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ज्ञानम् आत्म प्रदीपाय

Genetic Engineering 1
Practical Manual
Course No, CC-
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Dr. Prashant Shukla

Department of Biotechnology

School of Science and Technology

The Neotia University

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Aim: Demonstration on isolation of DNA

Materials Required: Extraction buffer, Microfuge tubes, Mortar and Pestle, Liquid Nitrogen, Absolute Ethanol (ice cold), 70 % Ethanol (ice cold), 7.5 M Ammonium Acetate 65° C water bath Chloroform : Iso Amyl Alcohol (24:1) Water (sterile), TE Buffer (10 mMTris, pH 8, 1 mM EDTA).

Composition of extraction buffer (For 5 ml)

component	Stock concentration	Final Concentration	Volume taken from stock
Tris	1M	0.1 M	500 µl
EDTA	0.5M	20 mM	200 µl
NaCl	5M	1.4 M	1.40 ml

+ CTAB 0.1g (2%) + PVP 0.1g (2%) (Heatat 65° Ctill dissolved)+ β-mercaptoethanol 10µl (0.2%)

Principle:

Isolation of DNA using CTAB exploits that polysaccharides and DNA have different solubilities in CTAB depending on the concentration of sodium chloride. At higher salt concentrations, polysaccharides are insoluble, while at lower concentrations DNA is insoluble. Consequently, by adjusting salt concentration in lysates containing CTAB, polysaccharides and DNA can be differentially precipitated.Polyphenols are compounds that contain more than one phenolic ring (e.g., tannin), a structure that binds very efficiently to DNA. They are naturally occurring in plants, but are also generated when plants have tissue damage (browning). Upon the homogenization of plant tissues, polyphenols are synthesized by liberated polyphenol oxidase. The addition of polyvinyl pyrrolidone prevents the interaction of DNA and phenolic rings by binding up the polyphenols.

Procedure:

1. Weigh 200 mg leaves.

2. Crush with liquid nitrogen.
3. Add 1 ml extraction buffer.
4. Mix well and incubate at 65oC for 30 min.
5. Cool down to room temperature.
6. Add equal volume of chloroform :isoamylalcohol (24:1).
7. Centrifuge at 10000 rpm for 10 min. at 4˚C.
8. Take out upper aqueous phase in fresh tube.
9. Add 0.6 volume of chilled isopropanol.
10. Incubate at -20o C for 1 hour.
11. Centrifuge at 12000 rpm for 15 min. at 4˚C.
12. Discard supernatant and add 1ml 70% ethanol.
13. Centrifuge at 10000 rpm for 10 min. at 4˚C.
14. Discard supernatant and air dry pellet at room temperature.
15. Add 50 µl of TE (10:1) and store at 4˚C for overnight.

Result:

Precautions:

- Material finely ground in liquid nitrogen should be immediately transferred into the extraction buffer.
- In chloroform :isoamyl alcohol extraction, the aqueous phase should be carefully removed and organic phase re-extracted to ensure full recovery of DNA. If no separation is observed between the two phases, may be due to high concentration of DNA and /or cell debris in aqueous phase, dilution with more digestion buffer and re-extraction is the solution.
- Care should be taken to do the operations as gently as possible. Vortexing, pipetting using fine tips etc. should be avoided to prevent the shearing of DNA.
- DNA should not be over dried as resuspension in TE become difficult.
- All the glassware, plastic ware, pestles and mortars etc. should be decontaminated properly. Care should be taken to prevent cross-contamination.
- Blank extraction controls are carried out along with normal extractions to check for any contamination.

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 $CaCl_2$ is used for this purpose.

MATERIALS: LB medium (Liq.), 100 mM $CaCl_2$ sol., 250 ml conical flask, 1.5 ml centrifuge tube, microtips and sterile polypropylene tubes

INSTRUCTIONS:

1. Inoculate a single colony of *E. coli* (DH5a) and raise 2 ml culture in LB broth (no antibiotic) at 37^0 C for overnight at 180 rpm.

2. Inoculate 300 ml (1%) of the overnight culture to 30 ml of LB medium (in a 250 ml conical flask) and leave it at 37^0 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 4^0 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 $CaCl_2$ gently and incubate in ice for 30 min.

6. Spin at 5000 rpm at 4^0 C for 5 min.

7. Discard the supernatant and resuspend the pellet very gently in 3 ml of ice-cold 100 mM $CaCl_2$. Take care to suspend the pellet gently as the cells become fragile after $CaCl_2$ treatment. Dispense 200 ml in each 1.5 ml centrifuge tube.

8. Store the competent cells in ice for atleast 30 min. before use

What is the role of $CaCl_2$ 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 $CaCl_2$. Pipette mix. Do not vortex. Dispense 200 ml in each eppendorf tube and store at -70^0 C.

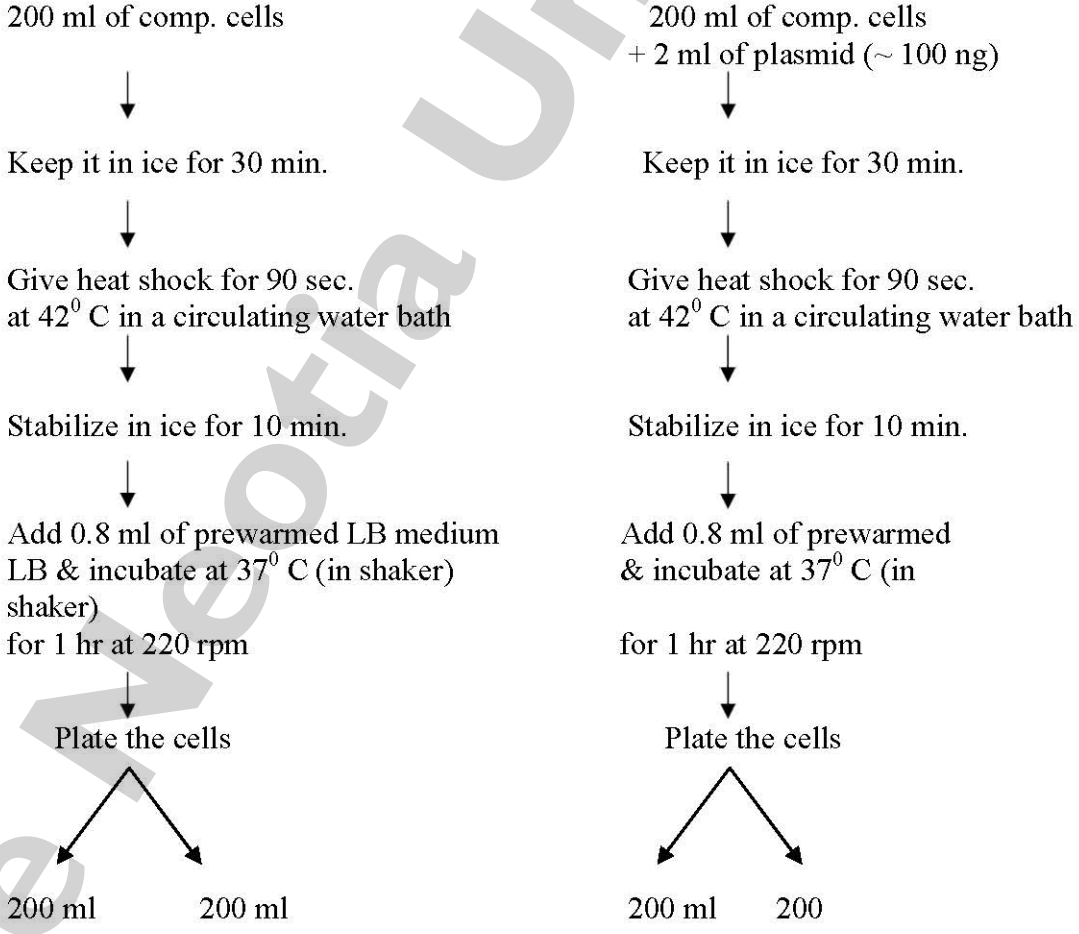
AIM: Transformation of competent cells of *E. coli* with plant transformation vectors.

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 $CaCl_2$ 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 42^0 C (HEAT SHOCK TREATMENT).

MATERIALS: Competent cells (200 ml), plant transformation vectors (~100 ng), LB medium (Liq. and solid), appropriate antibiotics, sterile petridishes and sterile microtips

INSTRUCTIONS:

Take two aliquots of 200 ml of competent cells (one as control and the other to be transformed) and thaw them in ice.



ml Incubate the plates at 37^0 C overnight (approx. 16 hrs.)

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 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 mg. 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 37^0 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 ml of **Solution I** (Tris, EDTA, Glucose) (Suspend well by vigorous vortexing)

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

Add 150 ml 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 ml of RNAse (10 mg/ml) to each individual tube, mix by inverting, give a pulse spin, incubate at 37^0C (water bath) for 1 hr

Add equal volume of phenol (200 ml) and then chloroform : isoamyl alcohol (200 ml), 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 ml) of Propan –2– ol and then 0.1 volume (40 ml) of Sodium acetate (pH 5.2) to each tube, mix by inversion, keep in
-20^0C (Over night).

–

Spin at 12000 rpm, 4^0 C for 15 mins.

–

Discard the supernatant, add 200 ml of ice cold 70% ethanol, mix by inverting, spin at 12000 rpm, 4^0C 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 ml TE (10 mM Tris+1 mM EDTA), mix by tapping and give a short spin. Store at -20^0 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 4^0 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 100^0C for 15 min. in a boiling water bath (to denature Dnase). Allow to cool slowly to room temperature. Dispense into aliquots and store at -20^0C.

Phenol

Melt phenol at 65^{0}C, distill phenol without water circulation and collect between 160^0 C and 182^0 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^{0}C.

TE (0.1X) pH 8.0 100 ml:

Tris HCl (1 mM)---------- 100 ml from 1 M stock (pH 8.0)
EDTA (0.1 M)---------- 20 ml from 0.5 M stock (pH 8.0)
Sterile double distilled water 98.8 ml.

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 intercalcating 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 60^0C, 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 occured, 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 50^0 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 ml 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 4^0 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)

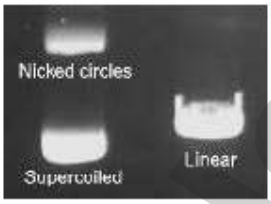
Ethidium Bromide Stock:

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

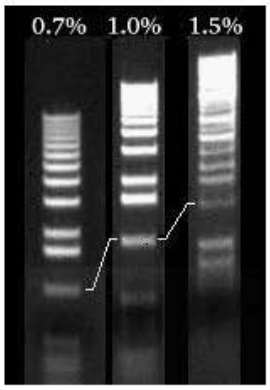


Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:

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

The image in the below 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 that 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.

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 -20^0C. 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 mg 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 (ml)
3	Plasmid DNA (1 mg)	
2	10X buffer	
1	Sterile water	
4	Restriction enzymes (10 units/mg DNA)	
Total Volume		**ml**

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 37^0C) 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/ mg)

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 37^0 C for 4 hours. Run a gel to confirm the digestion.

Aim: Qualitative and quantitative analysis of DNA by UV spectrometry

Principle

Analysis of UV absorption by the nucleotides provides a simple and accurate estimation of the concentration of nucleic acids in a sample. Purines and pyrmidines in nucleic acid show absorption maxima around 260nm (eg., dATP: 259nm; dCTP: 272nm; dTTP: 247nm) if the DNA sample is pure without significant contamination from proteins or organic solvents. The ratio of OD_{260}/OD_{280} should be determined to assess the purity of the sample. This method is however limited by the quantity of DNA and the purity of the preparation. Accurate analysis of the DNA preparation may be impeded by the presence of impurities in the sample or if the amount of DNA is too little. In the estimation of total genomic DNA, for example, the presence of RNA, sheared DNA etc. could interfere with the accurate estimation of total high molecular weight genomic DNA.

Procedure

1. Take 1 ml TE buffer in a cuvette and calibrate the spectrophotometer at 260nm as well as 280nm.
2. Add 10 ml of each DNA sample to 900ml TE (Tris-EDTA buffer) and mix well.
3. Use TE buffer as a blank in the other cuvette of the spectrophotometer.
4. Note the OD_{260} and OD_{280} values on spectrophotometer.
5. Calculate the OD_{260}/OD_{280} ratio.
6. The amount of DNA can be quantified using the formula:
   DNA concentration (mg/ml) = $\frac{OD_{260} \times 100 \text{ (dilution factor)} \times 50 \text{ mg/ml}}{1000}$

Inferences:

- A ratio between 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids.
- A ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers.
- A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (<1.8 or >2.0) it is advisable to re-precipitate the DNA.

Spectrophotomteric Conversions for Nucleic Acids:

1 A 260 of ds DNA = 50 mg/ml
1 A 260 of ss oligonucleotides = 33 mg/ml
1 A 260 of ss RNA = 40 mg/ml

Quantitative estimation of DNA

100 mg of calf thymus DNA was dissolved in 100ml distilled water (1mg/ml Primary stock solution) then pipette out 1ml Primary stock solution of and make up the volume upto 10ml with distilled water. Now prepared different dilution ranging from 20-100µg/ml. The absorbance was measured at 260 nm by using UV-Spectrophotometer. In this method, the absorbance of the unknown sample in a 1-cm cuvette was measured at

260 and 280 nm. The A260\A280 values were determined

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 100µl. 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 µM. 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 Cycle

INSTRUCTION:

1. To perform several parallel reactions, prepare a master mix containing water, buffer, $MgCl_2$, 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.