Manual

of

Standard Operating Procedures (SOP)

In

Botany

Department of Botany

Ramakrishna Mission Vivekananda Centenary College

Content

Exp. No:	Name of the experiment	Page Number
1	Gram Staining Procedure	3
2	Endospore staining procedure	9
3	Sterilization techniques	11
4	Isolation of plant genomic DNA	14
5	Isolation of genomic DNA using seedlings	18
6	Agarose Gel electrophoresis	20
7	Quantification of DNA using spectrophotometer	23
8	Polymerase Chain Reaction (PCR)	26
9	Isolation of Genomic DNA from E. coli	29

Experiment 1: **Gram Staining Procedure**

The Gram stain was first used in 1884 by Hans Christian Gram (Gram, 1884) for visual identification of bacteria depending on their staining properties.

A. Materials Required:

Materials:

1.	**Clean glass slides**
2.	**Bunsen burner/ Spirit lamp**
3.	**Blotting paper**
4.	**Microscope**
5.	**18 to 24 hour cultures of organisms**

Reagents:

1.	**Primary Stain**	**Crystal Violet**
2.	**Mordant**	**Grams Iodine**
3.	**Decolourizer**	**Ethyl Alcohol**
4.	**Secondary (counter) Stain**	**Safranin**
5.	**Washing agent**	**Distilled water**

B. Principle/ Theory:

In this staining method Crystal violet is the principle stain and safranin is the counter stain. The Gram positive bacteria, because of their thick peptidoglycan layer can retain the crystal violet stain and appears as dark violet in colour under the microscope. On the contrary, gram negative bacteria because of their different cell wall structure (thin peptidoglycan layer as well as presence of lipopolysaccharide made outer membrane) cannot retain the colour of crystal violet but take the colour of counter stain safranin. For that reason, Gram negative bacteria appear as red to pink in colour under microscope.

C. Procedure:

Part 1: Preparation of the glass microscopic slide

The slides were washed properly with detergent and then by alcohol to make it grease free.

Part 2: Labelling of the slides

The slides were marked properly to designate the side where smear has to be done.

Part 3: Preparation of the smear

The provided culture is properly smeared over the surface of the grease free slide by the help of another slide placing at around 45° angle to the first slide and at an unidirectional movement.

Part 4: Heat Fixing

Heat fixing kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains.

- The slide is allowed to air dry
- After the smear has air-dried, the entire slide was passed through the flame of a Bunsen burner two to three times with the smear-side up.

Part 5: Gram Stain Procedure

1. The slide was placed with heat fixed smear on staining tray.
2. The smear was gently flooded with crystal violet and let stand for 1 minute.
3. The slide was tilted slightly and gently rinsed with tap water or distilled water using a wash bottle.
4. The smear was gently flooded with Gram's iodine and let stand for 1 minute.
5. The slide was tilted slightly and gently rinsed with tap water or distilled water using a wash bottle. The smear appeared as a purple streak on the slide.
6. The slide was decolorized using 95% ethyl alcohol or acetone. The slide was tilted slightly and the alcohol was applied drop by drop for 5 to 10 seconds until the alcohol runs almost clear.
7. The slide was immediately rinsed with water.
8. The slide was gently flooded with safranin to counter-stain and let stand for 45 seconds.
9. The slide was tilted slightly and gently rinsed with tap water or distilled water using a wash bottle.
10. The slide was blotted to dry with tissue paper.
11. View the smear using a light-microscope.

D. Observations:

1. Morphology of bacteria: The bacteria are appeared as rod/ round in shape and hence the morphology of the bacteria of the supplied specimen is bacilliform/ coccoid.

2. Staining property: The bacteria are appeared as dark violet/ pink to red in colour and hence the staining property of the bacteria of the supplied specimen is Gram positive/ Gram negative in nature.

Detailed (For viva voce only)

History: The Gram stain was first used in 1884 by Hans Christian Gram (Gram,1884). Gram was searching for a method that would allow visualization of cocci in tissue sections of lungs of those who had died of pneumonia. Already available was a staining method designed by Robert Koch for visualizing turbercle bacilli. Gram devised his method that used Crystal Violet (Gentian Violet) as the primary stain, an iodine solution as a mordant followed by treatment with ethanol as a decolorizer. This staining procedure left the nuclei of eukaryotic cells in tissue samples unstained while the cocci found in the lungs of those who had succumbed to pneumonia were stained blue/violet. Gram found that his stain worked for visualizing a series of bacteria associated with disease such as the "cocci of suppurative arthritis following scarlet fever". He found however that Typhoid bacilli were easily decolorized after the treatment with crystal violet and iodine, when ethanol was added. We now know that those organisms that stained blue/violet with Gram's stain are **gram-positive** bacteria and include *Streptococcus pneumoniae* (found in the lungs of those with pneumonia) and *Streptococcus pyogenes* (from patients with Scarlet fever) while those that were decolorized are **gram-negative**bacteria such as the *Salmonella* Typhi that is associated with Typhoid fever.

Purpose: The Gram stain is fundamental to the phenotypic characterization of bacteria. The staining procedure differentiates organisms of the domain Bacteria according to cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. Gram-negative cells have a thin peptidoglycan layer and stain red to pink.

Theory: The Gram stain, the most widely used staining procedure in bacteriology, is a complex and differential staining procedure. Through a series of staining and decolorization steps, organisms in the Domain Bacteria are differentiated according to cell wall composition. Gram-positive bacteria have cell walls that contain thick layers of peptidoglycan (90% of cell wall). These stain purple. Gram-negative bacteria have walls with thin layers of peptidoglycan (10% of wall), and high lipid content. These stain pink. This staining procedure is not used for Archeae or Eukaryotes as both lack peptidoglycan. The performance of the Gram Stain on any sample requires four basic steps that include applying a primary stain (crystal violet) to a heat-fixed smear, followed by the addition of a mordant (Gram's Iodine), rapid decolorization with alcohol, acetone, or a mixture of alcohol and acetone and lastly, counterstaining with safranin.

Details of the chemical mechanism of the Gram stain were determined in 1983 (Davies et al.,1983 and Beveridge and Davies, 1983). In aqueous solutions crystal violet dissociates into CV^+ and Cl^-ions that penetrate through the wall and membrane of both gram-positive and gram-negative cells. The CV^+ interacts with negatively charged components of bacterial cells, staining the cells purple. When added, iodine (I^- or I_3^-) interacts with CV^+ to form large CVI complexes within the cytoplasm and outer layers of the cell. The decolorizing agent, (ethanol or an ethanol and acetone solution), interacts with the lipids of the membranes of both gram-positive and gram-negative Bacteria. The outer membrane of the gram-negative cell is lost from

the cell, leaving the peptidoglycan layer exposed. Gram-negative cells have thin layers of peptidoglycan, one to three layers deep with a slightly different structure than the peptidoglycan of gram-positive cells (Dmitriev, 2004). With ethanol treatment, gram-negative cell walls become leaky and allow the large CV-I complexes to be washed from the cell. The highly cross-linked and multi-layered peptidoglycan of the gram-positive cell is dehydrated by the addition of ethanol. The multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the cell. After decolorization, the gram-positive cell remains purple in color, whereas the gram-negative cell loses the purple color and is only revealed when the counterstain, the positively charged dye safranin, is added. At the completion of the Gram stain the gram-positive cell is purple and the gram-negative cell is pink to red.

Some bacteria, after staining with the Gram Stain yeild a pattern called gram-variable where a mix of pink and purple cells are seen. The genera *Actinomyces, Arthrobacter, Corynebacterium, Mycobacterium,* and *Propionibacterium* have cell walls particularly sensitive to breakage during cell division, resulting in gram-negative staining of these gram-positive cells. In cultures of *Bacillus, Butyrivibrio,* and *Clostridium* a decrease in peptidoglycan thickness during growth coincides with an in increasing number cells that stain gram-negative (Beveridge, 1990). In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain.

Some bacteria do not stain as expected with the Gram stain. For example, members of the genus*Acinetobacter* are gram-negative cocci that are resistant to the decolorization step of the Gram stain.*Acinetobacter* spp. often appear gram-positive after a well prepared Gram stain (Visca et al. 2001). For *Mycobacterium* spp., the waxy nature of the coat renders the bacteria not readily stainable with dyes used in the Gram stain, though the bacteria are considered to be gram positive (Saviola and Bishai, 2000). *Gardnella* has an unusual gram-positive cell wall structure that causes bacteria of this genus to stain gram-negative or gram-variable (Sadhu et al 1989).

Misinterpretation of the Gram stain has led to misdiagnosis or delayed diagnosis of infectious disease (Visca et al., 2001, Noviello et al., 2004)

Recipe: (Gephardt et al., 1981) This is Hucker's modification of the Gram Stain method. Gram originally used Gentian Violet as the primary stain in the Gram stain. Crystal violet is generally used today. In Hucker's method ammonium oxalate is added to prevent precipitation of the dye (McClelland, 2001) and uses an alcoholic solution of the counterstain. Burke's modification of the Gram Stain adds sodium bicarbonate to the crystal violet solution. Sodium bicarbonate prevents the acidification of the solution as iodine oxidizes (McClelland, 2001) and uses an aqueous solution of Safranin for the counterstain (Gephardt et al., 1981).
The reagents listed below can be made or purchased commercially from biological supply houses

1. Primary Stain: Crystal Violet Staining Reagent.

Solution A for crystal violet staining reagent
Crystal violet (certified 90% dye content), 2g
Ethanol, 95% (vol/vol), 20 ml

Solution B for crystal violet staining reagent
Ammonium oxalate, 0.8 g
Distilled water, 80 ml
Mix A and B to obtain **crystal violet staining reagent.** Store for 24 h and filter through paper prior to use.

2. Mordant: Gram's Iodine
Iodine, 1.0 g
Potassium iodide, 2.0 g
Distilled water, 300 ml
Grind the iodine and potassium iodide in a mortar and add water slowly with continuous grinding until the iodine is dissolved. Store in amber bottles.

3. Decolorizing Agent
Ethanol, 95% (vol/vol)

*Alternate Decolorizing Agent
Some professionals prefer an acetone decolorizer while others use a 1:1 acetone and ethanol mixture. Commercially, a variety of mixtures are available, most using 25 – 50% acetone with the ethanol. A few include a small quantity of isopropyl alcohol and/or methanol in the formulation.
Acetone, 50 ml
Ethanol (95%), 50 ml

4. Counterstain: Safranin
Stock solution:
2.5g Safranin O
100 ml 95% Ethanol
Working Solution:
10 ml Stock Solution
90 ml Distilled water

Typical Gram-negative bacteria:

1. ***Bordetella pertusis***, the causative agent of whooping cough
2. ***Salmonella typhi***, the causative agent of typhoid
3. ***Vibrio cholera***, the causative agent of cholera
4. ***Escherichia coli***, the normally benign, ubiquitous, gut-dwelling bacteria

Typical Gram-positive bacteria:

1. *Staphylococci* such as ***Staphylococcus epidermidis*** and ***Staphylococcus aureus*** which is a common cause of boils.
2. *Streptococci* such as the many species of oral streptococci, ***Streptococcus pyogenes*** which causes many a sore throat and scarlet fever and ***Streptococcus pneumoniae*** which causes lobar pneumonia.
3. *Clostridia* such as ***Clostridium tetani***, the causative agent of tetanus (lockjaw).
4. *Actinomyces* such as ***Actinomyces odontolyticus*** which is found in mouth.
5. Species of the genus Bacillus such as ***Bacillus subtilis*** which are common microbes living in soil.

Generally, cocci are Gram-positive but there are exceptions. The most significant from a clinical point of view is the gonococcus, ***Neisseria gonorrhoea*** which typically appears as a Gram-negative diplococcus looking very much like a pair of kidney bean.

Experiment 2: **Endospore staining procedure**

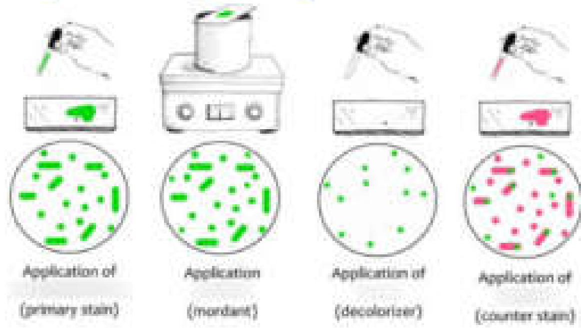
Endospore Staining is a technique used in bacteriology to identify the presence of endospores in a bacterial sample, which can be useful for classifying bacteria.

Preparation of microscope slide:

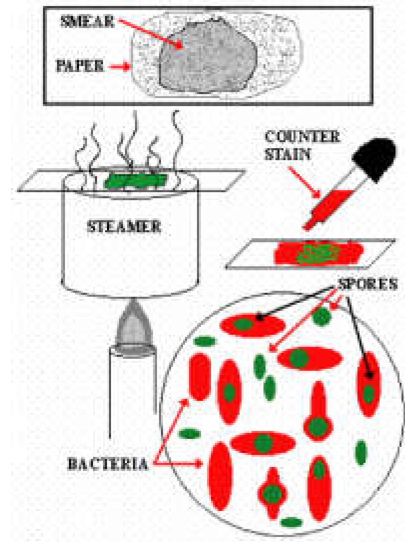
- Clean slide with a Kimwipe and alcohol to remove any fingerprints.
- Draw two circles with your Sharpie on the bottom of the slide.
- Using your inoculation loop, put two small drops of water in each circle.
- Using aseptic technique, remove a very small amount of bacteria from the culture tube. Make sure you flame the tube before and after you enter.
- Smear the bacteria in the drop of water on your slide. You may go out of the perimeter of your circles!
- Let the slide air dry completely.
- Heat-fix the slide by running it through the flame 3-4 times with the 'smear' side up. Do not flame the side with the bacteria!
- Let the slide cool completely and you are ready to stain it.

Staining procedure:

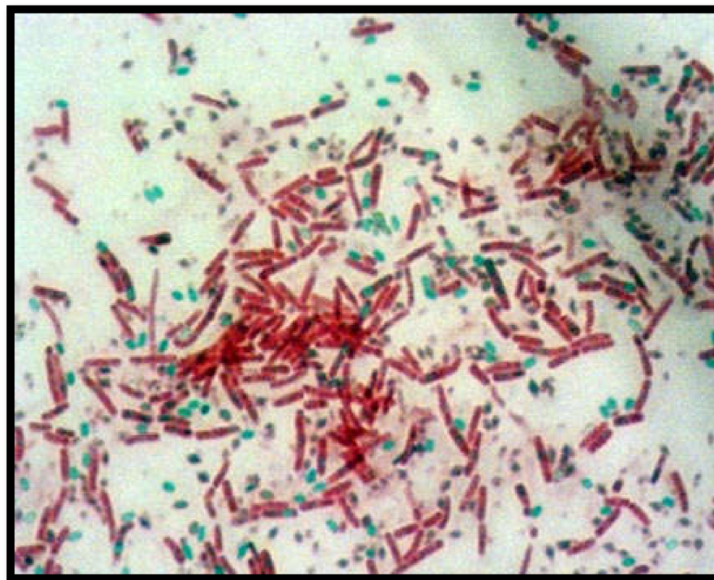
- Cover the smears with a piece of paper towel within the border of the slide.
- Place the slide over a beaker of steaming water. Do not let the beaker boil dry
- Flood the paper towel with malachite green and let the slide steam for 3-5 minutes.
- Complete the rest of the procedure at the back sinks.
- Remove the stained paper towel and discard it in the trash can, not in the sinks.
- Gently rinse the slide with water to remove any pieces of a loose paper towel and tap dry.
- Counterstain with safranin for 1 minute.
- Rinse with water and tap dry.
- Blot gently with bibulous paper.
- Dry the bottom of the slide before placing it on the stage of the microscope and view with the oil immersion lens.

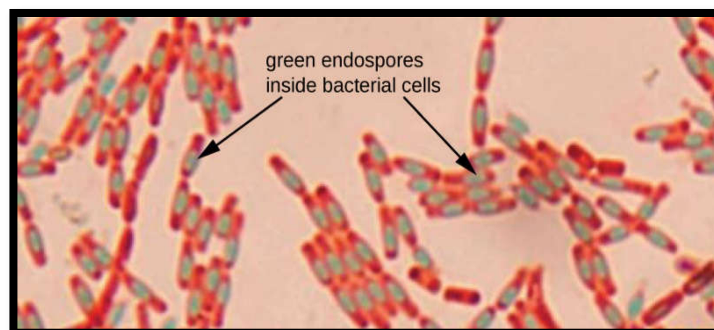


Endospore Staining: Principle, Procedure, Results
Application of
(primary stain)
Application
(mordant)
Application of
(decolorizer)
Application of
(counter stain)



SMEAR
PAPER
COUNTER
STAIN
STEAMER
SPORES
BACTERIA





green endospores
inside bacterial cells

Experiment 3: **Sterilization techniques**

Introduction

Sterilization is the process of killing all microorganisms (bacterial, viral, and fungal) with the use of either physical or chemical agents. A disinfectant is a chemical substance that kills microorganisms on inanimate objects, such as exam tables and surgical instruments. Skin can never be completely sterile. Sterilization in the microbiological laboratory denotes sterilization process implemented in preparation of culture media, reagents and equipment where the work warrants maintaining sterile condition. Sterilization in microbiology laboratory is done by following methods Physical method i.e., use of heat, filters, radiation Chemical method i.e., by use of chemicals and Heat sterilization

a. Dry heat sterilization

Inoculation loops or needle are sterilized by heating to 'red' in Bunsen burner or spirit lamp flame. Sterilization in hot air oven is performed at a temperature of 160C and maintained or holding for one hour. Spores are killed at this temperature and this is the most common method of sterilization of glassware, swab sticks, pestle and mortar, mineral oil etc. Dry heat sterilization causes protein denaturation, Oxidative damage, toxic effect of elevated electrolyte in absence of water.

b. Wet heat or moist heat sterilization: Moist heat sterilization is accomplished by

1). **Boiling** at 100°C for 30 minutes is done in a water bath. Syringes, rubber goods and surgical instruments may be sterilized by this method. Almost all bacteria and certain spores are killed in this method

2). **Steaming** at 100°C for 20 to 30 minutes under normal atmospheric pressure are more elective than dry heat at the same temperature because bacteria are more susceptible to moist heat, Steam has more penetrating power and sterilizing power as more heat is given up during condensation. Suitable for sterilizing media which may be damaged at a temperature higher than 100°C

3).**Tyndallisation** (Fractional Sterilization) is the steaming process performed at 100°C is done in steam sterilizer for 20 minutes followed by incubation at 37°C overnight and this cycle is repeated for successive 2 days. Spores, if any, germinate to vegetative bacteria during incubation and are destroyed during steaming on second and third day. Heat labile media containing sugar, milk, gelatin can be sterilized using this method.

4). **Autoclaving** is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. This is achieved by employing a higher

pressure. The autoclave is closed and made air-tight for pressure development and at 15 lbs per sq. inch pressure, 121°C temperatures will be reached and this temperature is given as sterilizing holding time for further 15 minutes. This process kill spores and this works like a pressure cooker and one of the most common methods of sterilization.

5). **Pasteurization** is another one method of moist heat sterilization which works below 100°C heat. This process is used in heating of milk and other liquid food. The product is held at temperature and for a period of time to kill pathogenic bacteria that may be present in the product. This process does not destroy complete organism including spores. All these moist heat sterilization causes denaturation and coagulation of protein, breakage of DNA strands, and loss of functional integrity of cell membrane.

c). **Filtration**: This method of sterilization is used for media particularly heat labile in nature (e.g. sera an media containing proteins or labile metabolites. If the study warrants bacteria- free filtrates it can be obtained through 0.45micron sized filter membranes and if the study requires viral particle free solution, then 0.22micron sized filter membranes are use. In earlier days absorptive filters of asbestos or diatomaceous earth were replaced by unglazed porcelain or sintered glass are used. Nowadays these are replaced by nitrocellulose membrane filters of graded porosity, PVDF etc.

d). **Ultraviolet Radiation:** at wavelength between 330nm and 400nm causes sterilizing effect. This method is used in surface sterilization of laminar airflow, biosafety cabinet and in certain cases in laboratory. In microbiology laboratory autoclaving, hot air oven sterilization, filtration and UV radiation are commonly used.

Standard operating procedure for the setting up of autoclave

- Pack your media, reagents, plastic wares, in their appropriate autoclavable resistant polypropylene or borosilicate glassware
- Screw the lid of the tube and leave one thread loose in case of closed containers or plastics
- Stack at random autoclavable indicators for each run in any of the items to be autoclaved
- Check for the water level in the autoclave machine
- Donor jam pack the items in the autoclave machine
- Switch on the machine
- Keep the lid of the machine tightly closed with one valve open until it reaches boiling

• Leave heated air to escape for few minute through valve

• Completely close the valve and wait to reach the temperature for 121 ^{0}C at 15lbs pressure.

• Hold the sterilization cycle for 15 minutes

• Once the sterilization cycle end, switch off the heating and leave the machine to reach to 65^{0}C

• Then open the lid and take out the items back after sterilization

Standard operating procedure for the setting up of hot air oven

• Pack all the glassware such as pipette with pipette can, glass Petri dishes, sample dish, test tubes, pestle and mortar, mineral oil to be sterilized by hot air oven sterilization with suitable wrapping

• Switch on the hot air oven until to reach 160^{0}C

• Hold on in that temperature for 1 hour

• Switch off the heating of hot air oven and open the door once come below 65^{0}C

Standard operating procedure for the setting up of filtration

• Once the bio safety cabinet is ready for filtration

• Switch on the blower

• Filtration unit should be inside the cabinet

• Vacuum or positive pump should be kept outside of the cabinet

• Filtration assembly should be with the suitable filters

• Pour the media or reagents to be sterilized in the top of the filtration assembly

• Connect the bottom assembly to vacuum pump or top of the assembly to the positive pump

Experiment 4: **Isolation of plant genomic DNA**

Introduction: DNA extraction from plant tissues is an art and always remains difficult due to the presence of a rigid cell wall (polysaccharide, pigments, secondary metabolites etc). Various factors like selection of source, type of material used and the concentration of metabolites present in the plant decide the isolation procedure. DNA is basically isolated from any living (root, leaves, seed, seedlings etc) or dead tissue (dried part of plant). The biochemical composition of tissues of different species is expected to vary greatly with respect to temporal and spatial variability in their metabolic pathway, however the most common sources and method for DNA isolation in plants is leaf and the CTAB (Cetyl trimethyl ammonium bromide) method (Murray and Thompson, 1980), respectively.

Principal: CTAB is a cationic detergent, which forms a complex with proteins and most of the acidic polysaccharides, but will not precipitate nucleic acid when used as extraction buffer. The extraction process involves breaking or digestion of cell wall in order to release the cellular constituents, which is brought about by disruption of the tissue in a mortar and pestle aided either by liquid nitrogen or by extraction buffer followed by precipitation and purification. The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, lipids, phenols, tannins and pigments which may interfere with the extracted DNA and difficult to separate. Most proteins are removed by denaturation and precipitation from the extract using chloroform. RNAs on the other hand are normally removed by treatment of the extract with RNase A. The DNA is precipitated and washed in organic solvents before re-dissolving in aqueous solution.

Function of different chemicals involved in DNA isolation:

1. **Tris-HCL:** It acts as stabilizing buffer during isolation of DNA, which maintain the pH of buffer.
2. **EDTA:** It is required for chelation of Mg^{2+} which is an important co-factor for activity of DNases. Hence, addition of EDTA stops the action of DNases located in the cytoplasm of cells that get released in abundance in the cell lysate. These DNases, DNA cutting enzymes if not taken care of, can destroy digest the genomic DNA and reduce the yield.
3. **NaCl:** It helps to remove proteins that are bound to the DNA. It also helps to keep the proteins dissolved in the aqueous layer so they don't precipitate in the alcohol along with the DNA.
4. **CTAB:** It is a cationic detergent used to break open plant cells and solubilize the plant cell wall and lipid membranes of internal organelles, denatures proteins (enzymes) and separate polysaccharides.
5. **Dichloromethane:** It aids in denaturing proteins and separating them. The organic solvent is dense. The non-aqueous parts of the cell (lipids, some proteins, etc) will be

dissolved in this this hydrophobic liquid while the DNA stays immersed in an aqueous layer.

6. **Beta mercaptoethanol:** It is a reducing agent which helps in denaturing proteins by breaking the di-sulphide bonds between the cysteine residues and for removing the tanins and polyphenols.
7. **Absolute Ethanol or Isoproponal**: DNA is insoluble in ice-cold absolute ethanol or isopropanol, it will aggregate together and precipitates to form pellet upon centrifugation.
8. **70% Ethanol:** It is used to wash the water and ethanol soluble impurities from the DNA pellet. It incur little loss of DNA quantity as a meagre amount of DNA is lost as water soluble fraction in this process but it improves the purity of reaming major DNA fraction.
9. **TAE**: It is used for resuspension of purified DNA. It contains ten parts of Tris-HCL, which maintains ambient pH for DNA and one part of EDTA, which chelate the Mg^{2+} (if any) thus maintaining ideal condition for long term DNA storage.

Activity 1

Chemical preparation for plant genomic DNA isolation

Preparation of stock solutions for plant genomic DNA isolation

S.No.	Chemicals	Stock Conc.	M. wt.	Quantity (g)	pH	pH adjustment	Volume (ml)
1.	Tris. Base	1.0 M	121.14	12.1	8.0	Con. HCl	100
2.	#EDTA	0.5 M	372.24	18. 6	8.0	NaOH pellets	100
3.	NaCl	5.0 M	54.41	44.25	-	-	150
4.	*CTAB	10 %		10g	-	-	100

Note: Dissolve each chemical separately in about 75 ml distilled water (dH_2O). Adjust the pH as per requirement and then makeup the final volume using dH_2O. Finally autoclave and store at room temperature (RT).

#EDTA doesn't dissolve until its pH is adjusted.

*Heat gently under stirring, autoclave and store at RT.

How to make working solutions from stocks:

1. 100mM Tris-HCl (100ml)

$N_1V_1 = N_2V_2$

$1M \times V_1 = 100mM \times 100\ ml \div 1000mM$

$V_1 = 10ml$

2. 20mM EDTA (100ml)

$N_1V_1 = N_2V_2$

$0.5\ M \times V_1 = 20\ mM \times 100\ ml \div 500mM$

$V_1 = 4ml$

3. 1.4M NaCl (100ml)

$N_1V_1 = N_2V_2$

$5\ M \times V_1 = 1.4\ M \times 100\ ml \div 5M$

$V_1 = 28\ ml$

4. 2% CTAB

$N_1V_1 = N_2V_2$

$10 \times V_1 = 2 \times 100 \div 10$

$V_1 = 20ml$

Note: N_1: Concentration of stock solution

V_1: Volume of stock solution to be taken

N_2: Required concentration for working solution

V_2: Volume of working solution to be made.

Preparation of 2 % (w/v) CTAB (working solution) for DNA isolation in jute, mesta & flax

S. No.	Chemicals	Stock Conc.	Working Conc.	Stock for 50 ml	Stock for 100 ml	Stock for 200 ml
1.	2 % CTAB Powder (g)/10 % stock	-	-	1.0g / 10 ml	2.0g / 20 ml	4.0g /40 ml
2.	Tris-HCl (ml)	1.0 M	100 mM	5.0	10.0	20.0
3.	EDTA (ml)	0.5 M	20 mM	2.0	4.0	8.0
4.	NaCl (ml)	5.0 M	1.4 M	14.0	28.0	56.0
5.	0.2 % (v/v) β-mercaptoethanol (µl)			250	500	1000
6.	H_2O (ml)			17.750	35.500	71.0

Note: Add the required stock one by one followed by CTAB powder/stock and adjust the final volume using dH_2O. No need to autoclave and store at RT.

Optional: β-mercaptoethanol can be added separately in each tube and mixed thoroughly before incubating the samples at 60 °C for 1 hr.

Preparation of $T_{10}E_{1.0}$ pH 8.0 (100 ml)

1. 10mM Tris-HCl

$N_1V_1 = N_2V_2$

$1M \times V_1 = 10mM \times 100\ ml \div 1000mM$

$V_1 = 1ml$

2. 1mM EDTA

$N_1V_1 = N_2V_2$

$0.5\ M \times V_1 = 1\ mM \times 100\ ml \div 500mM$

$V_1 = 0.2\ ml$

Note: Add 1ml of 1M Tris HCl and 0.2 ml 0.5 M EDTA and makeup with sterile distilled water

Preparation of RNase stock solution (10mg/ml)

Dissolve 100 mg RNase in 10 mM Tris, 15 mM NaCl and boil it for 15 min, cool to RT divide in aliquots and **store at -20º C.**
Note: ready to use is available.

Experiment 5: **Isolation of genomic DNA using seedlings**

Seed germination: Wash mature seeds in running tap water thoroughly for 5-10 min and germinate in magenta boxes containing moist germination paper till they sprout (1-2 days) in dark. Once germinated keep those under natural light for next 5-6 days.

Plant sample: Take fresh, young and tender leaves (150-200 mg) from 5-7 day old seedlings and cut the leaf material.

Materials required:
Pestle and mortar, liquid nitrogen, plant sample, CTAB, 1M Tris-HCl, 5 M NaCl , 0.5 M EDTA, dichloromethane, isopropanol, 100 % and 70% Ethanol, TE Buffer, pipettes, centrifuge**,** 1.5 and 2.0 ml Eppendrof tubes, RNase enzyme (10mg/ml), β-mercaptoethanol, scissors, forceps and tissue paper.

Methodology:

Day I

1. Crush about 150-200 mg fresh young leaves in 1.0 ml of pre- heated (60 °C) warm CTAB extraction buffer using autoclaved mortar and pestle.

Note: Liquid nitrogen may be used for grinding (optional).

Lysis phase:
2. Transfer the extract in 2 ml Eppendrof centrifuge tube using clean spatula and mix gently by inversion.
3. Incubate the samples at 60 °C in a water bath for an hour (i.e., 60 minutes) with gentle mixing after every 10 min for uniformity.
4. After 1 hr cool the tubes by keeping them at room temperature (RT) for 5-10 min.

Phase separation:
5. Add 1.0 ml Dichloromethane or 1.0 ml chloroform: isoamyl alcohol (24:1) as per availability and mixed by twisting (by hand), until it is mixed properly.
6. Centrifuge the tubes at 12000-13000 rpm for 15 min.
7. Transfer the top aqueous phase (upper layer) into a new 1.5 ml Eppendrof tube and add 4-5 ul RNase and mix by inversion and incubate for 10-15 min at 37 °C.

DNA precipitation:
8. Add equal volume i.e., 1.0ml of chilled isopropanol (or double the volume of absolute EtOH) and mix gently to precipitate the DNA. The DNA appears as white fluffy thread like structure.
9. Fish out the precipitated DNA with a Pasteur pipette/tooth pick or **spin down** at 12000 rpm for 10 min and discard the supernatant.
10. Add about 500 µl 100 % EtOH to the pellet.

Note: The protocol can be stopped here and can be continued on the second day if required, however store the tubes at -20 °C.

Day II

Washing:

11. Centrifuge at 12 000 rpm for 5 min and discard 100% EtOH.
12. Re-dissolve the obtained DNA in 500 µl of 70 % EtOH and centrifuge at 12 000 rpm for 5 min.
13. Discard 70 % EtOH and air dry the samples for 30 minutes in a laminar flow clean air work station.

Re-suspension of purified DNA:

14. Add about 50 µl (based on the size of DNA pellet) of TE buffer and leave overnight at room temperature (RT) or incubated at 60 °C in a water bath for 2 hours for re-suspending the pellet.

Storage of the DNA:

For short-term storage (24-48 hrs) of the DNA, 2 to -8 °C temperature is recommended. For long term storage, -20 °C or lower temperature (-80 °C) is recommended. Avoid repeated freezing and thawing of the sample which may cause degradation of DNA. The re-suspended buffer will help to stabilize the DNA at these temperatures.

Note: The same protocol can be used for genomic DNA isolation for mesta, flax and sunhump using seedlings.

References:

Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard RW (1984). Ribosomal spacer length polymorphism in barly. Mendelian inheritance, chromosomal location and population dynamic. Proceeding of the National Academy of Science of USA., 81: 8014-8019.

Experiment 6: **Agarose Gel electrophoresis**

Activity 1

Assessment of purity and quantity of isolated genomic DNA by agarose gel

Introduction: Many molecular biology experiments involving PCR, restriction digestion, Southern hybridization, gene cloning etc mainly relies on purity and quantity of isolated genomic DNA. DNA yield can be assessed using various methods including absorbance (optical density), agarose gel electrophoresis, or use of fluorescent DNA-binding dyes. Quality and quantity of DNA isolated dependence upon method used to isolate the DNA, source, age and size of the sample. Quantity and quality of DNA from stored samples many vary in comparison to DNA isolated form fresh samples. Agarose gel provides approximate idea about DNA concentration in samples when run with suitable control DNA (λ-uncut) of known concentration. Purity and concentration is finally judged based on visual characteristics, which reveal whether DNA is intact/sheared, presence or absence of RNA contamination and to know approximate concentration of DNA samples. Nucleic acids running on an electrophoresis are detected by staining the gels with EtBr, which fluorescent with an orange colour that is easily seen by the naked eye under 300-nm UV light.

Material required: 0.8 % agarose gel, running buffer, 6x gel loading dye (containing sucrose, Bromophenol blue dye), DNA standards (like λ- uncut) and DNA samples.
Note: Agarose gel with 0.8 % concentration forms appropriate pore size to separate high molecular weight molecules like genomic DNA for quantification.

Methodology:

1. Weigh 0.8g agarose in 100 ml 0.5 X TAE/TBE buffer and prepare the gel.
2. Mix DNA sample (say: 2 µl) with gel loading dye (1-2 µl) on a strip of paraflim and load the sample one by one in each well.
3. Also load different concentration of standard (50, 100, 150, 200 ng) in nearby wells and then exposed to an electric field at 80 V initially and then at 100 V for 1 hr and documented by a gel documentation system.
4. Concentration and yield can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantification standard.

For example, if a 2µl sample of crude DNA loaded on the gel and if it has approximately the same intensity as the 100 ng standard loaded, then the concentration is approximately 50 ng/µl (100 ng divided by 2µl). In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide is required.

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. Proteins cannot be visualized as a protein does not bind EtBr. In case of RNA, a portion will be degraded due to high pH of TAE/TBE buffer, another portion will move out of the gel due to their small size and rest will produce a low intensity smear throughout the lane or as a blotch at the end of the lane.

Observation and Result: Visualize the DNA bands under Gel Documentation system and calculate the yield and purity using UV Spectrophotometer.

Chemical preparation for agarose gel electrophoresis:

Preparation of gel electrophoresis buffer

The two most common buffers for nucleic acids are Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE), where DNA fragments migrate with different rates due to differences in their ionic strength. Buffers not only establish an ideal pH, but provide ions to support conductivity. In general, the ideal buffer should produce less heat, have a long life and a good conductivity.

Buffer preparation:

Chemicals	Tris-base	Glacial acetic acid	Boric acid	EDTA / 0.5 M EDTA solution
Stock (10 X TAE)				
1000 ml	44.4 g	11.4ml	-	3.7g or 20 ml
500 ml	22.2 g	5.7 ml	-	1.85 g or 10 ml
Stock (10 X TBE)				
1000 ml	108 g	-	55.0 g	9.4 g or 40 ml
500 ml	54 g	-	27.5 g	4.65 g or 20 ml

Working 0.5 X / 1.0 X			
Total volume to be prepared	**1000 ml**	**500 ml**	**100 ml**
Vol. to be taken from stock (10X)	25/50 ml	12.5/25 ml	2.5/5 ml

*For stock preparation dissolve the contents one by one in doubled distilled water and then make up using ddH_2O.

Preparation of Ethidium Bromide

Stock concentration: 10 mg/ml

Dissolve 100 mg of Ethidium Bromide in 10 ml of autoclaved ddH_2O and mixed thoroughly on a magnetic stirrer. It should be **stored in amber colored bottle at RT**.

Working Concentration is 0.5 µg/ml of buffer.

Note: Ethidium bromide is a mutagenic agent, wear gloves and protect your eyes while its preparation and usage.

Preparation of 6x DNA gel loading buffer

S.No.	Chemical	Quantity
1	80% Glycerol	93.6 ml
2	0.5M EDTA	3.0 ml
3	Bromophenol blue	0.3 gms
4	Xylene cyenol	0.3 gms
	Total	250 ml

*Dissolve the contents in ddH_2O, mix and make up using ddH2O, aliquots and store in fridge.

Experiment 7: **Quantification of DNA using spectrophotometer**

Objective: Assessment of purity and quantity of DNA using spectrophotometer

Introduction: The most common technique to determine DNA yield and purity is measurement of absorbance. Absorbance measurement is simple, and requires commonly available laboratory equipment spectrophotometer equipped with a UV lamp, UV-transparent cuvettes (depending on the instrument) and a solution of purified DNA. Absorbance readings are measured at 260nm (A_{260}) where DNA absorbs light most strongly, and this is the value used to estimate the concentration of the sample.

Material required: DNA samples, spectrophotometer, pipettes, tips, dH_2O, cuvettes, beaker, tissue paper.

Methodology:

1. For quantification of DNA, samples are diluted in 2: 98 ratio (i.e, 2µl DNA: 98 µl nuclease free H_2O) and quantified using UV- visible Spectrophotometer. This gives a dilution facto of 50.
2. The cuvettes of spectrophotometer are washed with distilled water followed by nuclease free water.
3. Take 100 µl of nuclease free water in the cuvette as blank for auto-zero at 280 and 260 nm visible light.
4. Take 2µl of DNA sample and 98 µl of nuclease free water, mix and measure absorbance at A_{260} and A_{280} and the values for the sample DNA are recorded.
5. Repeat the same for other samples.

DNA concentration is estimated by Wallace formula:

Concentration (µg/ml) = (reading at A_{260}) × dilution factor × 50µg/ml

Note:

1. 1 OD_{260} Unit = 50µg/ml for double stranded DNA
2. 1 OD_{260} Unit = 40 µg/ml for single stranded RNA
3. 1 OD_{260} Unit = 33 µg/ml for single stranded DNA (ssDNA)
4. 1 OD_{260} Unit = 20 µg/ml for single stranded oligo (ssOligo)

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield (µg) = DNA concentration × total sample volume (ml)

DNA is not the only molecule that absorbs light at 260nm. RNA also absorbs light at 260nm, whereas aromatic amino acids present in protein absorbs light at 280nm. Presence of both RNA and protein contaminants in isolated DNA samples, will contribute to the total measurement at 260nm. The most common ratio of the absorbance for DNA purity calculation is measured at 260 and 280nm.

1. An absorbance of 1.0 at 260 nm corresponds to approximately 50 µg/ml of DNA.
2. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0.

3. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present.
4. If the ratio of A260/A280 is >2.0 = it means contamination with RNA (samples showing ratio greater than 2.0 requires RNA treatment).

Activity 3

Objective: Treatment of DNA samples contaminated with RNA

Introduction: For certain PCR applications (such as quantitative PCR or qPCR), the use of gDNA template should be as pure as possible. The RNase A is used to remove RNA during the isolation procedures of genomic DNA. The enzyme is very active under a wide range of reaction conditions and difficult to inactivate. However, under high salt concentrations (>300 mM NaCl), the RNase A specifically cleaves single-stranded RNA.

Material required:

RNase A enzyme (10.0mg/ml), centrifuge, 37 °C incubator or water bath

Methodology:

1. If the isolated genomic DNA is contaminated with RNA then add about 4-5 µl enzyme RNase A (Sigma R4875-500 MG [Ribonuclease A type 1A from Bovine Pancreases] to DNA sample dissolved in TE buffer in the ratio of 10:1 i.e., 10µg DNA with 1 µg RNAase (10.0mg/ml).

2. Centrifuge tubes at 12000-13000 rpm for 30secs and incubate for 1 hour at 37 °C for complete RNA digest.
3. Add iso-propanol (500µl in each Eppendrof) and repeat all the steps used for DNA isolation till addition of TE buffer.
4. Check the samples on agarose gel.

Trouble shoot for plant genomic DNA

S. No.	Problem	Probable Cause	Solution
1	Carbohydrate contamination in the sample	Grinding of the midrib along with the leaf material	Remove the midrib from the leaf before grinding, removal of the midrib is not important in case of very young leaves.
2	DNA appears degraded (as a smear running down the gel)	DNA appears fragmented or broken	DNA being a large molecule can be broken by shear forces if treated vigorously. Therefore mix the samples gently, never vortex the DNA. To minimize shearing always use a wide bore pipette tip for mixing

3	Difficult to dissolve DNA in Elution Buffer	This is due to over-drying of DNA pellet	The DNA should not be allowed to over-dry at any stage during the preparation as it hinders the resuspension and solubilization in elution buffer
4	Low DNA yield	Incomplete lysis	Decrease the amount of starting material. Be sure to add Proteinase K during lysis. Increase the length of incubation at room temperature.
5	A_{260}/A_{280} of genomic DNA sample is < 1.7 0r > 1.9	Ratio of < 1.7 means protein may be present and ration of > 1.9 means RNA may be present	To remove protein contaminants treat the samples with proteinase K or phenol extraction followed by EtOH precipitation and proper washing of the pellet. For RNA contamination RNase can be used, however for some application this RNA doesn't interfere.

Experiment 8: **Polymerase Chain Reaction (PCR)**

Introduction: The polymerase chain reaction (PCR) is a laboratory technique for DNA amplification that allows a "target" DNA sequence to be selectively amplified in vitro. PCR can use the smallest sample of the DNA to be cloned and amplify it to millions of copies in just few hours. Discovered in 1985 by Kerry Mullis, PCR has become both and essential and routine tool in most biological laboratories.

Ingredients of PCR:

1. **DNA template** that will be copied.
2. **Primers** are short stretches of single stranded DNA (often 20-30 base pairs), which are complementary to the 3' ends of the sense and anti-sense strands of the target sequence.
3. **dNTPs (Deoxynucleotide triphosphates)** are building blocks of DNA and are needed to construct the new strand of DNA.
4. **Taq DNA polymerase** is a thermostable enzyme, which adds new DNA bases to synthesize new strand of DNA complementary to the offered template strand.
5. **Buffer** provides optimal conditions for DNA denaturation and renaturation; also important for polymerase activity, stability and fidelity. It also ensures the right condition for the reaction.

Procedure of PCR:

All the PCR components are mixed together and are taken through series of 3 major cyclic reactions conducted in an automated, self-contained thermocycler machine. These three processes of thermal cycling are repeated 20-40 times to produce lots of copies of the DNA sequence of interest.

1. **Denaturation or stand separation** (94-95 °C): At this stage the cocktail containing the template DNA and other ingredients is heated at 94-95 °C. During the process high temperature breaks the hydrogen bonds between the bases in two strands of template DNA and separates the two strands. This single strands act as template for the production of the new strands of DNA. It is important that the temperature is maintained at this stage for long enough to ensure that the DNA strands have separated completely. This step involves initial heating for 4-5 min and cyclic denaturation for 30s-1 min.
2. **Annealing of primers** (50-65 °C): During this stage the reaction is cooled to 50-65 °C. This enables the primers to attach to a specific location on the single-stranded template DNA by way of hydrogen bonding (the exact temperature depends on the melting temperature of the primers you are using). Primers serve as the starting point for DNA synthesis. The polymerase enzyme can only add DNA bases to a double strand of DNA. Only once the primer has bound can the polymerase enzyme attach and start making the new complementary strand of DNA from the loose DNA bases. The two separated strands of DNA are complementary and run in opposite directions (from one end - the 5' end – to the other - the 3' end); as a result, there are two primers – a forward primer and a reverse primer. This step usually takes about 45 seconds to 1 minute.

3. **Extension/elongation stage** (72-80 °C): During this final step, the heat is increased to 72 °C to enable the new DNA to be made by Taq DNA polymerase enzyme which adds DNA bases. It attaches to the primer and then adds DNA bases to the single strand one-by-one in the 5' to 3' direction. The result is a brand new strand of DNA and a double-stranded molecule of DNA.

Dilution of DNA Samples:

We generally dilute the DNA samples to 10-25 ng/ µl for PCR analysis.
Example: If **stock concentration** of DNA is say 2.0 µg/ µl
That means 2000 ng/ µl
Working concentration: say 25 ng/ µl is required, then

N1	X	V1	=	N2	X	V2
2000 ng	X	?	=	25	X	100 µl
			= 2500 ÷ 2000			
			= 1.25 µl			

So, add 1.25 µl DNA from stock and add 98.75 µl nuclease water or TE

Dilution of primers:

Stock preparation:

Based upon the instructions given by the company from which oligos are synthesized add the appropriate volume of either TE buffer (pH 8.0) or Tris (pH 7.5) or nuclease free water to the mother stock. Spin the vials and allow them to stand overnight in refrigerator. And finally store at -20 °C

This will give you concentration of primer as = 100 pmole/µl (100 µM)

For example: Consider a primer **X** with an amount of 32.1 nmol, then add about 321 µl of TE/Tris/nuclease free water to X primer (mother stock). This gives a concentration of 100pmole/µl or 100 µM to X primer.

Working concentration:

Thaw the primers (mother stock) and spin for second so that all the content pool at the bottom.

From this take 1µl of primer and add 9 µl of TE/Tris/nuclease free water (1:9 ratio), which will be 10 pmole/µl.

Note: Always use ice while working with primers.

PCR reaction mix and PCR programme:

Details of PCR programme for different marker system

	ISSR	RAPD	SCoT	SSR
Initial denaturation	95 °C (4 min)	95 °C (5 min)	94 °C (5 min	95 °C (5 min)
Denaturation	95 °C (30s)	95 °C (45s)	94 °C (1 min	95 °C (45s-1min)
Annealing	As per primer Tm (1 min)	36 °C (1 min)	45-50 °C (1 min)	As per primer Tm (1 min)
Extension	72 °C (2 min)	72 °C (2 min)	72 °C (2 min)	72 °C (1 min)
Final extension	72 °C (10min)	72 °C (8 min	72 °C (10 min)	72 °C (10 min
Hold	4 °C (∞)	4 °C (∞)	4 °C (∞)	4 °C (∞)
Cycles	43	45	45	35
Reaction volume (µl)	**25**	**25**	**25**	**20**

PCR components and PCR reaction

S. No.	PCR components	Stock conc.	Working conc.	Vol. of reagents	
				20 µl reaction	**25µl reaction**
1.	PCR Buffer (15mM $MgCl_2$)	10x	1x	2.0µl	2.5µl
2.	$MgCl_2$	25mM	2.5mM	1.6 µl	2.0 µl
3.	dNTPs	10mM	0.2mM	0.4 µl	0.5 µl
4.	Primer	10µM	0.2µM	0.4 µl	0.5 µl
5.	Taq Polymerase	5U/ µl	1U	0.2 µl	0.3 µl
6.	Mili Q H_20	-	-	13.4 µl	17.2 µl
7.	Template DNA	25 ng/ µl	50 ng	2.0 µl	2.0 µl
	TOTAL			**20.0 µl**	**25.0 µl**

Note: Always use ice and wear gloves while working on PCR.

Experiment 9: **Isolation of Genomic DNA from E. coli**

Aim: To isolate the genomic DNA from E .coli DH5α cells.

Principle: The isolation and purification of DNA from cells is one of the most common procedures in contemporary molecular biology and embodies a transition from cell biology to the molecular biology (from in vivo to in vitro). The isolation of DNA from bacteria is a relatively simple process. The organism to be used should be grown in a favorable medium at an optimal temperature, and should be harvested in late log to early stationary phase for maximum yield. The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) is used to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol.

Materials Required:

- LB Broth
- E. coli DH5α cells
- TE buffer (pH 8.0)
- 10% SDS
- Proteinase K
- Phenol-chloroform-isoamyl alcohol mixture (25:24:1)
- 5M Sodium Acetate (pH 5.2)
- Isopropanol
- 70% ethanol
- Autoclaved Distilled Water
- Eppendorf tubes 1.5/2 ml
- Micropipette
- Microtips
- Microfuge

Preparation of Reagents:

1. TE BUFFER (pH 8.0): 10 mm Tris HCl (pH 8.0), 1 mm EDTA (pH 8.0)

2. 10% SDS: Dissolve 10 g of SDS in 100 ml autoclaved distilled water.

3. PROTEINASE K: Dissolve 10 mg of Proteinase K in 1 ml autoclaved distilled water.

4. PHENOL – CHLOROFORM MIXTURE: The pH is very important. For RNA purification, the pH is kept around pH 4, which retains RNA in the aqueous phase preferentially. For DNA purification, the pH is usually 7 to 8, at which point all nucleic acids are found in the aqueous phase. Mix equal volume

of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it on ice.

5. 5M SODIUM ACETATE: Dissolve 41 g of sodium acetate in 100 ml distilled water and adjust pH with dilute acetic acid (pH 5.2). 6. ISOPROPANOL 7. 70% ETHANOL.

PROCEDURE:

- 2 ml overnight culture is taken and the cells are harvested by centrifugation for 10 minutes
- 875 µl of TE buffer is added to the cell pellet and the cells are resuspended in the buffer by gentle mixing.
- 100 µl of 10% SDS and 5 µl of Proteinase K are added to the cells.
- The above mixture is mixed well and incubated at 37º C for an hour in an incubator.
- 1 ml of phenol-chloroform mixture is added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4º C.
- The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube.
- The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
- 100 µl of 5M sodium acetate is added to the contents and is mixed gently.
- 2 ml of isopropanol is added and mixed gently by inversion till white strands of DNA precipitates out. ¾ The contents are centrifuged at 5,000 rpm for 10 minutes.
- The supernatant is removed and 1ml 70% ethanol is added.
- The above contents are centrifuged at 5,000 rpm for 10 minutes.
- After air drying for 5 minutes 200 µl of TE buffer or distilled water is added.
- 10 µl of DNA sample is taken and is diluted to 1 or 2 ml with distilled water.
- The concentration of DNA is determined using a spectrophotometer at 260/280 nm.
- The remaining samples are stored for further experiments. PRECAUTIONS:
- Cut tips should be used so that the DNA is not subjected to mechanical disruption.
- Depending on the source of DNA the incubation period of Proteinase K should extended.
- The phenol chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA.
- DNase free plastic wares and reagents should be used.