

- In diagnosis of infectious agents.
- Diagnosis of genetic disorders.
- Diagnosis and monitoring of hereditary diseases.

### 2. Forensic Sciences

To simply create multiple copies of a portion of DNA which is very rare. For example, a forensic scientist may needs to amplify a small fragment of DNA from a crime scene.

### 3. In DNA Sequencing and Fingerprinting.

### 4. Evolutionary Biology.

### Procedure

The following steps are involved in the procedure.

### I. PCR Amplification

The PCR amplification is done as follows.

### Materials Required

**Chemicals and Reagents:** 10X Assay Buffer, Template DNA, Forward Primer (10 nM), Reverse Primer (10 nM), 25 mM $MgCl_2$, 2.5 mM dNTP Mix containing all Four dNTPs, Taq DNA Polymerase, Double Distilled Water, Ethium Bromide (10 mg/ml).

**Glassware:** Measuring Cylinder, Beaker.

**Instruments:** Thermocycler (PCR Machine), Electrophoresis Apparatus, UV Transilluminator, Vortex Mixer.

**Other Requirements:** PCR Tubes, Micropipettes, Tips, Adhesive Tape, Microwave/ Hotplate / Burner, Crushed Ice.

### Steps to do PCR

1. Preparation of Master Mix for PCR.

To a PCR Tube add all the following ingredients in order:

| Sr. No. | Ingredients for PCR | Stock Concentration | Working Concentration | Volume in µl |
|---|---|---|---|---|
| 1 | Molecular Biology Grade Water | | | 30.5 µl |
| 2 | 10X Assay Buffer | 10X | 1X | 5 µl |
| 3 | Template DNA | 5 µg/ml | 100 ng | 1 µl |
| 4 | Forward Primer | 10 nM | 0.2 nM | 1 µl |
| 5 | Reverse Primer | 10 nM | 0.2 nM | 1 µl |
| 6 | $MgCl_2$ | 25 mM | 2.5 mM | 5 µl |
| 7 | dNTP Mix | 2.5 mM | 0.25 mM | 5 µl |
| 8 | Taq DNA Polymerase | | 1-2 Units | 0.5 µl |
| | Total Volume | | | 50 µl |

2. Tap the PCR tube for 1–2 seconds to mix the contents gently and keep them onto crushed ice until put in thermocycler.
3. Place the tube in the thermocycler block and set the program to get DNA Amplification.

   **Note:** Add 25 µl of Mineral Oil in the tube to avoid evaporation of the contents.
4. After the completion of Amplification Cycle switch off the PCR machine. Take out the sample and visualize the PCR product on Agarose Gel Electrophoresis.

## 4. VISUALIZATION OF PCR PRODUCT (AMPLICON) BY AGAROSE GEL ELECTROPHORESIS

After **performing PCR**, the tubes are taken out from the thermocycler and then **Amplicon** is visualized by **Agarose Gel Electrophoresis**. Agarose Gel Electrophoresis is the easiest and most popular way of separating and analyzing DNA. The **Phosphate backbone** of **DNA molecules** is negatively charged therefore negatively charged DNA molecules move from negatively charged electrode (cathode) towards positively charged electrode (anode) when placed in an electric field. Shorter molecules move faster and migrate farther than longer ones because shorter molecules move easily through the small pore size of the gel. This phenomenon is sieving. In order to **visualize** the **DNA** in the **Agarose Gel**, staining with an intercalating dye, such as **Ethidium Bromide** or **SYBR Green** is required.

### Materials Required

**Chemicals and Reagents:** 1% Agarose, Ethidium Bromide (10 mg/ml), Electrophoresis Buffer (1X TAE Buffer, pH 8.0), Distilled Water, Gel Loading Buffer, DNA Sample, DNA Ladder Appropriately Sized.

**Glassware:** Conical Flask, Measuring Cylinder.

NOTES

**Instruments:** Horizontal Gel Electrophoresis Tank (including Gel Casting Tray, Comb, Power Supply), External Power Supply, Transilluminator.

**Other Requirements:** Microwave/Burner, Adhesive Tapes, Micropipettes, Tips, Gloves.

**Steps to do Agarose Gel Electrophoresis**

**Preparation of 1X TAE (Tris-Acetate EDTA Buffer):** To prepare 500 ml of 1X TAE Buffer, Add 10 ml of 50X TAE Buffer to 490 ml of Sterile Distilled Water. Mix well before use.

**50X Concentrated TAE Electrophoresis Buffer (40 mM Tris-Acetate, 2 mM EDTA):** Add the following to Distilled $H_2O$ to give a final volume of 1 liter:

242 g Tris-Base

57.1 ml Glacial Acetic Acid

100 ml 0.5 M EDTA (pH 8.0)

**Procedure**

1. Prepare Gel Tray by sealing the ends with adhesive tape. Place Comb in Gel Tray about 1 inch from one end of the tray and position the Comb vertically such that the teeth are about 1-2 mm above the surface of the tray.
2. To prepare 50 ml of 0.8% Agarose Solution, measure 0.4 g Agarose into a Glass Beaker or Flask and add 50 ml 1X TAE Buffer.
3. Heat the mixture on a microwave or hot plate, swirling the Glass Beaker / Flask occasionally, until Agarose is dissolved completely. Ensure that the lid of the flask is loose to avoid buildup of pressure.
4. Allow solution to cool down to about 55-60°C. Add 2.5 ml Ethidium Bromide (to get final Concentrated 0.5 µg/ml from 10 mg/ml stock), mix well and pour the gel solution into the Gel Tray to a depth of about 5 mm. Allow the gel to solidify for about 30 minutes at room temperature.
5. To start the run, carefully remove the adhesive tape from both the ends of the Gel Tray, place the tray in Electrophoresis Chamber, and fill the chamber (just until wells are submerged) with 1X TAE Electrophoresis Buffer. Gently lift the Comb, ensuring that the wells remain intact.
6. Load 3 ml of ready to use DNA Ladder into the first well. Add 2 µl of 6X Gel Loading Buffer to 10 µl of PCR Product. Load the PCR samples next to ladder.
7. Connect the power cord to the Electrophoretic Power Supply according to the conventions - Red-Anode and Black-Cathode.
8. Electrophorese at 100-120 volts and 90 mA.

9. Switch off the power supply once the tracking dye from the wells reaches 3/4th of the gel which takes approximately 45 minutes.
10. Observe the PCR Product on a UV Transilluminator.
11. Compare the Amplified Product with the ladder and determine its size.
12. Store the PCR Product at -20°C. It gets degraded otherwise.
13. Also observe for the presence of any other band apart from the Amplified Product, to see if any non-specific band is present.

## Observation and Results

As observed on **Agarose Gel, PCR Amplification** of the template using specific primers results in a specific product of a around **800 base pairs (bp)**.

No non-specific band was observed.

Figure 2 illustrates the PCR Amplified Product Electrophoresed on Agarose Gel, in which,

Lane 1: 1Kb DNA Ladder

Lane 2: PCR Product

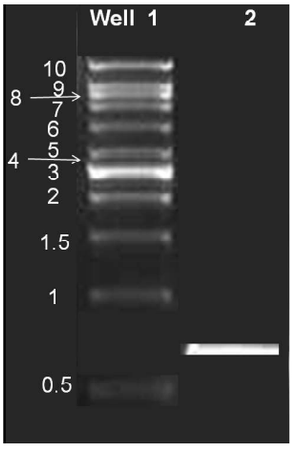



***Fig. 2:*** *PCR Amplified Product Electrophoresed on Agarose Gel*

## Interpretation

After performing Agarose Gel Electrophoresis, we checked the Amplification of a specific PCR product. The optimized conditions result in the Amplified PCR product of 800 bp. Following PCR, the product yield is in microgram quantity, which is approximately a million copies of the target sequence (100 ng), highlighting the fact that PCR is a very sensitive technique.

PCR is carried out in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperature required at each step of the reaction. Thermal cycler permits both heating and cooling of the block holding the PCR

tube simply by reversing the electric current. Most thermocycler have heated lids to prevent condensation at the top of the reaction tube. Older thermal cycler lacking a heated lid requires a layer of Mineral Oil on the top of the Reaction Mixture.

**Precautions**

1. Minimize damage to template DNA by avoiding vortexing or vigorous mixing to avoid Template DNA Damage.
2. Take the same amount of template DNA and dNTPs as specific in procedure to avoid template DNA or dNTPs concentration inappropriate.
3. Keep all the materials in ice while performing the experiment.
4. Ensure proper functioning of Thermocycler. Run positive control with every reaction.
5. Mix the reaction mixture using a micropipette, avoid air bubble to avoid inadequate mixing of the reaction tube.
6. Use recommended concentration of primers and dNTPs to avoid Primer-Dimer observed.
7. Make sure that the Agarose is fully dissolved in the Buffer. If it is not dissolved well, again melt it some more time to dissolve completely.
8. Before casting the Gel, the Tray and Comb should be wiped with Ethanol.
9. Check that no air bubbles are under or between the teeth of the Comb.
10. The Gel should be between 3 - 5 mm thick.
11. Make sure that the Gel in the Chamber is immersed in the TAE Buffer.
12. Electrophoresis apparatus should always be covered to protect against electric shocks. Avoid use of very high voltage which can cause trailing and smearing of DNA Bands in the Gel, particularly with High-Molecular-Weight DNA.
13. Monitor the temperature of the Buffer periodically during the run. If the buffer becomes heated, reduce the voltage. Melting of an Agarose Gel during Electrophoresis is a sign that the voltage is too high, that the buffer may have been incorrectly prepared or has become exhausted during the run.
14. Always wear protective eyewear when observing DNA on a Transilluminator to prevent damage to the eyes from UV Light.
15. Because Ethidium Bromide is a known Mutagen, precautions need to be taken for its proper use and disposal. So wear gloves while handling. All items that were in contact with Ethidium Bromide must be disposed off in the designated waste container, marked with 'Ethidium Bromide Waste' and within the Gel-Doc Area, put the Gels, Tissue Paper used to clean the Table, and Nitrile Gloves.

**NOTES**

# FOOD AND DAIRY MICROBIOLOGY

**Food microbiology** is the study of the **microorganisms** that inhibit, create, or contaminate food, including the study of microorganisms causing food spoilage, pathogens that may cause disease especially if food is improperly cooked or stored. Specifically those food items that are used to produce fermented foods, such as cheese, yogurt, bread, beer, and wine, and those with other useful roles, such as producing probiotics.

As per the definition, the term '**Microbiology**' refers to a branch of biology that deals with the study of microorganisms and their different activities. Since ages, these microorganisms are playing a potential role in human welfare both as useful and harmful biological agents. This leads to an extensive study of these micro-organisms with the aim of understanding their growth and nutritional requirements for their production or destruction both. **Food safety** is a key emphasis of **food microbiology**. Numerous agents of disease, pathogens, are readily transmitted via food, including bacteria, and viruses. Microbial toxins are also possible contaminants of food. However, microorganisms and their products can also be used to combat these pathogenic microbes. The bacteriophages, viruses that only infect bacteria, can be used to kill bacterial pathogens.

**Microorganisms** are living organisms that are individually too small to see with the naked eye. The unit of measurement used for microorganisms is the micrometer (μm); 1 mm = 0.001 millimeter; 1 nanometer (nm) = 0.001 mm. Microorganisms are found everywhere (ubiquitous) and are essential to many of our planets life processes. With regards to the food industry, they can cause spoilage, prevent spoilage through fermentation, or can be the cause of human illness. There are numerous classes of microorganisms, of which bacteria and fungi (yeasts and moulds), the bacterial viruses or bacteriophage are the most common forms.

## EXPERIMENT 16: RESAZURIN DYE REDUCTION TEST

### AIM: Resazurin Reduction Test (RRT)

### Theory

In this test, the **Resazurin Dye** undergoes **reduction** through a series of colour shades, such as **Blue**, **Purple**, **Lavender**, and **Pink** before finally getting reduced to **Colourless (White)**. This **colour change** is dependent upon the extent of **depletion of oxygen** by **metabolic activity** of **microbes**. Resazurin dye which is blue in colour (redox potential of +0.3 volts) undergoes an irreversible change to **Resorufin** (redox potential +0.2 volts) a pink colour compound. Further redox potential reduction (+0.1 volts or less), the dye colour changes to **Dihydroresorufin**, a colourless form in a reversible reaction. The colour change

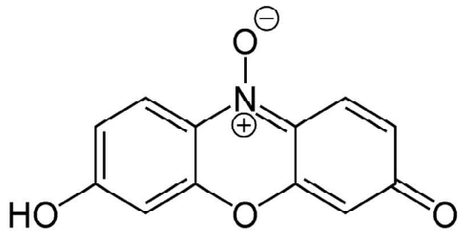
is measured with the help of a **Lovibond colour comparator** and a standard **Resazurin Disc**.

### Chemical Structures and Molecular Formula of Resazurin and Resorufin

### Resazurin

Molecular Formula of Resazurin - $C_{12}H_7NO_4$

Chemical Structure of Resazurin

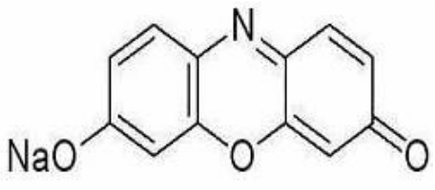



Resazurin (7-Hydroxy-3H-Phenoxazin-3-one 10-oxide)

### Resorufin

Molecular Formula - $C_{12}H_7NO_3$

Chemical Structure of Resorufin




Resorufin (7-Hydroxy-3H-Phenoxazin-3-one, Sodium Salt)

### Materials Required

**Milk Sample:** Raw and Pasteurized Milk.

**Reagents:** Resazurin Solution 0.05%, Lovibond Colour Comparator.

**Equipment:** Resazurin Colour Disc, Water Bath at 37.5°C, Screw Cap Tubes 10 ml, Pipettes 10 ml and 1 ml.

### Resazurin Dye Solution

To make a Standard Solution of 0.05%, dissolve 0.05 g of Resazurin Powder in 100 ml Distilled Water and Boil for 30 minutes. Standard solution should be stored in dark bottle at cool place. To make Working Solution of 0.005%, Dilute 1 ml of Standard Solution with 10 ml of Distilled Water before use.

### Procedure

- Take 10 ml of each Milk Sample into a Sterile Screw Cap Tube.
- Add 1 ml of Resazurin Solution.
- Cap the tubes to prevent oxygen entry.
- Mix the solution by inverting the tubes 2-3 times.
- Place the test tubes in a Water Bath at 37°C and incubate for 1 hour.
- Take the tube out after the incubation and match the colour of the milk with one of the colour standards of Resazurin Disc.

### Result

| Colour of Sample | Quality of Milk |
|---|---|
| 1. Blue (No Colour Change) | Excellent |
| 2. Lilac | Very Good |
| 3. Mauve | Good |
| 4. Pink Mauve | Fair |
| 5. Mauve Pink | Poor |
| 6. Pink | Bad |
| 7. White | Very Bad |

### Advantages

- Test is cheaper and can be measured in a shorter time.
- Microbial activity is measured rather than the number of bacteria.

### Disadvantages

- Continuous observation is required until reduction takes place.
- Not suitable for milk samples with low bacterial counts (less than $10^5$ /ml).
- Does not indicate for the type of microorganisms present in the milk sample.

## EXPERIMENT 17: PHOSPHATASE TEST

### AIM: Alkaline Phosphatase Test

### Theory

**Alkaline Phosphatase (ALP)** is a **heat sensitive enzyme** in **milk** that is used as **indicator** of **pasteurization**. If milk is properly pasteurized, Alkaline Phosphatase is inactivated. Alkaline Phosphatase induces the **Hydrolysis** of **p-Nitrophenyl Phosphate** at **Alkaline pH**, and liberates **para-Nitrophenol**, a yellow colored complex whose intensity is directly proportional to the concentration

NOTES

of ALP in the sample, and can be measured at 405 nm. The colour intensity is measured by direct comparison with standard colour discs in a Lovibond comparator.

### Materials Required

**Equipment:** Test Tubes, Pipettes – 1 ml, 5 ml and 10 ml, Water Bath at 37°C, Lovibond Comparator Discs of standard for Nitrophenol, p-Nitrophenyl Phosphate (pNpp) Disc.

### Reagents

1. p-Nitrophenyl Phosphate (pNpp) Solution.
2. Sodium Carbonate-Bicarbonate Buffer: Dissolve 3.5 g of Anhydrous Sodium Carbonate and 1.5 g of Sodium Bicarbonate in one litre of Distilled Water.
3. Buffer Substrate: Dissolve 1.5 g of Disodium p-Nitrophenyl Phosphate in one litre of Sodium Carbonate-Bicarbonate Buffer. This solution is stable if stored in a refrigerator at 4°C or less for one month but a colour control test should be carried out on such stored solutions.

### Procedure

1. Pipette 5 ml of Buffer Substrate into two clean, dry test tubes.
2. Add 1 ml of the Raw Milk to be tested in one and Pasteurized Milk in second tube for control.
3. Stopper the tubes and invert mix 2-3 times.
4. Place the tubes in the Water Bath and incubate for 30 minutes.
5. Remove the tubes from the Water Bath and invert mix each tube.
6. Observe/Read the **colour developed** or match the colour of the **Milk** with one of the colour standards of **pNpp Disc**.

### Result

Development of yellow colour in the tubes indicate bad quality of milk whereas white unchanged colour of milk after incubation indicates good quality.

## EXPERIMENT 18: LITMUS MILK REACTIONS

### AIM: Litmus Milk Reactions

### Theory

Milk is an excellent medium for the **growth of microorganisms** because it contains the **Milk Protein Casein**, the **Sugar Lactose**, **Vitamins**, **Minerals** and **Water**.

The major **milk substrates** capable of **transformation** are the **Milk Sugar Lactose** and the **Milk Proteins Casein**, the **Lactalbumin** and the **Lactoglobulin**. These milk components can all be metabolized by different types of Bacteria based on various metabolic reactions in **Litmus Milk**, including Reduction, Fermentation, Clot Formation, Digestion, and the Formation of Gas. To distinguish among the metabolic changes produced in milk, a pH indicator, the **Oxidation-Reduction Indicator Litmus**, is incorporated into the medium. This '**Litmus Milk**' then forms a good differential medium to distinguish between different species of bacteria, i.e., it is a milk-based medium used for distinguishing the different species of bacteria. The Lactose (Milk Sugar), Litmus (pH Indicator), and Casein (Milk Protein) contained within the medium can all be metabolized by different types of bacteria.

**Lactose Fermentation:** It is demonstrated by production of acids, such as the Pyruvic Acid and Lactic Acid which in turn reduces the pH to 4 turning the Litmus colour Pink from Purple (at neutral pH).

**Litmus Reduction:** Under anaerobic fermentative conditions, the Litmus is in the oxidized state and appear to be Purple in colour which on accepting Hydrogen from a substrate get reduced and turn White or Milk coloured. The oxidation of Lactose present in the Milk, under anaerobic condition produces Lactic Acid, Butyric Acid, $CO_2$ and $H_2$. Excess Hydrogen released during the process reduces Litmus colour to White.

**Curd Formation:** Under acidic conditions, Casein is precipitated as Calcium Caseinate to form an insoluble Hard Clot which does not retract from the walls of the test tube. This Acid Curd remains immobile even if the tube is inverted. However, Rennin produced by some organisms produces Paracasein, which on being converted to Calcium Paracaseinate forms soft semisolid clot also known as Rennet Curd, which can flow slowly on tilting the tube.

**Proteolysis (Peptonization):** Degradation of Milk Proteins primarily Casein by Proteolytic Enzymes into Amino Acids is accompanied by the evolution of Ammonia which increases the pH of the medium making it Alkaline turning the Litmus colour Deep Purple in the upper portion of the tube, while the medium begins to lose body and appear Translucent, Brown and Whey like.

**Gas Formation:** Production of end products, such as $CO_2 + H_2$ by Lactose Fermentation may be seen as cracks or fissures within the curd as gas rises to the surface.

### Materials Required

**Samples:** Trypticase Soy Broth (24 to 48 Hour Grown), Cultures of *Escherichia coli, Alcaligenes faecalis, Lactococcus lactis* and *Pseudomonas aeruginosa*.

**Equipment:** Bunsen Burner, Inoculating Loop, Test Tubes, Test Tube Rack, Marking Pen.

**Media:** Litmus Milk Broth (pH 6.8) Medium. Take Skim Milk Powder 100 g, Litmus 0.075 g. Dissolve in 500 ml Distilled Water to make up the Volume up to 1 Liter. Autoclave at 12 lb pressure for 15 minutes.

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### Procedure

1. Take 5 ml of autoclaved Litmus Milk Broth in Sterile Test Tubes.
2. Aseptically, inoculate each experimental organism from overnight grown culture into labeled tube by loop inoculation.
3. Label one un-inoculated Litmus Milk Tube as 'Control' for comparison.
4. Incubate the cultures for 24-48 hours at 37°C.
5. Observe the Litmus Cultures Colour and consistency changes.

### Observations and Results

Note the observations in the following table.

**Observation Table**

| S. No. | Bacterial Species | Litmus Milk Reactions |
|---|---|---|
| 1. | *Escherichia coli* | |
| 2. | *Lactococcus lactis* | |
| 3. | *Alcaligenes faecalis* | |
| 4. | *Pseudomonas aeruginosa* | |

### Positive Test

- **Acid pH:** Pink to Red Colour
- **Alkaline pH:** Purplish-Blue Colour
- **Reduction:** White
- **Acid Curd:** Hard Curd with Clear Supernatant (Whey)
- **Rennet Curd:** Soft Curd followed by Peptonization (Alkaline pH, Supernatant Brown)
- **Gas Production:** Bubbles in Coagulated Milk

### Negative Test

- Colour and consistency remain same.

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# EXPERIMENT 19: POTABILITY ANALYSIS OF DRINKING WATER

**AIM: Potability Analysis of Drinking Water / Standard Qualitative Analysis of Water**

**Theory**

Inadequate sanitation practices lead to a number of **water borne diseases** caused by **Enteropathogenic Microorganisms**, such as ***Vibrio cholera*** **(Cholera)**, ***Salmonella typhi*** **(Typhoid Fever)**, and ***Entamoeba histolytica*** **(Amoebic Dysentery)**. The common feature of these diseases is that the infectious organisms are shed in the feces of sick individuals. Fecal contamination through untreated or inadequately treated sewage effluents entering lakes, rivers, or ground waters that in turn serve as municipal water supplies creates conditions for rapid dissemination of these pathogens.

The **public health** importance of **clean drinking water** requires **water quality testing** to evaluate the effectiveness of **water treatment procedures** and to establish **drinking water safety standards**.

Although many of these pathogens can be detected directly, but the environmental microbiologists have generally used ***indicator organism*** as an **index of possible water contamination** by **human enteropathogens**. The most frequently used indicator organism for fecal contamination is non-pathogenic ***Escherichia coli***, which inhabit the intestinal tract (colon) of humans and other animals in large numbers. Thus, the presence of coliforms in water may indicate fecal contamination and establish the possibility of the presence of **enteropathogenic microorganisms**.

**Standard Qualitative Analysis of Water**

**Multiple Tube Fermentation Technique/Most Probable Number (MPN):** This technique is used to determine the **potability of water**. It determines whether the water sample is focally contaminated or not. It includes three basic tests to detect **Coliform Bacteria in Water**: **Presumptive, Confirmed** and **Completed**. The tests are performed sequentially on each sample under analysis. They detect the presence of Coliform Bacteria (Indicators of Fecal Contamination), the Gram Negative, Non-Spore-Forming, Rod Shaped Bacteria that Ferment Lactose with the production of Acid and Gas that is detectable following a 24-hour incubation period at 37°C. The following tests are performed.

1. **Presumptive Test:** This test is used to determine the **Most Probable Number (MPN)** of all **Coliforms** present in **water**. Because these bacteria are capable of using **Lactose** as a **Carbon Source** (the other enteric organisms are not), their detection is facilitated by the use of **Lactose Broth**

**Medium**. Tubes of this Lactose medium are inoculated with 10 ml, 1 ml, and 0.1 ml aliquots of the water sample. The series consists of at least three groups, each composed of five tubes of the specified medium. The tubes in each group are then inoculated with the designated volume of the water sample to be tested.

Coliforms produce both Acid and Gas. **Phenol Red (pH indicator)** present in **Lactose Fermentation Broth** detects the presence of **Acid**. At **neutral pH**, **Phenol Red** is of **Orange Red colour** but at **pH less than 6.8**, it turns **Yellow** in colour. It turns **Pink** in color under **Alkaline Conditions**. To detect the presence of **Gas**, an **inverted Durham's Tube** is inserted within the tube. After incubation, if Gas is produced then it gets trapped at the top of Durham Tube indicating Gas Formation. The tubes in which both Acid and Gas is produced, show **Positive Presumptive Test**. These tubes are used to find Most Probable Number (MPN) and make statistical table. If only Acid or Gas is produced, it is a **Negative Presumptive Test**. If the **presumptive test is negative**, **no further testing** is performed and the **water source** is considered **microbiologically safe**. The **potable water** should have **MPN 4/100 ml**. In US, the potable water should have MPN of 1/100 ml.

The presumptive test also enables the microbiologist to obtain some idea of the number of coliform organisms present by means of the Most Probable Number (MPN) test. The MPN is estimated by determining the number of tubes in each group that show Gas following the incubation period.

2. **Confirmed Test:** The presence of a **Positive** or **Doubtful Presumptive Test** immediately suggests that the **water sample** is **non-potable**. Confirmation of these results is necessary because **Gas Formation** is the **characteristic of fecal coliforms**, such as *Salmonella, Shigelle* and *Escherichia coli* but also of the non-fecal coliforms, such as *Enterobacter aerogenes* and some *Klebsiella*. In this test, three different types of selective (most **Gram Negative Organisms** grow well in these medium, while growth of **Gram Positive Organisms** is inhibited) and differential media are used: Eosin–Methylene Blue (EMB) Agar, m-Endo Agar, and Brilliant Green Lactose Bile (BGLB) Broth.

   **EMB Agar**: Eosin–Methylene Blue Agar contains the dye Methylene Blue, which inhibits the growth of Gram Positive Bacteria. It also contains **Lactose** (for Coliforms) and **Sucrose** (for Non-Coliforms). Three different types of colonies appear on EMB Agar.

   (i) In the presence of an Acid Environment, EMB forms a complex that precipitates out onto the Coliform Colonies, producing dark centers and a green metallic sheen. Metallic green sheen colonies produced by fecal coliforms, such as *Escherichia coli* the major indicator of fecal pollution.

(ii) Pink colonies formed by Gram Negative, Coliform Bacteria other than *Escherichia coli*.

(iii) Colourless colonies by non-lactose fermenters.

**m Endo Agar:** It contains Deoxycholate and Lauryl Sulfate, which serve as inhibitory agents against Gram Positive Microorganisms; Lactose, which is a source of Fermentable Carbohydrate; Peptones and Yeast Extract, which provide necessary growth nutrients; **Basic Fuchsin** acts as the pH indicator. Microorganisms capable of Lactose Fermentation produce Acetaldehyde, which reacts with Basic Fuchsin and Sodium Sulfite to form a Red Zone surrounding the colonies. Coliform Organisms produce Red Colonies with a characteristic Golden-Green Metallic Sheen. The development of a metallic sheen occurs when the organism produces Aldehydes during the rapid Fermentation of Lactose. If the inoculum is too heavy, the sheen will be suppressed. Non-fecal coliforms, such as *Enterobacter* produce Reddish Colonies while Non-Lactose Fermenter forms clear colourless colonies.

**BGLB Broth:** Brilliant Green is an Acid Base Indicator which also inhibits Gram Positive Bacteria. Bile Salts inhibit the growth of Non-Enteric Bacteria (Non-Fecal Coliforms). Only Gram Negative, Enteric Coliforms (Fecal Coliforms) which can Ferment Lactose grow in this medium.

3. **Completed Test:** This test is performed only if the positive result is obtained in the confirmed test. In this test an Isolated Green Metallic Sheen Colonies obtained from **EMB** and **m-Endo Agar Medium** is inoculated into a tube of **Lactose Broth** and streaked on a **Nutrient Agar Medium** to perform a **Gram Stain**. Following incubation, tubes showing Acid and Gas in the Lactose Broth and presence of Gram Negative, Non-Spore Forming Bacilli are further confirmation of the presence of *Escherichia coli*, and they are indicative of a positive completed test.

## Clinical Applications

Water used for human consumption and recreational use is routinely analyzed for safety. Water sources are regularly tested for the presence of *Escherichia coli* to determine the quality and safety of municipal water supplies. Several testing methods are available for this purpose including the Most Probable Numbers (MPN), ATP Testing, Membrane Filtration, and the use of Pour Plates.

## Materials Required

**Cultures:** Water Samples from Sewage Plant, Pond, and Municipal Supply Water (Tap Water), RO Water, Bisleri Water.

**Media:** Double Strength Lactose Fermentation Broths (LB2X) and Single Strength Lactose Fermentation Broths (LB1X), Nutrient Agar Slants, EMB Agar Plates, m-Endo Agar Plates, BGLB Broth.

**NOTES**

**Equipment:** Laminar Air Flow, Incubator, Microscope.

**Reagents:** Gram Staining Reagents (Crystal Violet, Gram's Iodine, 95% Ethyl Alcohol, and Safranin).

**Glassware and Miscellaneous:** Bunsen Burner, Test Tubes, Test Tube Stands, Sterile 10 ml Pipettes, Sterile 1 ml Pipettes, Sterile 0.1 ml Pipettes, Glassware Marking Pencil (Marker), Inoculating Loop, Glass Slides, Durham's Tube, and Bunsen Burner.

**Procedure**

The following procedures are involved.

**Presumptive Test**

1. Set up three separate series consisting of three groups, a total of 15 tubes per series, in a test tube rack; for each tube, label the water source and volume of sample inoculated as illustrated below.

| | |
|---|---|
| Series 1: Sewage Water | 5 Tubes of LB2X-10 ml |
| | 5 Tubes of LB1X-1 ml |
| | 5 Tubes of LB1X-0.1 ml |
| Series 2: Pond Water | 5 Tubes of LB2X-10 ml |
| | 5 Tubes of LB1X-1 ml |
| | 5 Tubes of LB1X-0.1 ml |
| Series 3: Tap Water | 5 Tubes of LB2X-10 ml |
| | 5 Tubes of LB1X-1 ml |
| | 5 Tubes of LB1X-0.1 m |
| Series 4: RO Water | 5 Tubes of LB2X-10 ml |
| | 5 Tubes of LB1X-1 ml |
| | 5 Tubes of LB1X-0.1 m |
| Series 5: Bisleri Water | 5 Tubes of LB2X-10 ml |
| | 5 Tubes of LB1X-1 ml |
| | 5 Tubes of LB1X-0.1 m |

**NOTES**

2. Mix Sewage Plant Water Sample by shaking thoroughly.
3. Flame bottle and then, using a 10 ml pipette, transfer 10 ml aliquots of water sample to the five tubes labeled LB2X-10 ml.
4. Flame bottle and then, using a 1 ml pipette, transfer 1 ml aliquots of water sample to the five tubes labeled LB1X-1 ml.
5. Flame bottle and then, using a 0.1 ml pipette, transfer 0.1 ml aliquots of water sample to the five tubes labeled LB1X-0.1 ml.

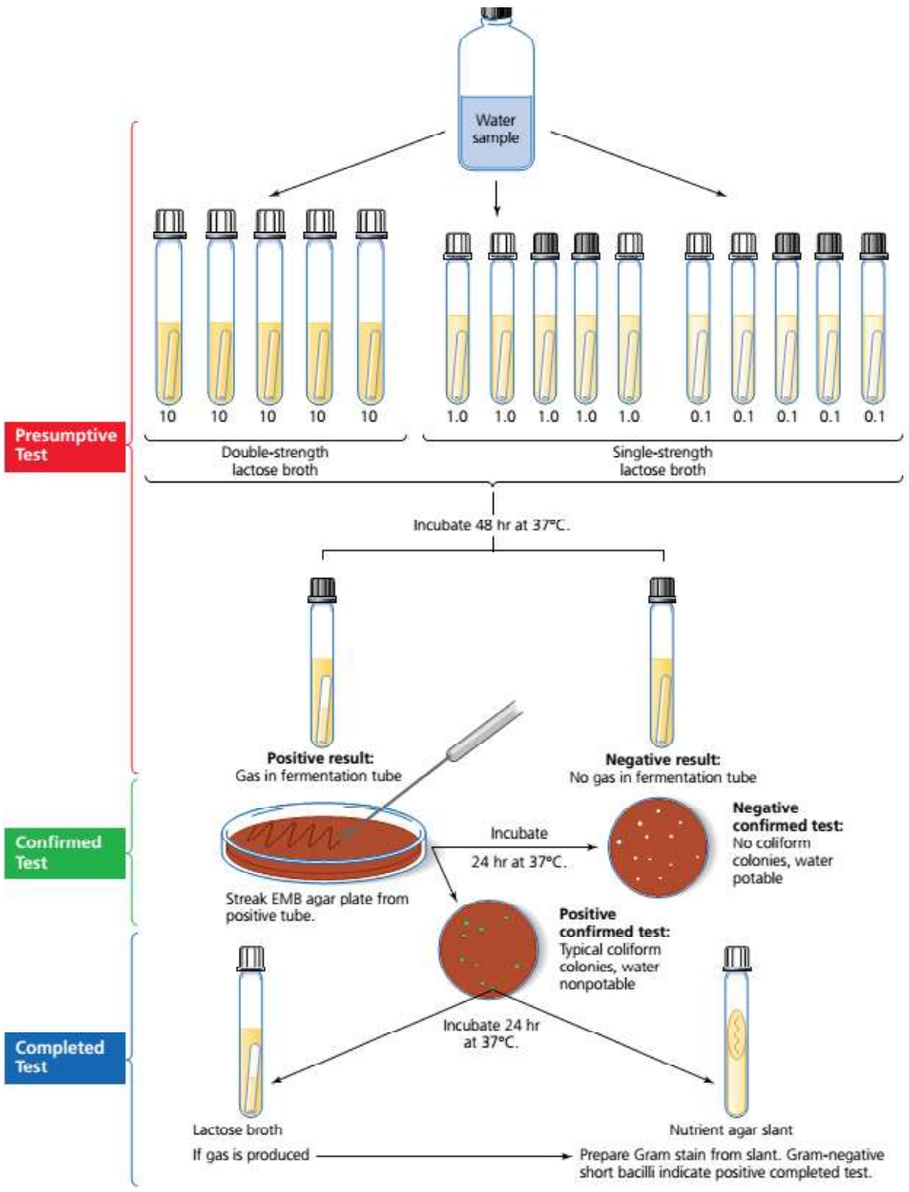


***Fig. 1:*** *Water Sample Contamination Test Results*

6. Repeat Steps 2 through 5 for all types of water samples.
7. Incubate all tubes for 48 hours at 37°C.
8. Examine the tubes after 24 and 48 hours of incubation. Your results are POSITIVE if the Durham Tube fills 10% or more with Gas in 24 hours, doubtful if Gas develops in the tube after 48 hours, and NEGATIVE if there is No Gas in the tube after 48 hours, as shown below in Figure 1.

## Observation and Results

Record the observations in the following table to determine and record the MPN, refer Table 1 given as example.

**Example:** If gas appeared in all five tubes labeled LB2X–10, in two of the tubes labeled LB1X–1, and in one labeled LB1X–0.1, the series would be read as 5-2-1. From the MPN table, such a reading would indicate approximately 70 microorganisms per 100 ml of water, with a 95% probability that between 22 and 170 microorganisms are present.

**Observation Table**

| | **GAS** | | | | | | | | | | | | | | | **Reading** | **MPN** | **95% Probability Range** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **LB2X-10** | | | | | **LB1X-1** | | | | | **LB1X-0.1** | | | | | | | |
| | **Tube** | | | | | **Tube** | | | | | **Tube** | | | | | | | |
| Water Sample | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | | | |
| Sewage Water | | | | | | | | | | | | | | | | | | |
| Pond Water | | | | | | | | | | | | | | | | | | |
| Municipal (Tap Water) | | | | | | | | | | | | | | | | | | |
| RO Water | | | | | | | | | | | | | | | | | | |
| Bisleri Water | | | | | | | | | | | | | | | | | | |

## Discussion

Coliforms present in water sample (name them) utilized Lactose present in the medium to produce Acid and Gas. As Acid is produced, the pH of the medium decreased (make it acidic, less than 6.8) and the Phenol Red present in the medium turned Yellow in colour.

In some tubes slight Pink colour was observed. It was due to the utilization of Peptone as Carbon and energy source.

Comment on the MPN of each sample and their probability range.

Figure 2 illustrates the possible MPN Presumptive Test Results.


***Fig. 2:*** *Possible MPN Presumptive Test Results*

***Table 1:*** *The MPN Index Per 100 ml for Combinations of Positive and Negative Presumptive Test Results when Five 10 ml, Five 1 ml, and Five 0.1 ml Portions of Sample are Used*

| NUMBER OF TUBES WITH POSITIVE RESULTS | | | | | |
|---|---|---|---|---|---|
| FIVE OF 10 ML EACH | FIVE OF 1 ML EACH | FIVE OF 0.1 ML EACH | MPN INDEX PER 100 ML | 95% CONFIDENCE LIMITS | |
| | | | | LOWER | UPPER |
| 0 | 0 | 0 | <2 | 0 | 6 |
| 0 | 0 | 1 | 2 | <0.5 | 7 |
| 0 | 1 | 0 | 2 | <0.5 | 7 |
| 0 | 2 | 0 | 4 | <0.5 | 11 |
| 1 | 0 | 0 | 2 | 0.1 | 10 |
| 1 | 0 | 1 | 4 | 0.7 | 10 |
| 1 | 1 | 0 | 4 | 0.7 | 12 |
| 1 | 1 | 1 | 6 | 1.8 | 15 |
| 1 | 2 | 0 | 6 | 1.8 | 15 |
| 2 | 0 | 0 | 5 | <0.5 | 13 |
| 2 | 0 | 1 | 7 | 1 | 17 |
| 2 | 1 | 0 | 7 | 1 | 17 |
| 2 | 1 | 1 | 9 | 2 | 21 |
| 2 | 2 | 0 | 9 | 2 | 21 |
| 2 | 3 | 0 | 12 | 3 | 28 |
| 3 | 0 | 0 | 8 | 2 | 22 |
| 3 | 0 | 1 | 11 | 4 | 23 |
| 3 | 1 | 0 | 11 | 5 | 35 |
| 3 | 1 | 1 | 14 | 6 | 36 |
| 3 | 2 | 0 | 14 | 6 | 36 |
| 3 | 2 | 1 | 17 | 7 | 40 |
| 3 | 3 | 0 | 17 | 7 | 40 |
| 4 | 0 | 0 | 13 | 4 | 35 |
| 4 | 0 | 1 | 17 | 6 | 36 |
| 4 | 1 | 0 | 17 | 6 | 40 |
| 4 | 1 | 1 | 21 | 7 | 42 |
| 4 | 1 | 2 | 26 | 10 | 70 |
| 4 | 2 | 0 | 22 | 7 | 50 |
| 4 | 2 | 1 | 26 | 7 | 67 |
| 4 | 3 | 0 | 27 | 9 | 78 |
| 4 | 3 | 1 | 33 | 9 | 78 |
| 4 | 4 | 0 | 34 | 11 | 93 |
| 5 | 0 | 0 | 23 | 7 | 70 |
| 5 | 0 | 1 | 31 | 11 | 89 |
| 5 | 0 | 2 | 43 | 14 | 100 |
| 5 | 1 | 0 | 33 | 10 | 100 |
| 5 | 1 | 1 | 46 | 14 | 120 |
| 5 | 1 | 2 | 63 | 22 | 150 |
| 5 | 2 | 0 | 49 | 15 | 150 |
| 5 | 2 | 1 | 70 | 22 | 170 |
| 5 | 2 | 2 | 94 | 34 | 230 |
| 5 | 3 | 0 | 79 | 22 | 220 |
| 5 | 3 | 1 | 110 | 34 | 250 |
| 5 | 3 | 2 | 140 | 52 | 400 |
| 5 | 3 | 3 | 180 | 70 | 400 |
| 5 | 4 | 0 | 130 | 36 | 400 |
| 5 | 4 | 1 | 170 | 58 | 400 |
| 5 | 4 | 2 | 220 | 70 | 440 |
| 5 | 4 | 3 | 280 | 100 | 710 |
| 5 | 4 | 4 | 350 | 100 | 710 |
| 5 | 5 | 0 | 240 | 70 | 710 |
| 5 | 5 | 1 | 350 | 100 | 1100 |
| 5 | 5 | 2 | 540 | 150 | 1700 |
| 5 | 5 | 3 | 920 | 220 | 2600 |
| 5 | 5 | 4 | 1600 | 400 | 4600 |
| 5 | 5 | 5 | ≥2400 | 700 | --- |

**NOTES**

## Confirmed Test

The following is the procedure of confirmed test.

### Procedure

1. Label the covers of the three EMB Plates, and/or the three m-Endo Agar Plates with the source of the Water Sample (Sewage, Pond, and Tap).
2. Using a positive 24-hour Lactose Broth Culture from the Sewage Water Series from the Presumptive Test, streak the surface of one EMB or one Endo Agar Plate, to obtain discrete colonies.
3. Repeat Step 2 using the Positive Lactose Broth Cultures from the Pond and Tap Water Series from the Presumptive Test to inoculate the remaining plates.
4. Incubate all plate cultures in an inverted position for 24 hours at 37°C.
5. Examine all the plates for PRESENCE or ABSENCE of *Escherichia coli* colonies. Refer to Figure 3 for an illustration of *Escherichia coli* growth on EMB Agar.
6. Based on your results, determine whether each of the samples is POTABLE or NON-POTABLE.

The **Presence** of ***Escherichia coli*** is a **Positive Confirmed Test**, indicating that the water is **Non-Potable**.

The **Absence** of ***Escherichia coli*** is a **Negative Test**, indicating that the water is not contaminated with fecal wastes and is therefore **Potable**.

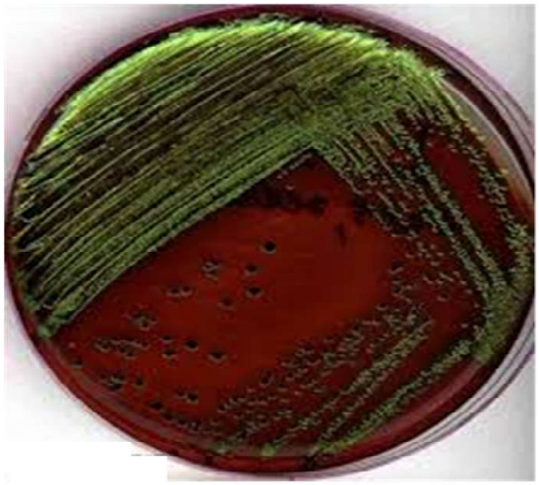

***Fig. 3:*** *Escherichia coli Growth on EMB Agar*

NOTES

## Observation and Results

| | Coliforms | | | |
|---|---|---|---|---|
| **Water Sample** | **EMB Plate** | **Endo Agar Plate** | **Potable** | **Non-Potable** |
| Sewage Water | | | | |
| Pond Water | | | | |
| Municipal (Tap Water) | | | | |
| RO Water | | | | |
| Bisleri Water | | | | |

## Discussion

Three types of colonies (Metallic Sheen, Pink Coloured and Colourless Colonies) were observed.

1. *Escherichia coli* Ferment Lactose to produce Organic Acids and stabilize these organic acids in its vicinity. The pH decreased to 4.0. At this pH, Eosine Yellow and Methylene Blue present in EMB Agar, formed a complex which is Green in colour, thus forming Metallic Green Sheen Colonies.
2. Other Coliforms Ferment Lactose to produce Organic Acids which get metabolized further to non-acidic products, such as Acetone, Ethanol, Acetyl Carbonyl, etc., so the pH of the medium remains same (around 6.0). At this pH no complex is formed but because Lactose was fermented, Pink coloured colonies are seen.
3. Non-Coliforms did not Ferment Lactose. They utilize Sucrose, so colourless colonies are formed.

## Completed Test

Following is the procedure for completed test.

## Procedure

1. Label each tube of Nutrient Agar Slants and Lactose Fermentation Broths with the source of its Water Sample.
2. Inoculate one Lactose Broth and one Nutrient Agar Slant with a positive isolated *Escherichia coli* colony obtained from each of the experimental water samples during the confirmed test.
3. Incubate all tubes for 24 hours at 37°C.
4. Examine all Lactose Fermentation Broth Cultures for the PRESENCE or ABSENCE of Acid and Gas.
5. Prepare a Gram Stain, using the Nutrient Agar Slant Cultures and examine the slides microscopically.

## Observation and Results

| | | GRAM STAIN | POTABILITY | |
|---|---|---|---|---|
| **Water Sample** | **Lactose Broth A/G (+) or (−)** | **Reaction/Morphology** | **Potable** | **Non-Potable** |
| Sewage Water | | | | |
| Pond Water | | | | |
| Municipal (Tap Water) | | | | |
| RO Water | | | | |
| Bisleri Water | | | | |

## Discussion

Gram Negative, Non-Spore Forming short Bacilli which could Ferment Lactose to produce Acid and Gas are indicative of *Escherichia coli* and thus Non-Potable Water.

## Comment on Quality and hence Potability of Different Water Samples as Evident from the Experiment

## Advantages

Presumptive Test allow us to obtain an Index indicating the possible number of organisms present in the sample under analysis.

## Disadvantages

- Lengthy and complex procedure. The inoculations and preparation of Broth Tubes for MPN is a laborious process. Hence, not feasible to test a large number of samples at a time.
- Does not detect the presence of waterborne pathogens directly.
- The initial amount of sample taken is too small (1 ml) to rely upon the results. This can be overcome by inoculating a larger amount of sample in proportionately more amount of broth.

## Precautions

1. Care in handling Sewage Waste Water Sample because Enteric Pathogens may be present.
2. Sewage or Pond Water should be mixed thoroughly by shaking prior to inoculation of Lactose Fermentation Tubes.
3. There must be no air bubble inside the Durham's Tube before starting the experiment.
4. The experiment should be done under sterile conditions.

# EXPERIMENT 20: BACTERIAL EXAMINATION OF WATER - QUALITATIVE AND QUANTITATIVE

NOTES

**AIM: Bacterial Examination of Water (Qualitative and Quantitative)**

## Theory

**Bacteriological Water Analysis** is a method of **analysing water** to **estimate** the numbers of **Bacteria Present**. It represents one aspect of **water quality**. This process is used, for example, to routinely confirm that water is safe for human consumption or that bathing and recreational waters are safe to use.

Although the **Sanitary Quality of Water** is judged by the presence of **Coliform Bacteria**, the total load of bacteria in water is an estimate of the overall extent of **microbial contamination**.

**Total Bacterial Count** by **Standard Plate Counting (SPC)** technique is useful to assess the efficiency of a particular water treatment process, such as Sedimentation, Filtration or Chlorination. A water sample containing less then **Hundred Bacteria Per Millilitre** is considered to be of **good quality**.

## Materials Required

**Sample:** Water Sample to be Tested.

**Media:** Trypytone Glucose Extract Agar/ Nutrient Agar (100 ml).

**Instruments:** Water Bath Maintained at 45-50°C, Incubator at 20-22°C and 37°C.

**Miscellaneous:** Sterile Petri Plates, Sterile 1 ml Pipette.

## Procedure

1. Place 6 tube containing molten and cooled 25 ml Sterile Trypytone Glucose Agar or Nutrient Agar Medium in a 45-50°C Water Bath.
2. Inoculate 1 ml of Water Sample from the $10^{-2}$, $10^{-3}$ and $10^{4}$ tubes into three tubes from Step 1.
3. Mix the contents and quickly pour in Sterile Petri Dishes.
4. Allow the medium to solidify.
5. Incubate the plates in an inverted position at 20-22°C for 72 hours.
6. Repeat Steps 2, 3, and 4 but keep at 37°C for 24 hours.
7. After incubation, the colony count is taken and multiplied by the dilution factor to obtain the total number of bacteria per ml of sample.

## Observation and Results

A Total Bacterial Count is made by calculating the number of colonies appearing Per Trypytone Glucose Extract Agar Plates incubated at 20°C and 37°C for 72 and 24 hours, respectively, to which aliquots of water sample are added.

CFU/ml is calculated by multiplying the number of colonies in the plate by the dilution used. The result is recorded in the following table.

| Temperature of Incubation | CFU/ml | | |
|---|---|---|---|
| | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| 20-22°C | | | |
| 37°C | | | |

## Discussion

The total number of colonies is referred to as the Total Viable Count (TVC). The unit of measurement is (or colony forming units per millilitre) and relates to the original sample.

To be effective, the original sample is diluted so that on average between 30 and 300 colonies of the target bacterium are grown. Fewer than 30 colonies makes the interpretation statistically unsound whilst greater than 300 colonies often results in overlapping colonies and imprecision in the count.

The bacteria appearing at 20-22°C include the natural flora and those growing at 37°C include all the microorganisms associated with the human body (i.e., fecal flora). Other methods that are used for examining water quality are membrane filtration and multiple tube method.

**Standard Quantitative Analysis by Membrane Filtration:** Most modern laboratories use a refinement of Total Plate Count (TPC) in which serial dilutions of the sample are vacuum filtered through purpose made membrane filters and these filters are themselves laid on Nutrient Medium. The methodology is otherwise similar to conventional Total Plate Count (TPC). Membranes have a printed millimetre grid printed on and can be reliably used to count the number of colonies under a binocular microscope.

**Standard Qualitative Analysis of Water by Multiple Tube Fermentation Technique/Most Probable Number (MPN):** MPN is used to determine the potability of water. It determines whether the water sample is contaminated by fecal or not. It includes three basic tests to detect Coliform Bacteria in Water: Presumptive, Confirmed, and Completed.

**Precautions**

1. The experiment should be done under sterile conditions.
2. Use disposable gloves when handling the water samples in this experiment.
3. Dilutions should be made carefully.
4. Be sure that water gets uniformly mixed in Molten Agar before pouring into the Petri Dishes.

## EXPERIMENT 21: MEMBRANE FILTRATION TECHNIQUE

### AIM: Membrane Filtration Technique

### Theory

In the **Membrane Filter Method**, a **water sample** is passed through a **Sterile Membrane Filter** (**pore size of 0.45 µm**) that is kept in a special filter apparatus contained in a **suction flask**. Following filtration, the filter disc that contains the **trapped microorganisms** is aseptically transferred to a Sterile Petri Dish containing an absorbent pad saturated with a selective, differential liquid medium. Following incubation, the colonies present on the filter are counted and expressed as **CFU/ml** of the sample.

The membrane filtration unit comprises of two glass or sterilizeable hard fibre compartments. The upper container is the reservoir of sample intake and lower one is for collection of filtrate. The two containers are separated by a sintered glass filter surface. The **milipore membrane filter** is placed on this glass surface just before filtration. A metal clamp is used for holding the assembly intact. A provision is made in the lower container to connect it to a vacuum pump for the filtration process can be enhanced by negative pressure.

This method enables a large volume of water to be tested more economically, results obtained are more accurate and are more quickly than by the multiple tube technique. This technique is routinely used to assess the efficiency of a treatment process by enumeration of organisms before and after treatment. It is widely used in mineral water plants and their packaged drinking water factories, on a routine basis.

### Materials Required

**Cultures:** Water Sample.

Media: One 20 ml Tube of m-Endo Broth, m-FC Broth, KF Broth.

**Equipment:** Sterile Membrane Filtration Apparatus, Sterile Membrane Filters (0.45 mm), 1 Liter Suction Flask, 44-45°C Water Bath.

NOTES

**Other Requirements:** Sterile Forceps, Sterile Water.

**Procedure**

1. Sterilize the glass assembly wrapped in parts, in a hot air oven.
2. Cool the glassware and aseptically assemble the unit. Fix the glass filter cum funnel to the lower container. Place the freshly unsealed membrane on the glass surface. Place the upper container in position and set the clamp to hold the assembly.
3. Shake the water sample and pour 100 ml of the water sample into the upper compartment.
4. Filter under vacuum.
5. Switch off the pump as soon as water is filtered, as there is a risk of tearing the membrane otherwise.
6. Add a sterile absorbent pad to a Sterile Petri Dish using a sterile forceps.
7. Aseptically add, using a Sterile Pipette, 2 ml of **m-Endo Broth** (for **Total Coliform Count, TCC**) to the pad to saturate it.
8. Remove the clamp and disassemble the unit. With the help of sterile forceps, carefully remove the membrane filter from the filter holder and aseptically transfer the membrane on the medium saturated pad in the Petri Dish.
9. Repeat the experiment with **m-FC Broth** (for **Fecal Coliform Count, FCC**) and **KF Broth** (for **Fecal Streptococcal Count, FSC**).
10. Incubate the plates in an inverted position at 37°C for 24 hours.
11. FCC plates sealed with waterproof tape and placed in a weighted watertight plastic bag, which is then submerged in a 44.5°C Water Bath for 24 hours.
12. Using membrane forceps, place each Dry Filter Disc into its Petri Dish cover. Keep the discs within the covers at all times for further observations.
13. Examine all filter discs under a dissecting microscope.
14. Perform **colony counts** on each set of discs as follows:
    (i) TCC: Count Colonies on m-Endo Agar that present a Golden Metallic.
    (ii) FCC: Count Colonies on m-FC Agar that are Blue.
    (iii) FSC: Count Colonies on KF Agar that are Pink to Red.
15. For each of the three counts, determine the number of organisms present in 100 ml of the Water Sample.

Figure 1 illustrates the Membrane Filter technique.

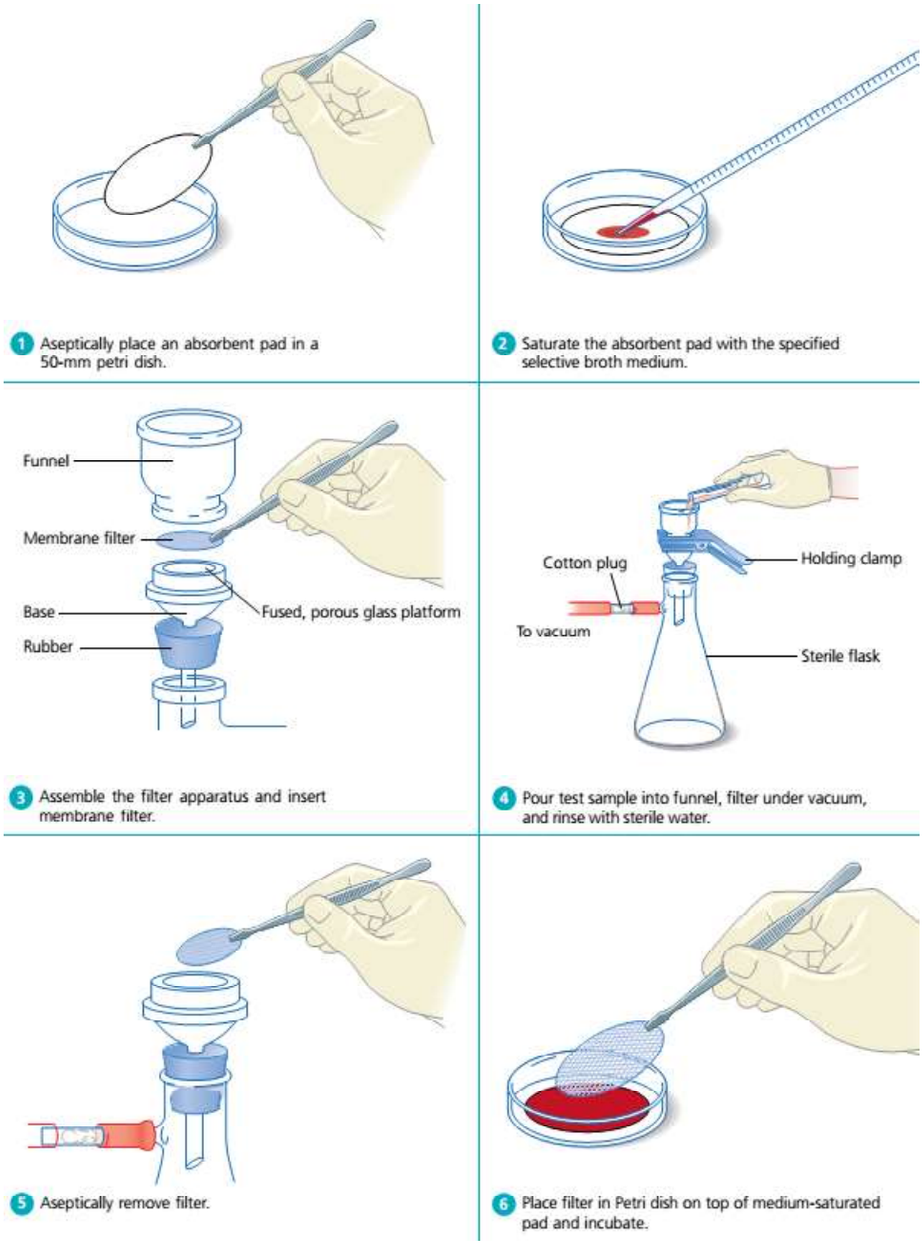


***Fig. 1:*** *Membrane Filter Technique*

## Observations and Results

Figure 2 illustrates the development of colonies on a membrane filter following incubation.

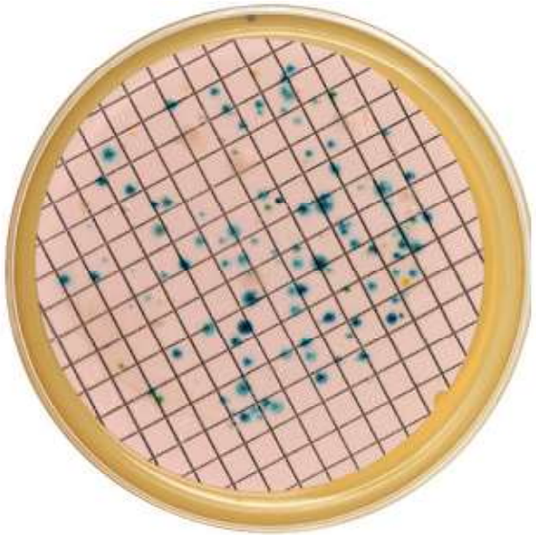

***Fig. 2:*** *Development of Colonies on a Membrane Filter Following Incubation*

1. Count the number of colonies that appear on the membrane for each plate and express your results per 100 ml of Water Sample by applying the formula:

   **No. of Colonies per 100 ml of Water**

$$= \frac{\text{Colony count}}{\text{volume of sample used}} \text{ X } 100$$

   Record the result in the following table.

**Observation Table**

| Sr. No. | Media Used | Number of Colonies per 100 ml |
|---|---|---|
| 1 | m-Endo Broth (TCC) | |
| 2 | m-FC Broth (FCC) | |
| 3 | KF Broth (FSC) | |

2. Based on your FC: FS Ratio, indicate the type of fecal pollution, if any. The ratio of the fecal coliforms to Fecal Streptococci Per Milliliter of sample is interpreted as follows:

   Between 2 and 4 indicates human and animal pollution.

   >4 indicates human pollution.

   <0.7 indicates poultry and livestock pollution.

**Discussion**

A Total Count of Coliform Bacteria determines the potability of the water sources. Water is considered safe for drinking if it contains fewer than Four Coliforms per 100 ml of Water.

Also, the types of fecal pollution, if any, are established by means of a Fecal Coliform Count (FCC), indicative of human pollution, and a Fecal Streptococcal Count (FSC), indicative of pollution from other animal origins.

No growth or Non-Coliforms indicate that water is safe for drinking.

Membrane filter method offer several advantages over the conventional, multiple-tube method of water analysis.

**Advantages**

(1) Membrane Filter Procedure is a rapid method in which results are available in a shorter period of time that isolates discrete bacteria that are able to be accurately counted, whereas the MPN method only allows for the approximate determination of the number of organisms and does not separate species without further testing.

(2) Larger volumes of sample can be processed.

(3) Because of the high accuracy of this method, the results are readily reproducible.

**Disadvantages**

(1) Processing of turbid specimens that contain large quantities of suspended materials, particulate matter clogs the pores and inhibits passage of the specific volume of water.

(2) Membrane filter assembly is expensive.

(3) The technique requires a new membrane for every test, which amounts to enhanced recurring expense.

## Precaution

1. Use disposable gloves when handling the water samples in this experiment.
2. Carefully remove a sterile membrane filter from its package, holding it only by its edge as shown in Figure 1.
3. Use blunt forceps to handle the membrane or wrap a little amount of cotton to the two tips of the forceps. This will prevent tearing of the membrane.
4. Never place a torn or damaged membrane for filtration as all the organisms will escape through the filter giving erroneous results.
5. When the entire sample has been filtered, wash the inner surface of the funnel with 100 ml of the sterile water.

# M.Sc. [Microbiology]
# 364 24

## LAB II – MICROBIAL GENETICS, MOLECULAR BIOLOGY & rDNA TECHNOLOGY, FOOD & DAIRY MICROBIOLOGY

**II - Semester**



**ALAGAPPA UNIVERSITY**

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