

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/266885873>

# PLANT BIOTECHNOLOGY LAB MANUAL

## Article

---

CITATIONS

2

---

READS

449

## 1 author:



[Lingaraj Sahoo](#)

Indian Institute of Technology Guwahati

105 PUBLICATIONS 1,368 CITATIONS

SEE PROFILE

## Some of the authors of this publication are also working on these related projects:



1. Development of abiotic stress resilient tropical pulses through tailoring of ABA receptor genes [View project](#)

# PLANT BIOTECHNOLOGY LAB MANUAL



Dr. Lingaraj Sahoo

Department of Biotechnology  
Indian Institute of Technology Guwahati

## CONTENT

SERIAL NO.	EXPERIMENT	PAGE NUMBER
1	Aseptic culture techniques for establishment and maintenance of cultures	3-4
2	Preparation of stock solutions of MS basal medium and plant growth regulator stocks.	5-7
3	Micropropagation of Tobacco plant by leaf disc culture	8-9
4	Micropropagation of Rice by indirect organogenesis from embryo	10-11
5	Preparation of competent cells of <i>E. coli</i> for harvesting plant transformation vector	12-13
6	Transformation of competent cells of <i>E. coli</i> with plant transformation vectors.	14-14
7	Small scale plasmid preparation from <i>E. coli</i>	15-18
8	DNA check run by Agarose Electrophoresis	19-22
9	Restriction digestion of insert plasmid) and binary vector	23-24
10	Electroelution of insert DNA from agarose gel slice.	25-25
11	Mobilization of recombinant Ti plasmid from common laboratory host ( <i>E. coli</i> ) to an <i>Agrobacterium tumefaciens</i> strain	26-27
12	<i>Agrobacterium tumefaciens</i> -mediated plant transformation	28-29
13	Direct DNA delivery to plant by Particle Bombardment	30-31
14	Isolation of plant genomic DNA by modified CTAB method	32-33
15	Molecular analysis of putative transformed plants by Polymerase Chain Reaction	34-35

# EXPERIMENT- 1

**AIM:** Aseptic culture techniques for establishment and maintenance of cultures

## **PRINCIPLE:**

### **Maintenance of aseptic environment:**

All culture vessels, media and instruments used in handling tissues as well as the explants must be sterilized. The importance is to keep the air surface and floor free of dust. All operations are carried out in laminar air-flow, a sterile cabinet. Infection can be classified in three ways:

1. The air contains a large quantity of suspended microorganisms in the form of fungal and bacterial spores.
2. The plant tissue is covered with pathogens on its surface.
3. The human body (a skin, breathe etc) carries several microorganisms.

In general, the methods of elimination of these sources of infection can be grouped under different categories of sterilization procedures:

1. Preparation of sterile media, culture vessels and instruments (sterilization is done in autoclave)
2. Preparation of sterile plant growth regulators stocks (by filter sterilization)
3. Aseptic working condition
4. Explants (isolated tissues) are sterilized using chemical sterilents, e.g.  $\text{HgCl}_2$  and  $\text{NaOCl}$ .

**Sterilization:** It follows that all the articles used in the plant cell culture must be sterilized to kill the microorganisms that are present.

**A. Steam or Wet sterilization (Autoclaving):** This relies on the sterilization effect of super-heated steam under pressure as in a domestic pressure cooker. The size of the equipment used can be as small as one litre or even as large as several thousand litres. Most instruments/ nutrient media are sterilized with the use of an autoclave and the autoclave has a temperature range of 115- 135<sup>0</sup>C. The standard conditions for autoclaving has a temperature of 121<sup>0</sup>C and a pressure of 15 psi (Pounds per square inch) for 15 minutes to achieve sterility. This figure is based on the conditions necessary to kill thermophilic microorganisms. The time taken for liquids to reach this temperature depends on their volume. It may also depend on the thickness of the vessel. The temperature of 121<sup>0</sup>C can only be achieved at 15 psi. The efficiency of autoclave can be checked in several ways:

The most efficient way is to use an autoclave tape. When the autoclave tape is autoclaved, a reaction causes dark diagonal strips to appear on the tape indicating that it is autoclaved.

### **Precautions:**

1. Excessive autoclaving should be avoided as it will degrade some medium components, particularly sucrose and agar breakdown under prolonged heating. Especially when under pressure and in an acidic environment. A few extremely thermoduraic microorganisms exist that can survive elevated temperature for sometime. But 15-30 minutes kill even those.
2. At the bottom of the autoclave the level of water should be verified.
3. To ensure that the lid of the autoclave is properly closed.
4. To ensure that the air- exhaust is functioning normally.

5. Not to accelerate the reduction of pressure after the required time of autoclaving. If the temperature is not reduced slowly, the media begin to boil again. Also the medium in the containers might burst out from their closures because of the fast and forced release of pressure.
6. Bottles, when being autoclaved, should not be tightly screwed and their tops should be loose. After autoclaving these bottles are kept in the laminar air-flow and the tops of these bottles are tightened on cooling.

**B. Filter sterilization:** Some growth regulators like amino acids and vitamins are heat labile and get destroyed on autoclaving with the rest of the nutrient medium. Therefore, it is sterilized by filtration through a sieve or a filtration assembly using filter membranes of 0.22  $\mu\text{m}$  to 0.45 $\mu\text{m}$  size.

**C. Irradiation:** It can only be carried out under condition where UV radiation is available. Consequently, its use is restricted generally to purchased consumables like petridishes and pipettes. UV lights may be used to kill organisms in rooms or areas of work benches in which manipulation of cultures is carried out. It is however, dangerous and should not be turned on while any other work is in progress. UV light of some wavelengths can damage eyes and skin.

**D. Laminar Airflow Cabinet:** This is the primary equipment used for aseptic manipulation. This cabinet should be used for horizontal air-flow from the back to the front, and equipped with gas corks in the presence of gas burners. Air is drawn in electric fans and passed through the coarse filter and then through the fine bacterial filter (HEPA). HEPA or High Efficiency Particulate Air Filter is an apparatus designed such that the air-flow through the working place flows in direct lines (i.e. laminar flow). Care is taken not to disturb this flow too much by vigorous movements. Before commencing any experiment it is desirable to clean the working surface with 70% alcohol. The air filters should be cleaned and changed periodically.

## EXPERIMENT- 2

**AIM:** Preparation of stock solutions of MS (Murashige & Skoog, 1962) basal medium and plant growth regulator stocks.

**PRINCIPLE:** The basal medium is formulated so that it provides all of the compounds needed for plant growth and development, including certain compounds that can be made by an intact plant, but not by an isolated piece of plant tissue. The tissue culture medium consists of 95% water, macro- and micronutrients, vitamins, aminoacids, sugars. The nutrients in the media are used by the plant cells as building blocks for the synthesis of organic molecules, or as catalysators in enzymatic reactions. The macronutrients are required in millimolar (mM) quantities while micronutrients are needed in much lower (micromolar,  $\mu\text{M}$ ) concentrations. Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions. Sugar is essential for *in vitro* growth and development as most plant cultures are unable to photosynthesize effectively for a variety of reasons. Murashige & Skoog (1962) medium (MS) is the most suitable and commonly used basic tissue culture medium for plant regeneration.

Plant growth regulators (PGRs) at a very low concentration (0.1 to 100  $\mu\text{M}$ ) regulate the initiation and development of shoots and roots on explants on semisolid or in liquid medium cultures. The auxins and cytokinins are the two most important classes of PGRs used in tissue culture. The relative effects of auxin and cytokinin ratio determine the morphogenesis of cultured tissues.

### MATERIALS:

- Amber bottles
- Plastic beakers (100 ml, 500 ml and 1000 ml)
- Measuring cylinders (500 ml)
- Glass beakers (50 ml)
- Disposable syringes (5 ml)
- Disposable syringe filter (0.22  $\mu\text{m}$ )
- Autoclaved eppendorf tubes (2 ml)
- Eppendorf stand
- Benzyl-aminopurine
- Naphthalene acetic acid

## INSTRUCTIONS:

### MS NUTRIENTS STOCKS

Nutrient salts and vitamins are prepared as stock solutions (20X or 200X concentration of that required in the medium) as specified. The stocks are stored at 4<sup>0</sup> C. The desired amount of concentrated stocks is mixed to prepare 1 liter of medium.

**Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473-497**

MS major salts	mg/1 L medium	500 ml stock (20X)
1. NH <sub>4</sub> NO <sub>3</sub>	1650 mg	16.5 gm
2. KNO <sub>3</sub>	1900 mg	19 gm
3. CaCl <sub>2</sub> .2H <sub>2</sub> O	440 mg	4.4 gm
4. MgSO <sub>4</sub> .7H <sub>2</sub> O	370 mg	3.7 gm
5. KH <sub>2</sub> PO <sub>4</sub>	170 mg	1.7 gm

MS minor salts	mg/1 L medium	500 ml stock (200X)
1. H <sub>3</sub> BO <sub>3</sub>	6.2 mg	620 mg
2. MnSO <sub>4</sub> .4H <sub>2</sub> O	22.3 mg	2230 mg
3. ZnSO <sub>4</sub> .4H <sub>2</sub> O	8.6 mg	860 mg
4. KI	0.83 mg	83 mg
5. Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.25 mg	25 mg
6. CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025 mg	2.5 mg
7. CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025 mg	2.5 mg

MS Vitamins	mg/1 L medium	500 ml stock (200X)
1. Thiamine (HCl)	0.1 mg	10 mg
2. Niacine	0.5 mg	50 mg
3. Glycine	2.0 mg	200 mg
4. Pyrodoxine (HCl)	0.5 mg	50 mg

Iron, 500ml Stock (200X)
Dissolve 3.725gm of Na <sub>2</sub> EDTA (Ethylenediaminetetra acetic acid, disodium salt) in 250ml dH <sub>2</sub> O. Dissolve 2.785gm of FeSO <sub>4</sub> .7H <sub>2</sub> O in 250 ml dH <sub>2</sub> O Boil Na <sub>2</sub> EDTA solution and add to it, FeSO <sub>4</sub> solution gently by stirring.

### **PLANT GROWTH REGULATOR STOCK**

The heat-labile plant growth regulators are filtered through a bacteria-proof membrane (0.22  $\mu\text{m}$ ) filter and added to the autoclaved medium after it has cooled enough (less than 60<sup>0</sup> C). The stocks of plant growth regulators are prepared as mentioned below.

<b>Plant Growth Regulator</b>	<b>Nature</b>	<b>Mol. Wt.</b>	<b>Stock (1 mM)</b>	<b>Soluble in</b>
Benzyl aminopurine	Autoclavable	225.2	mg/ ml	1N NaOH
Naphtalene acetic acid	Heat labile	186.2	mg/ ml	Ethanol

The desired amount of plant growth regulators is dissolved as above and the volume is raised with double distilled water. The solutions are passed through disposable syringe filter (0.22  $\mu\text{m}$ ). The stocks are stored at -20<sup>0</sup> C.



## EXPERIMENT- 3

**AIM:** Micropropagation of Tobacco plant by leaf disc culture.

**PRINCIPLE:** Plant cells and tissues are **totipotent** in nature i.e., every individual plant cell or tissue has the same genetic makeup and capable of developing along a "programmed" pathway leading to the formation of an entire plant that is identical to the plant from which it was derived. The totipotency of the plant cells and tissues form the basis for **in vitro cloning** i.e., generation or multiplication of genetically identical plants in *in vitro* culture. The ability to propagate new plants from a cells or tissues of parent plant has many interesting possibilities.

**Micropropagation** is used commercially to asexually propagate plants. Using micropropagation, millions of new plants can be derived from a single plant. This **rapid multiplication** allows breeders and growers to introduce new cultivars much earlier than they could by using conventional propagation techniques, such as cuttings. Micropropagation also can be used to establish and maintain **virus-free plant stock**. This is done by culturing the plant's apical meristem, which typically is not virus-infected, even though the remainder of the plant may be. Once new plants are developed from the apical meristem, they can be maintained and sold as virus-free plants.

Micropropagation differs from all other conventional propagation methods in that aseptic conditions are essential to achieve success. The process of micropropagation can be divided into four stages:

- 1. Initiation stage:** A piece of plant tissue (called an explant) is (a) cut from the plant, (b) disinfested (removal of surface contaminants), and (c) placed on a medium. A medium typically contains mineral salts, sucrose, and a solidifying agent such as agar. The objective of this stage is to achieve an aseptic culture. An aseptic culture is one without contaminating bacteria or fungi.
- 2. Multiplication stage:** A growing explant can be induced to produce vegetative shoots by including a cytokinin in the medium. A cytokinin is a plant growth regulator that promotes shoot formation from growing plant cells.
- 3. Rooting or preplant stage:** Growing shoots can be induced to produce adventitious roots by including an auxin in the medium. Auxins are plant growth regulators that promote root formation. For easily rooted plants, an auxin is usually not necessary and many commercial labs will skip this step.
- 4. Acclimatization:** A growing, rooted shoot can be removed from tissue culture and placed in soil. When this is done, the humidity must be gradually reduced over time because tissue-cultured plants are extremely susceptible to wilting.

Micropropagation has become more feasible with the development of growth media that contain nutrients for the developing tissues. These media have been developed in response to the needs of plant species to be multiplied. This laboratory exercise will use a growth medium (MS) that will contain the macronutrients, micronutrients, vitamins, iron and sucrose. A combination of cytokinin (BAP) and auxin (NAA) will be supplemented to basal medium (MS) for induction of multiple shoots from the leaf disc explant.

**MATERIALS:**

Beakers, Measuring cylinders, Conical flasks, Cotton plugs, Myoinositol, Sucrose, BAP (1mM stock), Agar Agar, Forceps, Blade Holder (No.3), Sterilized blades (No.11), NAA (1 mM stock), Micropipettes, sterilized microtips, cork borers, petridishes.

**INSTRUCTIONS:**

The shoot multiplication medium for tobacco leaf disc is MS basal + BAP (2.5  $\mu$ M) + NAA (0.5  $\mu$ M)

**Preparation of MS medium (1000 ml)**

- MS Major (20X)            50 ml
- MS Minor (200X)        5 ml
- MS Vitamin (200X)      5 ml
- Iron (200X)              5 ml
- Myoinositol              100 mg
- Sucrose                    30 gm (3%)

- Add BAP at this stage (Calculate, how much to add?)
- Make final volume to 1000 ml by double distilled water
- Set pH at 5.8
- Add agar agar 8 gm/L (0.8%), melt the agar agar in microwave oven
- Sterilize the media at 15 psi/121<sup>0</sup> C for 15 minutes
- After autoclaving, gently swirl the medium to mix the agar. When the agar is completely dissolved and mixed, the medium should appear clear and not turbid.
- Add filter sterilized NAA (desired amount, calculate?) once the temperature of the medium cools down to 60<sup>0</sup> C.

Cut the tobacco leaf into discs and culture tobacco leaf disc in the medium. Maintain the cultures under cool white fluorescent light in a 16 h photoperiod regime at 25 $\pm$ 2<sup>0</sup>C. Observe the cultures periodically.

## EXPERIMENT- 4

**AIM:** Micropropagation of Rice by indirect organogenesis from embryo.

**PRINCIPLE:** The regeneration of plants through an intermediate callus phase is termed as “Indirect regeneration”. The explants (meristematic tissue) dedifferentiate to form callus, an unorganized growth of dedifferentiated cells. Group of cells in callus reorganize to form meristemoid, similar to meristem tissue. Meristemoid redifferentiate to form shoot buds, which finally regenerate to plantlets.

This experiment will use a growth medium (MS) supplemented with 2,4-D (auxin) to induce callus. The whitish-friable calli will be selected for redifferentiation on MS medium containing the BAP (cytokinin). The healthy-growing calli with green spots will be subcultured on the fresh medium. The regenerating shoots will be transferred to basal medium for root induction.

### MATERIALS:

- Plastiware and glassware for medium preparation,
- MS stocks,
- 2,4-D,
- casein hydrolysate,
- culture vessels and
- rice seeds

### INSTRUCTIONS:

**Callus induction medium** from rice seeds: MS or N6 basal + 2,4-D (2.0 mg/L) + Casein hydrolysate (0.3-1.0 mg/L)

**Redifferentiation medium:** MS basal + BAP (3 mg/L)

**Rooting medium:** MS basal

#### A. Preparation of callus induction media

The carbon source in callus induction medium can be maltose or sucrose (30 g/L), and casein hydrolysate is used as an optional supplement. The concentrations are optimized for each variety. Usually, MS is used for rice var. *Indicas* and N6 for *Japonica*.

- Mix all the ingredients together (i.e. basal salt, carbon source, vitamins, hormones, etc.) in 700 ml ddH<sub>2</sub>O. Stir it until all they dissolve.
- Make final volume to 1000 ml by ddH<sub>2</sub>O
- Adjust the pH to 5.8, add agar agar and autoclave for 15 min.
- Dispense the media to sterile petridishes (20-25 ml each) inside laminar hood. Allow them to cool.

#### B. Dehulling, sterilization and plating of seeds

- Remove carefully the lemma and palea using forceps, avoiding any damage to the embryo.
- After dehulling, select the healthy and shiny seeds. Place them in a sterile flask and surface sterilize with 70% ethanol for 1-2 minutes. Rinse 3 times with sterile dH<sub>2</sub>O.
- Sterilize the seeds again in 50% Chlorox (Zonrox - a commercial bleach) for 25-30 minutes, preferably under vacuum or in a shaker. (A drop of Tween 20 or any surfactant can be added to enhance the effect of chlorox).

- Rinse 3-5 times with sterile dH<sub>2</sub>O to remove all of the chlorox. Place the seeds in sterilized filter paper for drying before plating.
- Put 10-15 seeds in each sterile petridish containing 30 ml of solidified callus induction medium and incubate them in the dark room for 30-40 days. Check the culture for contamination 3 days after inoculation, and every week thereafter.

### **C. Selecting calli for organogenesis**

- Select the embryogenic calli (whitish, globular, friable, dry, free of any differentiated structures such as root-like or shoot-like appearance).
- Transfer the healthy and growing embryogenic calli into MS regeneration media containing 3 mg/L BAP.

### **D. Regeneration and rooting**

- Transfer the healthy and growing embryogenic calli into MS regeneration media containing 3 mg/L BAP.
- Subculture the healthy and proliferating calli with green spots into culture bottles containing fresh regeneration media with same concentration of BAP.
- After one month, transfer the proliferated shoots (3-4 cm) to rooting media free or devoid of any hormone.
- Establish the rooted plantlets in pot containing soil.

## EXPERIMENT- 5

**AIM:** Preparation of competent cells of *E. coli* for harvesting plant transformation vector

**PRINCIPLE:** Most species of bacteria, including *E. coli*, take up only limited amounts of DNA under normal circumstances. For efficient uptake, the bacteria have to undergo some form of physical and/or chemical treatment that enhances their ability to take up DNA. Cells that have undergone this treatment are said to be COMPETENT.

The fact that *E. coli* cells that are soaked in an ice-cold salt solution are more efficient at DNA uptake than unsoaked cells, is used to make competent *E. coli* cells. Traditionally, a solution of  $\text{CaCl}_2$  is used for this purpose.

**MATERIALS:** LB medium (Liq.), 100 mM  $\text{CaCl}_2$  sol., 250 ml conical flask, 1.5 ml centrifuge tube, microtips and sterile polypropylene tubes

### INSTRUCTIONS:

1. Inoculate a single colony of *E. coli* (DH5 $\alpha$ ) and raise 2 ml culture in LB broth (no antibiotic) at 37<sup>0</sup> C for overnight at 180 rpm.
2. Inoculate 300  $\mu\text{l}$  (1%) of the overnight culture to 30 ml of LB medium (in a 250 ml conical flask) and leave it at 37<sup>0</sup> C for 3 to 4 hrs till it reaches an O.D. of 0.5 to 0.6 at 600 nm.
3. Transfer the culture to a sterile pre-chilled polypropylene tube and incubate in ice for 30 min.
4. Spin at 5000 rpm at 4<sup>0</sup> C for 5 min.
5. Discard the supernatant. Resuspend the cells into a fine suspension in the small volume of medium left behind and finally suspend the pellet in 30 ml of ice cold 100 mM  $\text{CaCl}_2$  gently and incubate in ice for 30 min.
6. Spin at 5000 rpm at 4<sup>0</sup> C for 5 min.
7. Discard the supernatant and resuspend the pellet very gently in 3 ml of ice-cold 100 mM  $\text{CaCl}_2$ . Take care to suspend the pellet gently as the cells become fragile after  $\text{CaCl}_2$  treatment. Dispense 200  $\mu\text{l}$  in each 1.5 ml centrifuge tube.
8. Store the competent cells in ice for atleast 30 min. before use

### QUESTINARE:

1. What is the role of  $\text{CaCl}_2$  solution in competent cell preparation?
2. How the competent cells are stored for future use?

**What is the role of CaCl<sub>2</sub> solution in competent cell preparation?**

- Divalent cations may shield the negative charges on DNA (from the phosphate groups) and on the outside of cell (from cell-surface phospholipids and lipopolysaccharide) so that the DNA come in close association with the cell
- Divalent cations cause the DNA to precipitate onto the outside of the cells, get attached to the cell exterior
- They may help to recognize the lipopolysaccharides away from the channels, they normally guard

**How the competent cells are stored for future use?**

Add 30% of 50% ice-cold glycerol (supplied) to the final volume of 100 mM CaCl<sub>2</sub>. Pipette mix. Do not vortex. Dispense 200 µl in each eppendorf tube and store at -70<sup>0</sup> C.

## EXPERIMENT- 6

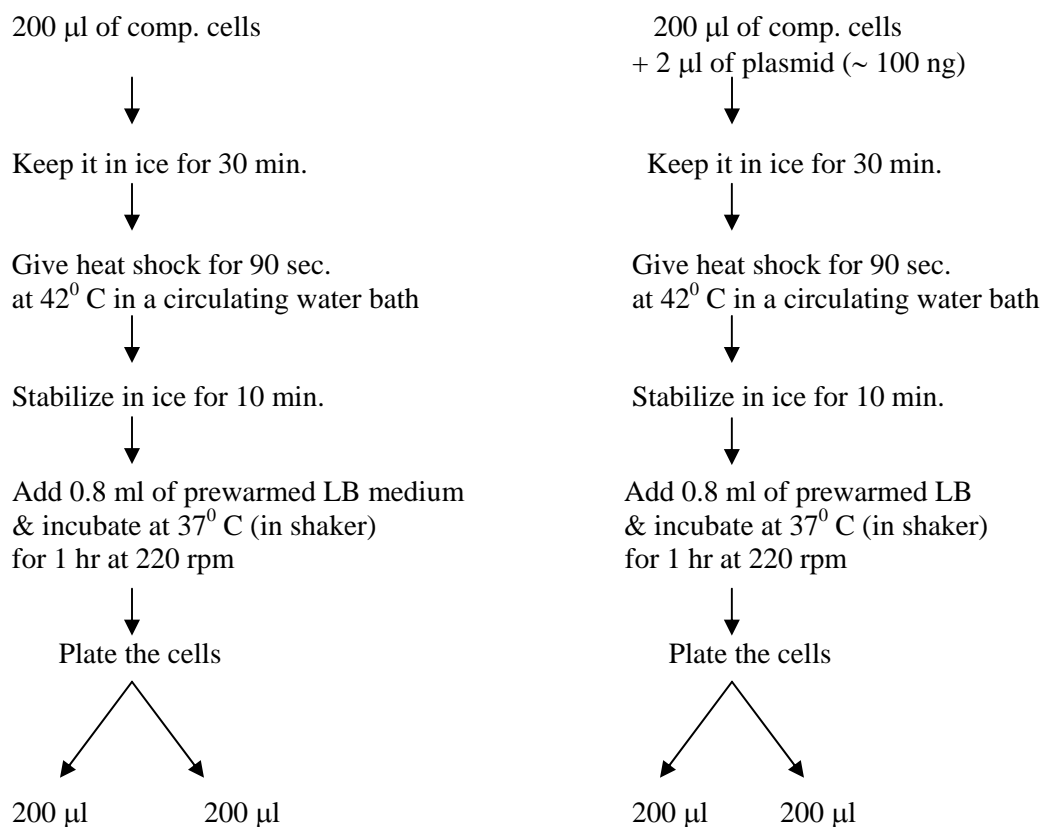
**AIM:** Transformation of competent cells of *E. coli* with plant transformation vectors.

**PRINCIPLE:** Transformation is broadly means uptake of any DNA molecule (plasmid) by living cell (bacteria). *E. coli* cells that are soaked in an ice-cold salt solution are more efficient at DNA uptake than unsoaked cells. Soaking in  $\text{CaCl}_2$  solution affects only DNA binding, and not the actual uptake into the cell. The actual movement of DNA into competent cells is stimulated by briefly raising the temperature to  $42^\circ\text{C}$  (HEAT SHOCK TREATMENT).

**MATERIALS:** Competent cells (200  $\mu\text{l}$ ), plant transformation vectors (~100 ng), LB medium (Liq. and solid), appropriate antibiotics, sterile petridishes and sterile microtips

### INSTRUCTIONS:

Take two aliquots of 200  $\mu\text{l}$  of competent cells (one as control and the other to be transformed) and thaw them in ice.



Incubate the plates at  $37^\circ\text{C}$  overnight (approx. 16 hrs.)

### QUESTINARE:

1. How does the heat shock aid in movement of DNA to the competent cells?

## EXPERIMENT- 7

**AIM:** Small scale plasmid preparation from *E. coli*

### PRINCIPLE:

Alkaline lysis plasmid miniprep is a procedure developed by Birnboim and Doly in 1979 (1) used to prepare bacterial plasmids in highly purified form. This method is used to extract plasmid DNA from bacterial cell suspensions. Plasmids are relatively small extrachromosomal supercoiled DNA molecules while bacterial chromosomal DNA is much larger and less supercoiled. Therefore, the difference in topology allows for selective precipitation of the chromosomal DNA, cellular proteins from plasmids and also RNA molecules. Under alkaline conditions, both nucleic acids and proteins denature. They are renatured when the solution is neutralized by the addition of potassium acetate. Chromosomal DNA is precipitated out because the structure is too big to renature correctly; hence plasmid DNA is extracted efficiently in the solution.

Previous works have shown that between pH 12.0-12.5, only linear DNA denatures (1). Supercoiled DNA remains and can then be purified. Birnboim and Doly employed this principle to develop alkaline lysis plasmid miniprep. According to the Molecular Cloning: A Laboratory Manual by Sambrook and Russell (2), the cells that contained the plasmids are treated with lysozyme, a protein discovered by Alexander Fleming in 1922 (3), which has the ability to weaken the cell wall. The cells are then lysed completely with sodium dodecyl sulfate (SDS) and NaOH. This is achieved by careful determination of the ratio of cell suspension to NaOH solution that allows a reproducible alkaline pH value without monitoring with a pH meter. Glucose is also used as a pH buffer to control the pH. Chromosomal DNA, which remained in a high molecular weight form, is selectively denatured. Acid sodium acetate is used to neutralize the lysate as the mass of chromosomal DNA renatures and coagulates to form an insoluble pellet. At the same time, high concentrations of sodium acetate also results in the precipitation of protein-SDS complexes and high molecular weight RNA. By now, three major contaminants: chromosomal DNA, protein-SDS complexes and high molecular weight RNA can be removed by spinning in a microcentrifuge. In order to recover plasmid DNA in the supernatant, ethanol precipitation is carried out. A mini prep usually yields 5-10 µg. This can be scaled up to a midi prep or a maxi prep, which will yield much larger amounts of DNA (or RNA). A gel electrophoresis analysis is conducted to verify the results.

Although plasmid minipreparation allows us to work with purified forms of DNA, contaminants (proteins) are not completely removed. Therefore, a combination of phenol/chloroform treatment followed by ethanol precipitation could yield us with higher purity of plasmid DNA (4). Plasmid DNA will be found in the aqueous phase, denatured proteins are collected at the interface, and lipids are found in the organic phase. An equal volume of phenol/chloroform/isoamyl alcohol is added to the plasmid suspended in TE. The mixture is then vortexed and centrifuged vigorously to make sure that sufficient plasmid DNA is extracted from the solution. Following phenol/chloroform extraction, the aqueous layer containing the plasmid DNA is carefully removed to a second centrifuge tube to carry out ethanol precipitation. Ethanol is able to expose the negatively charged phosphates by depleting the hydration shell from the nucleic acids (4). Sodium acetate is then added as the positively charged sodium binds to the exposed phosphate groups to form a precipitate. Centrifugation then removes the ethanol to yield a DNA pellet. The pellet will then be exposed to the air to allow all ethanol to evaporate. Pure DNA pellets are clear and difficult to observe, therefore, careful handling is necessary to ensure that the product is



obtained. The plasmid DNA pellet can then be resuspended in TE or distilled water for storage. Sometimes, low molecular weight RNA molecules are also removed using DNase-free RNase A to obtain a highly purified plasmid DNA.

### **MATERIALS:**

- Overnight grown bacterial culture
- Sterile eppendorf tubes
- Sterile microtips
- Micropipette
- Solution I, II and III
- RNase
- Phenol: chloroform: isoamyl alcohol
- Isopropanol
- Sodium acetate
- Ethanol
- TE buffer

### **INSTRUCTIONS:**

Grow 2 ml culture with appropriate antibiotic for 4-5 hrs at 37<sup>0</sup> C in a shaker till log phase (Check for the turbidity of the culture)



Take 1.5 ml culture from each tube in an eppendorf tube (1.5 ml), spin at 10 K for 2 min., remove the supernatant, spin down the rest of 3 ml culture in the same eppendorf tube, 1.5 ml at a time. (Final culture spun, 4-5 ml)



Resuspend the cells in 100 µl of **Solution I** (Tris, EDTA, Glucose) (Suspend well by vigorous vortexing)



Immediately add 200 µl of freshly prepared **Solution II** (0.4 N NaOH and 2% SDS, 1:1). Mix by inverting the tube.



Add 150 µl of **Solution III** (ice cold) to each tube, Mix by inverting, Spin at 12,000 rpm for 15 min.



Transfer the supernatant to a fresh tube (carefully by avoiding the interphase), add 5 µl of RNase (10 mg/ml) to each individual tube, mix by inverting, give a pulse spin, incubate at 37<sup>0</sup>C (water bath) for 1 hr



Add equal volume of phenol (200 µl) and then chloroform : isoamyl alcohol (200 µl), mix by vortexing vigorously (Do not vortex vigorously for plant genomic DNA)



Spin at 12,000 rpm for 15 mins.



Transfer the supernatant carefully to fresh eppendorf, add equal volume (400  $\mu$ l) of Propan –2– ol and then 0.1 volume (40  $\mu$ l) of Sodium acetate (pH 5.2) to each tube, mix by inversion, keep in  $-20^{\circ}\text{C}$  (Over night).



Spin at 12000 rpm,  $4^{\circ}\text{C}$  for 15 mins.



Discard the supernatant, add 200  $\mu$ l of ice cold 70% ethanol, mix by inverting, spin at 12000 rpm,  $4^{\circ}\text{C}$  for 5 mins



Discard the supernatant by using pipette, dry the pellet in Speed Vac for 2 min, 1200 rpm, ambient temp.



Dissolve the pellet in 40  $\mu$ l TE (10 mM Tris+1 mM EDTA), mix by tapping and give a short spin. Store at  $-20^{\circ}\text{C}$ .

## **REAGENTS:**

### **Solution I: 100 ml**

	(Mol. Wt)	(for 100 ml)
Tris (25 mM)	121.1	0.303 gm
EDTA (10 mM)	372.0	0.372 gm
Glucose (50 mM)	180.16	0.901 gm

Weigh the above salts and dissolve in 80 ml of dd water and adjust the pH 8.0 using 1 N HCl. Make up the volume to 100 ml. Autoclave and store at room temperature. (Do not over autoclave, glucose will be charred)

### **Solution II: (prepare fresh each time)**

NaOH	0.2 M
SDS	1.0%

Prepare 0.4 N NaOH and store in a plastic reagent bottle. Prepare 0.2% SDS and autoclave. Mix them in 1:1 ratio before use. Do not autoclave NaOH.

### **Solution III (3 M potassium acetate (pH 5.5))**

Weigh 29.4 gm of potassium acetate and dissolve in 25 ml to 30 ml double distilled water. Adjust the pH with glacial acetic acid and make up the volume to 100 ml. Autoclave and store at  $4^{\circ}\text{C}$ .

### **RNase**

Dissolve pancreatic RNase (Rnase A) at a concentration of 10 mg/ml (10 mM Tris pH 7.5, 15 mM NaCl), heat to  $100^{\circ}\text{C}$  for 15 min. in a boiling water bath (to denature Dnase). Allow to cool slowly to room temperature. Dispense into aliquots and store at  $-20^{\circ}\text{C}$ .

### **Phenol**

Melt phenol at  $65^{\circ}\text{C}$ , distill phenol without water circulation and collect between  $160^{\circ}\text{C}$  and  $182^{\circ}\text{C}$

**Chloroform : Isoamyl alcohol**

Prepare Chloroform: Isoamyl alcohol in 24:1 ratio.

**3M Sodium acetate (pH 5.2) 100 ml**

Weigh 24.61 gm of sodium acetate and dissolve in 80 ml of double distilled water. Adjust the pH with glacial acetic acid. Make up the volume to 100 ml. Autoclave it and store at 4<sup>0</sup>C.

**TE (0.1X) pH 8.0 100 ml:**

Tris HCl (1 mM) ----- 100 µl from 1 M stock (pH 8.0)

EDTA (0.1 M) ----- 20 µl from 0.5 M stock (pH 8.0)

Sterile double distilled water 98.8 ml.

**REFERENCES:**

1. Birnboim, H.C., J. Doly, (1979). 'A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA.' *Nucleic Acids Res* 7(6): 1513-1523
2. Sambrook, J., D. Russel. 'Molecular Cloning: A Laboratory Manual.' Cold Spring Harbour Laboratory Press 3rd Ed
3. <http://www.fordras.com/whatis.htm>
4. Serghini, M.A., C. Ritzenthaler, et al. (1989). 'A Rapid and Efficient Miniprep for Isolation of Plasmid DNA.' *Nucleic Acids Res* 17(9): 3604

## EXPERIMENT- 8

**AIM:** DNA check run by Agarose Electrophoresis

### PRINCIPLE:

Agarose gel electrophoresis separates DNA fragments according to their size. An electric current is used to move the DNA molecules across an agarose gel, which is a polysaccharide matrix that functions as a sieve to help "catch" the molecules as they are transported by the electric current.

The phosphate molecules that make up the backbone of DNA molecules have a high negative charge. When DNA is placed on a field with an electric current, these negatively charged DNA molecules migrate toward the positive end of the field, which in this case is an agarose gel immersed in a buffer bath. The agarose gel is a cross-linked matrix i.e., a three-dimensional mesh or screen. The DNA molecules are pulled to the positive end by the current, but they encounter resistance from this agarose mesh. The smaller molecules are able to navigate the mesh faster than the larger ones. This is how agarose electrophoresis separates different DNA molecules according to their size. The gel is stained with ethidium bromide so as to visualize these DNA molecules resolved into bands along the gel. Ethidium bromide is an intercalating dye, which intercalate between the bases that are stacked in the center of the DNA helix. One ethidium bromide molecule binds to one base. As each dye molecule binds to the bases the helix is unwound to accommodate the stain from the dye. Closed circular DNA is constrained and cannot withstand as much twisting strain as can linear DNA, so circular DNA cannot bind as much dye as can linear DNA.

Unknown DNA samples are typically run on the same gel with a "ladder." A ladder is a sample of DNA where the sizes of the bands are known. Unknown fragments are compared with the ladder fragments (size known) to determine the approximate size of the unknown DNA bands.

Approximately 10ng is visible in a single band on a horizontal agarose gel.

### MATERIALS:

- Agarose
- TBE buffer
- Gel casting tray, comb, power pack
- Sample DNA
- Loading dye
- Sterile microtips
- EtBr staining solution
- UV transilluminator or Gel Documentation System

### INSTRUCTIONS:

**For casting gel**, agarose powder is mixed with electrophoresis buffer (TBE) to the desired concentration, then heated in a microwave oven until completely melted. After cooling the solution to about 60<sup>0</sup>C, it is poured into a casting tray containing a comb and allowed to solidify at room temperature for nearly 45 min.

**After the gel has solidified**, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just immersed with buffer (TBE). DNA samples mixed with loading buffer are then pipeted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. The current flow is confirmed by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.

The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively.

When adequate migration (2/3 of the gel) has occurred, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on a ultraviolet transilluminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

#### **Preparation of 0.7% Agarose gel:**

Weigh 0.35 g agarose, add in 50 ml 1X TBE and melt agarose in a microwave oven for 2-3 min. Cool down to about 45 to 50<sup>0</sup> C (bearable warmth) and pour into the gel platform with the comb in position.

#### **Running gel:**

After solidification of the gel (approx. 45 min), place the gel in a gel tank with 1 X TBE buffer. Buffer should be filled to the surface of the gel. Load the samples in the well and run the gel at 60 V till the blue dye runs to the end.

#### **Staining the gel:**

Prepare staining solution by adding 10 µl of 10 mg/ml stock of Ethidium bromide in 100 ml of dd water. Place the gel in staining solution for 30 min and view the gel in UV transilluminator.

#### **Gel loading dye: 10X stock (10 ml)**

Bromophenol blue – 0.25%  
Ficoll – 25%

Weigh 25 mg of bromophenol blue and dissolve in 7 ml of sterile dd water, in a screw cap tube. Add 2.5 g of ficoll and dissolve completely (keep the tube in a shaker, overnight). Measure the volume using a pipette and make up to 10 ml using sdd water. Store at 4<sup>0</sup> C.

#### **10X TBE (pH 8.2): 1000 ml**

Tris – 107.78 g  
EDTA – 8.41 g  
Boric acid – 55 g

Dissolve in 600 ml of dd water. First allow the Tris to dissolve in water, then add EDTA. Make up the volume to one liter and autoclave. (Check and confirm the pH is about 8.2)

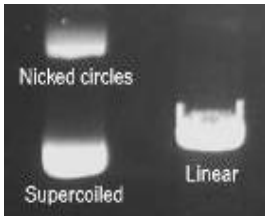
**Ethidium Bromide Stock:**

Stock 10 mg/ml. Working concentration 1 µg/ml.

**NOTES:**

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the  $\log_{10}$  of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the  $\log_{10}$  of either their molecular weights or number of base pairs, a roughly straight line will appear.

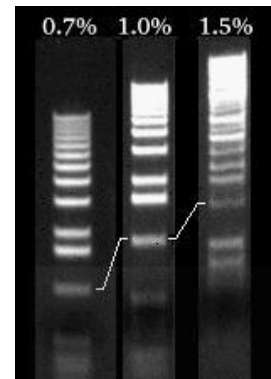
Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles. The image to the right shows an ethidium-stained gel with uncut plasmid in the left lane and the same plasmid linearized at a single site in the right lane.



Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:

**a. Agarose Concentration:** By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.

The image in the right shows migration of a set of DNA fragments in three concentrations of agarose, all of which were in the same gel tray and electrophoresed at the same voltage and for identical times. Notice how the larger fragments are much better resolved in the 0.7% gel, while the small fragments separated best in 1.5% agarose. The 1000 bp fragment is indicated in each lane.



**b. Voltage:** As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

**c. Electrophoresis Buffer:** Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

**d. Effects of Ethidium Bromide:** Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels, as shown by all the images on this page. As described above, it can be incorporated into agarose gels, or added to samples of DNA before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.

## EXPERIMENT- 9

**AIM:** Restriction digestion of pSIV (insert plasmid) and pRIN (binary vector)

### PRINCIPLE:

Restriction enzymes each have their own specific recognition site on double-stranded DNA, usually 6 to 8 bp in length and usually palindromic in sequence. These enzymes allow us to specifically cut DNA into fragments and manipulate them. Each restriction enzyme has a set of optimal reaction conditions, which are given in the catalogues supplied by the manufacturer. The major variables in the reaction are the temperature of incubation and the composition of the reaction buffer. Most companies supply 10x concentrates of these buffers with the enzymes. These 10x buffers are usually stored at  $-20^{\circ}\text{C}$ . Some enzymes also require a non-specific protein. Usually bovine serum albumin (BSA) is used for this and is also supplied as a concentrated solution.

One unit of enzyme is usually defined as the amount of enzyme required to digest 1  $\mu\text{g}$  of DNA to completion in 1 hour in the recommended buffer and temperature. In general, digestion for longer periods of time or with excess enzyme does not cause problems unless there is contamination with nucleases. Such contamination is minimal in commercial enzyme preparations. It is possible to minimize enzyme use (expensive reagent) by incubating for 2-3 hours with a small amount of enzyme.

### INSTRUCTIONS:

1. Calculate the amount of each component that your digest will require. Use the following chart as a reference order:

Order	Plasmid (vector) Digest	volume ( $\mu\text{l}$ )
3	Plasmid DNA (1 $\mu\text{g}$ )	
2	10X buffer	
1	Sterile water	
4	Restriction enzymes (10 units/ $\mu\text{g}$ DNA)	
<b>Total Volume</b>		<b><math>\mu\text{l}</math></b>

2. Using sterile pipette tips, add each component of the digest to a sterile microfuge tube. **The order of addition is important!** Put water in tube first, followed by buffer and DNA. **Add the enzyme last!!** Keep digest and enzyme on ice. Put enzyme back on ice or in freezer as quickly as possible. And make sure to use a clean tip for each addition.
3. Mix contents of tube by tapping with finger; microfuge briefly to bring contents to bottom of tube. Incubate reaction at appropriate temperature (usually  $37^{\circ}\text{C}$ ) for 1-3 hours, depending on amount of DNA and enzyme added.



**pSIV:**

Size of the pSIV = 7.551 kb

No. of HindIII sites = Two

Size of gene cassette (insert) = 4.887 kb

Size of the vector backbone = 2.664 kb

**pRIN:**

Size of the pRIN = 11.621 kb

No. of HindIII sites = One

Time duration of restriction digestion

Plasmid DNA = 4 hrs.

Order of digestion set up

- I. Sterilized double distilled water
- II. Plasmid DNA
- III. Buffer (10X)
- IV. Restriction enzyme (10 U/  $\mu\text{g}$ )

Add all the four components in order, tap, give a brief spin, and wrap parafilm around the cap of eppendorf tube. Incubate the tubes in waterbath at  $37^{\circ}\text{C}$  for 4 hours. Run a gel to confirm the digestion.

## EXPERIMENT- 10

**AIM:** Electroelution of insert DNA from agarose gel slice.

### PRINCIPLE:

The most popular method for the complete purification of DNA from agarose is electroelution. In the most straightforward form of electroelution, the band is excised from the gel and placed in a bag of dialysis membrane. This bag is then filled with electrophoresis buffer and placed in an electric field. The DNA migrates out of the gel slice and into the buffer, but it is too large to migrate out of the bag. Recovery is then just a matter of collecting the buffer from the bag and concentrating the DNA.

**MATERIALS:** Digested plasmid DNA, Activated dialysis bags, Dialysis clips, Flat shaped forcep, 0.5X TBE buffer, Sterilized dd. Water, Sterilized beaker and glass pipettes.

### INSTRUCTIONS:

1. Run the digested DNA sample and stain it with EtBr for approx. 30 min. View the gel using long wavelength 300-360 nm UV light (to minimize the DNA damage). Place the gel in the transilluminator over a plastic sheet and cut the gel slice with band of interest. Transfer into pretreated and washed dialysis bag (sealed one side with dialysis clip) filled with 0.5 X TBE.
2. Invert the bag with the gel piece so that only a minimal amount (200  $\mu$ l to 300  $\mu$ l) of 0.5 X TBE is remained in the bag. Care should be taken to avoid any air bubbles getting trapped in the bag.
3. Close the open end of the bag with another dialysis clip. Place the bag in a gel tank containing 0.5 X TBE. The bag should be completely immersed in the buffer. Run for 1 hr at 100 V. Visualize under long UV and ensure that DNA is completely eluted out of the gel and is attached to the dialysis bag.
4. Reverse the current and run for 20 sec at 100 V. Visualize under long UV. DNA attached to the dialysis membrane should come into the buffer. Squeeze gently.
5. Take out the bag and collect the solution completely in a microfuge tube.
6. Measure the volume and add 1/10<sup>th</sup> volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of 95 % ethanol. Mix well. Keep it at -20<sup>o</sup> C overnight.
7. Spin for 10 min at 4<sup>o</sup> C. Discard the supernatant. Add 500  $\mu$ l of cold 70 % ethanol and spin for 5 min at 4<sup>o</sup> C. Discard the supernatant.
8. Dry the pellet in a speed vac and dissolve in 10  $\mu$ l to 20  $\mu$ l of 0.1 X TE (pH 8.0)

## EXPERIMENT- 11

**AIM:** Mobilization of recombinant Ti plasmid (i.e. with gene of interest) from common laboratory host (*E. coli*) to an *Agrobacterium tumefaciens* strain.

**PRINCIPLE:** The plant transformation vectors are usually maintained in *E. coli* for the simple reasons i.e., due to ease of cloning and harvesting plasmids. Subsequently, the plant transformation vector with cloned gene of interest is mobilized to recipient *Agrobacterium* strain harboring *vir* plasmid.

The approach is simply to mate the donor strain (*E. coli* harboring Ti plasmid with gene of interest) with conjugal helper strain (*E. coli* harboring broad host range plasmid pRK2013) and recipient *Agrobacterium* strain (harboring *vir* plasmid). The Ti plasmid in *E. coli* is mobilized to recipient *Agrobacterium* strain due to the mobilization function of pRK2013 (broad host range plasmid). After mating, *Agrobacterium tumefaciens* strain harboring the engineered plant transformation vector (Ti plasmid with gene of interest) are selected by growth in the presence of antibiotics for which resistance is provided by genetic markers unique to those recipient *Agrobacteria* and Ti plasmid vector (Ti plasmid with gene of interest).

### MATERIALS:

- Donor *E. coli* strain harboring engineered Ti plasmid.
- Recipient *Agrobacterium tumefaciens* strain harboring *vir* plasmid.
- Conjugal helper strain i.e., *E. coli* harboring broad host range plasmid pRK2013.
- Inoculation loop
- LB plates, appropriate antibiotics
- 0.9 % NaCl (autoclaved)
- sterile eppendorf tubes

### INSTRUCTION:

The day on which triparental mating is performed is taken as Day 1.

#### Day: -4

Streak *Agrobacterium* strain on LB medium containing appropriate antibiotic and incubate at 28<sup>0</sup> C.

#### Day: -1

Streak *E.coli* harboring pRK2013 (helper strain) on LB medium containing kanamycin (50 mg/L) and incubate at 37<sup>0</sup> C.

Streak *E.coli* harboring plasmid of interest (donor strain) on LB medium containing appropriate antibiotic and incubate at 37<sup>0</sup> C.

#### Day: +1

Prepare one LB plate (without antibiotic). Take one colony each from *E.coli* pRK2013, *E.coli* harboring plasmid to be mobilized and *Agrobacterium tumefactions* (the recipient) with the help of loop, patch separately on the LB plate very close to each other. Mix the three colonies with a sterile loop and incubate the triparent mix at 28<sup>0</sup> C for 12 to 18 hrs.

**Day: +2**

Take six sterilized eppendorf tubes, add 0.9 ml of 0.9% sterile NaCl. Take the triparent scoop and perform serial dilution by taking 100  $\mu$ l from each dilution and plate on LB medium containing antibiotics for selection of donor and recipient plasmids. Incubate the plates at 28<sup>0</sup> C for 12-16 hrs.

**Day: +6**

At one or two dilutions, single colonies appear on LB medium which indicates that donor plasmid has been mobilized from *E. coli* to *Agrobacterium tumefactions* .

**Day: +7**

Streak six to eight single colonies of *Agrobacterium tumefactions* harboring the donor plasmid on appropriate antibiotic containing medium. Single colonies are seen after 4 days.

## EXPERIMENT- 12

**AIM:** *Agrobacterium tumefaciens*-mediated plant transformation.

**PRINCIPLE:** The pathogenic bacteria *Agrobacterium* have the capacity to transfer part of its plasmid DNA (called the T-DNA) into the nuclear genome of plants cells. Two types of *Agrobacterium* strains are used for plant genetic transformation. In the *A. tumefaciens* strains, the T-DNA genes encode oncogenes that will induce the formation of a tumor on the infected plant tissue. In the *A. rhizogenes* strains, the T-DNA genes encode oncogenes that will induce the production of adventitious roots called the hairy root tissue. This later is used to produce rapidly chimaeric plants with untransformed aerial part and transgenic roots cotransformed with the Ri T-DNA and the construct of interest.

The T-DNA transfer to the plant nucleus depends on the expression of the *Agrobacterium vir* genes that delimit the extent of the DNA sequence transferred to the nucleus, by recognizing specific sequences called T-DNA right and left borders (RB and LB). In between these borders any DNA sequence can be introduced and transferred into the plant genome. This forms the basis for the generation of transgenic plants.

For this, the oncogenes are deleted from the T-DNA and replaced by selectable marker gene and gene of interest. This T-DNA construct can be placed on another replicon (binary vector) than the *vir* genes, making the transformation system more versatile. The integration of the T-DNA in the genome probably depends on the plant DNA repair machinery. Generally one copy of the T-DNA is inserted randomly in the plant genome, and gene fusions studies indicated that these insertions preferably occur in transcribed regions or in their vicinity.

The steps involved are:

1. Infection of plant tissues with overnight grown *Agrobacterium* culture
2. Cocultivation
3. Post-cocultivation wash and Transient expression assay
4. Culture in selective medium
5. Selection of putative transformed plants
6. Molecular analysis of putative transformed plants

### MATERIALS:

- *In vitro* germinated seedlings
- *A. tumefaciens* culture
- Liquid plant growth medium
- Sterilized-petridishes
- Filter discs
- Mmicrotips
- GUS substrate
- Double distilled water

**WORKING PROTOCOL:**

1. Raise the desired *Agrobacterium* strain in 20 ml of LB medium with appropriate antibiotics, agitated overnight at 200 rpm at 28<sup>0</sup> C
2. Concentrate the cells at 5000 rpm for 5 min, resuspend the cells in liquid plant growth medium.
3. Prepare the explants. Submerge the explants in bacterial suspension for 10-20 min.
4. Blot-dry the explants and cocultivate them in tissue culture growth conditions for 2-3 days.
5. Wash the explants with sterile dd water to eliminate *Agrobacteria*.
6. Incubate few explants in GUS substrate (overnight in the dark at 37<sup>0</sup> C) after for detection of transient GUS expression.

**RESULT:** Strong expression of GUS (indigo blue color) was observed in the region of the explants from where the shoots developed. The endogenous GUS activity (color) was not detected in non-transformed (control) explants. GUS activity at the cut ends indicates the susceptibility of explants to *Agrobacterium* mediated transformation.

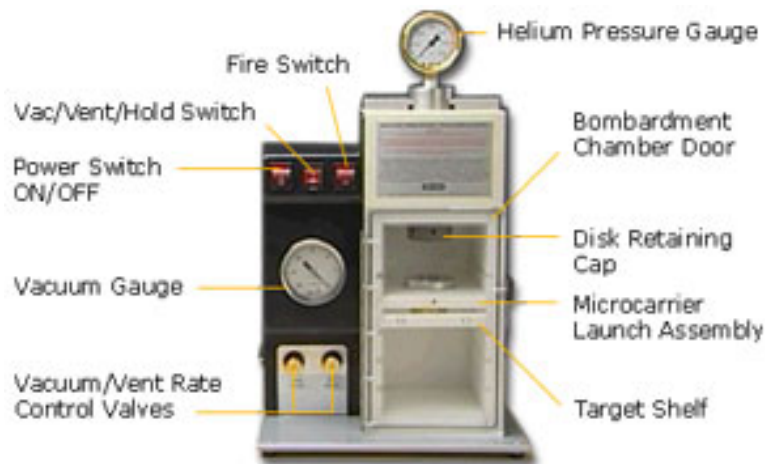
## EXPERIMENT- 13

**AIM:** Direct DNA delivery to plant by Particle Bombardment

**PRINCIPLE:** The fact that DNA could be delivered into plant cells by physical means and expressed in intact cells effectively, revolutionized genetic engineering of plants. Out of the available physical procedures for delivering DNA, particle bombardment is the most preferred method as it allows introduction of DNA directly into any plant cell type. With particle bombardment, the difficulties of using fragile protoplasts and host-range limitations associated with *Agrobacterium* are circumvented.

The basis of the particle bombardment process is the acceleration of DNA-coated microprojectiles (mainly particles of tungsten or gold, with 0.2 to 1.5  $\mu\text{m}$  in diameter) at high speed (about 1500 km/h) towards the living cells. After penetration in the cell, the DNA dissociates from the microprojectiles and integrates into the chromosome.

The BioRad PDS1000/He biolistic gun will be used for the said purpose, which is a gas (He) pressure-driven device. It uses plastic film as a macro carrier. The device is powered by a burst of helium gas that accelerates the supporting macrocarrier onto which DNA coated microcarriers are loaded. The pressure at which helium bursts is controlled by rupture disk (made up of kapton membrane). The rupture disk can be chosen such that helium could be allowed to burst at different pressures. Upon bursting of helium gas, the macrocarrier is instantly accelerated but is stopped by the stopping screen (metal perforated screen). The microcarriers then pass through the stopping screen due to their small size and move at desired velocity. The air in the chamber is evacuated by vacuum suction to reduce the air drag, which might slow down the velocity of the microcarriers.



**Biolistic Gun**

**MATERIALS:**

- Explants
- Microcarrier (gold particles)
- Plasmid DNA with reporter gene
- Macrocarrier
- Stopping screen
- Macrocarrier launch assembly
- Biolistic Gun

**INSTRUCTION:**

- Soak macrocarriers, holders, stopping screens, rupture disks in 95% ethanol for 15 min, then air dry.
- Coat plasmids over gold particle and prepare a suspension.
- Drip 6 ~ 10 µl of the suspension on to the macrocarrier.
- Open the valve on the steel cylinder, which contain the pressurized helium, rotate the black button (helium pressure regulator) to adjust the helium pressure (at least 200 psi higher than the desired pressure).
- After all the materials are in place, close the chamber door and apply vacuum.
- When appropriate vacuum is reached, activate the fire switch. The gas is held until the burst pressure of the rupture disk is reached.

**RESULT:** Strong expression of GUS (indigo blue color) was observed in the bombarded cells of the explants. The endogenous GUS activity (color) was not detected in non-transformed (control) explants i.e, explants bombarded with naked particles. GUS activity in the bombarded cells indicates the direct gene delivery to the target plant cells.



## EXPERIMENT- 14

**AIM:** Isolation of plant genomic DNA by modified CTAB method.

**PRINCIPLE:** The extraction of genomic DNA from plant material requires cell lysis, inactivation of cellular nucleases and separation of the desired genomic DNA from cellular debris. Ideal lysis procedure is rigorous enough to disrupt the complex starting material (plant tissue), yet gentle enough to preserve the target nucleic acid. The cetyltrimethylammonium bromide (CTAB) protocol (developed by Murray and Thompson in 1980) is appropriate for the extraction and purification of DNA from plants and plant derived foodstuff and is particularly suitable for the elimination of polysaccharides and polyphenolic compounds otherwise affecting the DNA purity and therefore quality.

Plant cells can be lysed with the ionic detergent CTAB, which forms an insoluble complex with nucleic acids in a low-salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away. The DNA complex is solubilised by raising the salt concentration and precipitated with ethanol or isopropanol.

**1. Lysis of cell membrane:** The first step of the DNA extraction is the rupture of the cell and nucleus wall. For this purpose, the **homogenized** sample is first treated with the extraction buffer containing EDTA, Tris/HCl and CTAB. The lysis of the membranes is accomplished by the detergent (CTAB) contained in the extraction buffer. When the cell membrane is exposed to the CTAB extraction buffer, the detergent captures the lipids and the proteins allowing the release of the genomic DNA. In a specific salt (NaCl) concentration, the detergent forms an insoluble complex with the nucleic acids. EDTA is a chelating component that among other metals bind magnesium. Magnesium is a cofactor for Dnase. By binding Mg with EDTA, the activity of present Dnase is decreased. Tris/HCl gives the solution a pH buffering capacity (a low or high pH damages DNA). After the cell and organelle membranes (such as those around the mitochondria and chloroplasts) have been broken apart, the purification of DNA is performed.

**2. Extraction:** In this step, polysaccharides, phenolic compounds, proteins and other cell lysates dissolved in the aqueous solution are separated from the CTAB nucleic acid complex. Under low salt concentration, the contaminants of the nucleic acid complex do not precipitate and can be removed by extraction of the aqueous solution with chloroform. The chloroform denatures the proteins and facilitates the separation of the aqueous and organic phases. Once the nucleic acid complex has been purified, precipitation can be accomplished.

**3. Precipitation:** In this final stage, the nucleic acid is liberated from the detergent. For this purpose, the aqueous solution is first treated with a precipitation solution comprising of Sodium acetate, which precipitates the nucleic acid. Under these conditions, the detergent, which is more stable in alcohol than in water, can be washed out, while the nucleic acid precipitates. The successive treatment with 70% ethanol allows an additional purification, or wash, of the nucleic acid from the remaining salt.

**MATERIALS:**

- Plant samples (leaf, callus etc.)
- Liquid nitrogen
- Sterile pestle and mortar
- Sterile spatulas
- Waterbath set at 65<sup>0</sup> C
- Sterile eppendorf tubes and desired reagents

**INSTRUCTION:****DNA EXTRACTION:**

1. Take 100 mg tissue, homogenate to powder with liquid nitrogen and transfer the powder to sterile eppendorf tube.
2. Add 1 ml of Extraction buffer (preheated at 65<sup>0</sup> C for 15 min.) to homogenate, mix vigorously and incubate in waterbath at 65<sup>0</sup> C for 1 hr.
3. Bring down the sample temperature to RT, add 667 µl chloroform:isoamyl alcohol (24:1), mix gently by inverting for a period of 15-20 min.
4. Spin at 10,000 rpm at 4<sup>0</sup> C for 10 min, collect the supernatant, add 2/3 ml of cold isopropanol. Mix gently to precipitate the nucleic acid. Spin for 5-10 min.
5. Wash with around 500 µl of 70% ethanol. Decant and dry the pellet at RT. Dissolve in 50 µl of 0.1X TE+ Rnase (100 µg/ml). Incubate 1 hr at 37<sup>0</sup> C.

**DNA PURIFICATION:**

6. Raise the volume of DNA sample to 250 µl with 0.1 X TE.
7. Add equal volume (250 µl) of chloroform:phenol:isoamyl alcohol (25:24:1), mix, keep for 5 min, spin for 15 min at 12000 rpm.
8. Take the upper layer (aqueous phase), add equal vol. of chloroform (to remove phenol), mix, spin for 15 min. Transfer the top layer in fresh tubes, measure the volume.
9. Add chilled absolute ethanol (1 ml), 1/10<sup>th</sup> vol. 3M sodium acetate (pH 5.2), keep in -20<sup>0</sup> C for 1 hr.
10. Spin for 10 min, perform ethanol (70%) wash, spin for 10 min, dry the pellet, dissolve in steril dd water.

**DNA CHECK RUN:**

11. Load 2-4 µl of isolated plant genomic DNA in 0.8% agarose gel and determine the quality and yield.

## EXPERIMENT- 15

**AIM:** Molecular analysis of putative transformed plants by Polymerase Chain Reaction.

**PRINCIPLE:** Detection of transgenes, which may not be being expressed at that time, can only be achieved by analysis of plant DNA. By their very nature, transgenes are novel, and can be distinguished from the surrounding host plant genome, but at the practical level this requires either some knowledge of the inserted DNA sequences.

The most common strategy employed for screening of transgene presence is PCR-based detection of transgenes followed by gel electrophoresis and comparison with standard samples. The process uses the enzyme Taq DNA Polymerase to amplify minute quantities of transgene DNA from plant material to a detectable level. A major advantage of a PCR-based detection-strategy is that it is extremely sensitive.

PCR is usually conducted in microtubes or microtitre plates, and reaction volumes vary from 10 to 100 $\mu$ l. The quantity of template DNA used also varies considerably. PCR reaction schemes differ with respect to times, temperatures, and numbers of amplification cycles, often for the same assay in different laboratories. Most PCR tests are assessed by agarose gel electrophoresis, and results are scored visually as the presence or absence of a DNA fragment of the appropriate size.

The quantity of template plant DNA used in the PCR ranges from 5 - 100ng. Primer concentrations used vary from 2 - 10.0  $\mu$ M. In practice, primer lengths are normally around 20 - 25 nucleotides (the shortest reported being 16). PCR reaction schemes are broadly similar, reaction times varying with the thermocycler used, and ramping rates being set as fast as possible. Primer annealing temperatures for most assays are standardised at approximately 55°. Most assays use 30-35 amplification cycles, although some labs use particular assays of 45-50 cycles. This may increase the sensitivity of the test, but care is necessary in these extended runs as the effect of minor contamination or PCR artefacts is significantly amplified. PCR results are assessed by gel electrophoresis (1.4 - 4.0% agarose).

A positive PCR result only means that a product has been successfully amplified, but the host plant DNA template may not necessarily be the source. Likewise, a negative PCR result only indicates that a product has not been amplified. It does not necessarily imply that the transgene is not present. These problems are addressed by the use of duplicate samples and appropriate controls. Each PCR run performed includes the following controls:

- Verified positive control
- Verified negative leaf sample
- No-DNA blank controls

### **MATERIALS:**

- Genomic DNA isolated from control plant (untransformed)
- Genomic DNA isolated from putative transformed plants
- PCR components
- Thermal Cycler

**INSTRUCTION:**

1. To perform several parallel reactions, prepare a master mix containing water, buffer, MgCl<sub>2</sub>, dNTPs, primers and *Taq* DNA Polymerase in a single tube, which can then be aliquoted into individual tubes.
2. Add the desired amount of master mix to the template DNA. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.
3. Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube.
4. Set the conditions in Thermal cycler, place the samples and start PCR.