LABORATORY MANUAL

for

5-Year Integrated *(B.Sc. - M. Sc.)* Botany

PLANT BIOTECHNOLOGY

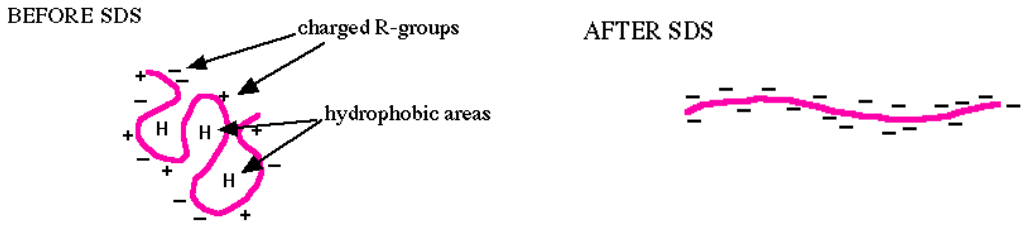
DEPARTMENT OF BOTANY

UNIVERSITY OF KASHMIR, NORTH CAMPUS, DELINA, BARAMULLA

Protein Quantification – SDS-PHAGE

SDS – PAGE:

SDS (sodium dodecyl sulfate) is a detergent that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it. Therefore, if a cell is incubated with SDS, the membranes will be dissolved and the proteins will be soluablized by the detergent, plus all the proteins will be covered with many negative charges. So a protein that started out is the one shown in before SDS will be converted into the one shown in after SDS of figure. The end result has two important features: 1) all proteins contain only primary structure and 2) all proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.



If the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. So we need to put the proteins into an environment that will allow different sized proteins to move at different rates. The environment of choice is polyacrylamide, which is a polymer of acrylamide monomers. When this polymer is formed, it turns into a gel and we will use electricity to pull the proteins through the gel so the entire process is called polyacrylamide gel electrophoresis (**PAGE**). A polyacrylamide gel is not solid but is made of a laberynth of tunnels through a meshwork of fibers.

Concentration, molecular weight & intactness of the purified crystal protein is checked by SDS PAGE

1. To know presence of the protein of expected size based on protein marker (molecular weight).
2. To analyse the protein profile of given sample.

3. To isolate and determine molecular mass of crystal proteins of Bt strains and to know about the intactness of Bt proteins.

Principle

1. To separate and characterize proteins by applying electric current.

Reagents

- **Tris-SDS pH 8.8:-** 1.5M Tris SDS pH 8.8-100ml

  Take 18.2g of Tris with 50ml of single distilled water and adjust the pH to 8.8 with conc. HCl then add 0.4g of SDS and make up the volume to 100ml.

- **Tris-SDS pH 6.8:** 0.5M Tris SDS pH 6.8-100ml

  Take 6.055g of Tris with 50ml of single distilled water and adjust the pH to 6.8 with conc. HCl then add 0.4g of SDS and make up the volume to 100ml.

- **Acrylamide Mix:**

  Take 29g of Acrylamide + 1g of Bis Acrylamide in 50ml of single distilled water, dissolve the entire content thoroughly and make up the volume to 100ml.

- **Running Buffer:- 1000ml of 10X - stock**

  0.25M Tris : 30 g of Tris is dissolved in 100ml of single distilled water,

  1.92M Glycine : 144 g is dissolved in the contents thoroughly

  10% SDS : 10g

10X. Running buffer. Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of **SDS** in 1000 ml of H_2O. The pH of the **buffer** should be 8.3 and no pH adjustment is required. Store the **running buffer** at room temperature and dilute to 1X before use.

1X. Running Buffer: (100ml)

10 ml of 10X buffer is diluted with 90 ml of distilled water

- **APS 10%**

  0.1g of APS (ammonium persulphate) is dissolved in 1ml distilled water and vortex it completely. **It should be prepared freshly.**

- **0.8% Agarose**

0.8 g of Agarose is dissolved in 100 ml of distilled water and boiled completely.

- **Sample loading buffer: (4X)**

0.25M Tris HCl pH 6.8 (2.5ml from 0.5M Tris-SDS actually using for SDS PAGE)
8% SDS – 0.8g in 100 ml
40% glycerol - 4ml
0.5% Bromophenol blue – 0.05g

Add all the ingredients step by step and then finally add 1.5ml of water → this should be dispensed in cryovials 1ml each, while using this dye add 200µl / 1ml of sample loading buffer.

Staining solution

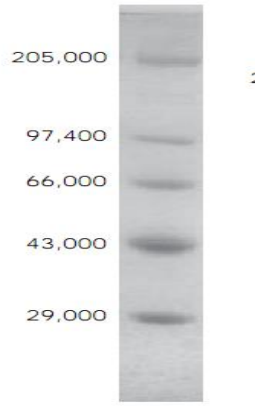
Coomassie Brilliant Blue (R250)
25mg in 100ml of destaining solution

Destaining solution: (for 100ml)

Double distilled water - 50ml
Methanol - 40 ml
Glacial acetic acid - 10 ml

High molecular weight marker (range 29 –205 Kda)

Myosin	205 **Kda**
Phosphorylase	97 **Kda**
Bovine (BSA)	68 **Kda**
Ovalbumin	43 **Kda**
Carbonic anhydrase	29 **Kda**



Steps in preparing a PAGE Gel

Gel sealing, Gel casting (separating and stacking), Well cleaning, Gel loading, Staining, Destaining

Gel Sealing

1. The gel glass plates are cleaned thoroughly with water followed by alcohol.

2. The plates are assembled by placing the appropriate spacer at sides and then sealed with cellophane tape at the sides and bottom.
3. For sealing 0.8% Agarose is used at each side.

Gel casting

The gels are casted as per the recipe given in the tables.

1. Separating 2. Stacking

Separating: 9%

1. Separating gel should be prepared accordingly based on the given composition.
2. The separating gel is overlaid with a film of alcohol to accelerate polymerization.
3. After polymerization, the alcohol layer is removed and blotted with filter paper.

Separating: - (7.5 cm) 7.5ml – (1mm spacer) – 7.5ml

Stacking: 4%

1. Stacking gel (always 4%) should be prepared based on the given composition.
2. The stacking gel is poured and the comb is placed on top of the sandwich and allows it to polymerize for 20- 30 min.
3. After polymerization the comb is carefully removed from the slots stacking gel.
4. Stacking: - (1mm spacer) – 3ml
5. The stacking gel is poured and the comb is placed on top of the sandwich.
6. After 20-30 min the comb is carefully removed from the slots after polymerization of the stacking gel.
7. **Well cleaning:** Slots are rinsed with electrode buffer before loading the samples and damage of wells should be noted by releasing the airflow through syringe.
8. **Gel loading:** *Bt* – crystal protein are mixed with 4x loading dye boiled for 1 min 45 sec. and then loaded.

Sl. No.	Sample Name	Sample Volume (µl)	Sample buffer (µl)	Water (µl)	Total (µl)
1.	HMW	2	5	13	20
2.	Clone	4	5	11	20
3.	Indigenous	4	5	11	20

Gel running

1. The gel is initially run at constant current of 15mA till the dye front reached the separating gel.
2. The current supply is increased to a constant supply of 25mA till dye reaches bottom.

Staining:

After the completion of electrophoresis, the gel unit is dismantled and separating gel alone is stained overnight in a staining solution.

Destaining:

Destain the overnight stained gel till the background become colourless and document for records.

Electrophoresis of proteins in polyacrylamide gels is carried out

1. in buffer gels (non-denaturing) relies on both the charge and size
2. as well as SDS gels (denaturing) gels of the protein
3. Chain Initiator – TEMED (Tetramethyl ethylene diamine) High conc. Of TEMED - the rate of polymerization is also high.
4. SDS – Sodium dodecyl sulphate (is an anionic detergent which binds strongly to and denatures proteins
5. Heat sample solutions in boiling water for 2-3 min to ensure complete interaction between proteins and SDS (protein binding)
6. Sample buffer – Bromophenol Blue → facilitate easy loading of the samples
7. Stacking gel helps concentration of the samples. Proteins absorb the Coomassie brilliant blue.
8. Destaining: Dye that is not bound to proteins is thus removed.
9. Acrylamide + Bis Acrylamide → Polymerization

SDS-PAGE

Separating Gel

Percentage and Composition	**Big gel (9cm)**			**Small gel (7.5cm)**	
	1mm wide spacer	1.5mm wide spacer	1 mm narrow spacer	1mm wide spacer	1.5mm wide spacer
	10 ml	**15 ml**	**23 ml**	**7.5 ml**	**11.5 ml**
8%					
Water	4.7 ml	7.05 ml	10.81 ml	3.525 ml	5.405 ml
Acrylamide mix	2.7 ml	4.05 ml	6.71 ml	2.025 ml	3.105 ml
Tris-SDS pH 8.8	2.5 ml	3.75 ml	5.75 ml	1.875 ml	2.875 ml
APS 10 %	0.1 ml	0.15 ml	0.23 ml	0.075 ml	0.115 ml
TEMED	0.006 (6µl)	0.009 (9 µl)	0.0138(14µl)	0.0045 (5µl)	0.0069 (7µl)
9%					
Water	4.356 ml	6.534 ml	10.018 ml	3.267 ml	5.069 ml
Acrylamide mix	3.037 ml	4.556 ml	6.98 ml	2.278 ml	3.490 ml
Tris-SDS pH 8.8	2.5 ml	3.75 ml	5.75 ml	1.875 ml	2.875 ml
APS 10 %	0.1 ml	0.15 ml	0.23 ml	0.075 ml	0.115 ml
TEMED	0.006 (6µl)	0.009 (9 µl)	0.0138(14µl)	0.0045 (5µl)	0.0069 (7µl)
10%					
Water	4.1 ml	6.15 ml	9.43 ml	3.075 ml	4.715 ml
Acrylamide mix	3.3 ml	4.95 ml	7.59 ml	2.475 ml	3.795 ml
Tris-SDS pH 8.8	2.5 ml	3.75 ml	5.75 ml	1.875 ml	2.875 ml
APS 10 %	0.1 ml	0.15 ml	0.23 ml	0.075 ml	0.115 ml
TEMED	0.006 (6µl)	0.009 (9 µl)	0.0138(14µl)	0.0045 (5µl)	0.0069 (7µl)

Stacking Gel

Percentage and Composition	**Big gel (9cm)**			**Small gel (7.5cm)**	
	1mm wide spacer	1.5mm wide spacer	1 mm narrow spacer	1mm wide spacer	1.5mm wide spacer
	3 ml	**5 ml**	**7.5 ml**	**3 ml**	**4.5 ml**
Water	2.10 ml	3.500 ml	5.25 ml	2.10 ml	3.15 ml
Acrylamide mix	0.50 ml	0.833 ml	1.249 ml	0.50 ml	0.75 ml
Tris-SDS **pH 6.8**	0.38 ml	0.633 ml	0.949 ml	0.38 ml	0.57 ml
APS 10 %	0.030 ml	0.050 ml	0.075 ml	0.030 ml	0.045 ml
TEMED	0.003 (3µl)	0.005 (5µl)	0.0075 (8µl)	0.003 (3µl)	0.0045 (5µl)

Note:

10% APS should be prepared freshly,

Tris–SDS pH should be 8.8 and 6.8 for separating and stacking gel respectively.

QUANTIFICATION OF PROTEIN BY LOWRY'S METHOD

Principle

The blue colour developed by the reduction of the phosphomolybdic–phosphotungstic compounds in the Folin ciocalteau reagent by the amino acids tyrosin and tryptophan present in the protein plus, the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method.

Reagents

Reagent A : 2% sodium carbonate in 0.1 N NaOH.

Reagent B : 0.5% $CuSO_4$. $5H_2O$ (copper sulfate) in % potassium sodium tartrate.

Reagent C : Alkaline copper solution: 50 ml of A and 1 ml of B prior to use.

Reagent D : Folin – ciocalteau reagent –1X from 2X.

Protein solution (Stock standard)

50 mg bovine serum albumin is dissolved in distilled water and made upto 50 ml in standard flask.

Working standard

2.5 ml of the stock solution is diluted to 25 ml with distilled water (one ml of this solution contain 100 µg of protein).

Procedure

- A measured quantity of the solubilized protein is taken in a clean test tube and the volume is made upto 1.0 ml with distilled water.
- 5.0 ml of reagent C is added to the above and is mixed well.
- After 10 min, 0.5 ml of reagent D is added and mixed well.
- The contents are incubated in dark for 30 min, after which absorbance is taken at 660 nm in a spectrophotometer.
- The standard graph is prepared in the range of 0-100 µg (intervals of 10 µg) of BSA and the amount of solubilized protein present per ml of sample is calculated from this graph.

Isolation of genomic DNA from gram positive strains

1. Inoculate a single colony of Bacterium into 5 ml LB broth and incubate over night in a shaker at 180 rpm, 30°C.
2. Harvest the cells from 2 ml culture by centrifugation at 5000 X g (~8000rpm) for 5 min.
3. Discard the supernatant and wash the cells with washing (5000 X g (~8000rpm) for 5 min) solution 1 ml of TES
4. Then suspend in 600 µl of resuspension solution containing 1 mg / ml lysozyme and incubated at 37°C for 1h.
5. Add 150 µl of 10% SDS and incubated at 50°C for 15 min.
6. Add 250 µl of 5M NaCl and incubated at 50°C for 5 min followed by overnight incubation.
7. Centrifuge the cell extract at 10000 X g (~12000rpm) for 5 min at 4°C, DNA in the supernatant is precipitated with equal volume of ice cold-isopropanol and dry the pellet.
8. Dissolve the pellet (DNA) in 100 µl of TE buffer containing 1mM NaCl and 10 µg of RNase per ml and incubate at 37°C for 30 min.
9. Add Proteinase K (0.6 mg/ ml) to the mixture and incubated at 37 °C for 30 min.
10. Extract the mixture with equal volume of phenol and chloroform (1:1) at 10000 X g for 5 min at 4°C.
11. Precipitate the DNA with 200 µl of absolute ethanol and 1/10th volume of 3M sodium acetate (pH 5.2) by incubating at 70˚C for 30 minutes and centrifuge at 10000 X g for 5 min. wash the pellet with 70 per cent ethanol and air dry.
12. Dissolve the dried pellet with 50-100 µl of TE buffer and stored at –20°C for further use.

TES solution :

Tris-HCl (pH 8.0)	10 mM
EDTA	1 mM
Sodium Chloride	100 mM

Resuspension solution

Tris - HCl	25 mM
Sucrose	25%
EDTA	25 mM
Lysozyme	1mg / ml

TE Buffer

Tris (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

10% SDS 10g in 100 ml of water
5M NaCl 29.22 g in 100ml of water

Agarose Gel Electrophoresis

Conc. of Agarose	Preparation	Use
0.8%	Take 0.8gm of agarose in 100ml 1x TAE	Genomic DNA & Plasmid DNA conc. checking, restriction digested product, elution, Conc. checking of eluted product.
1.2%	Take 1.2gm of agarose in 100ml 1x TAE.	Checking PCR product of size >1000bp
2%	Take 2gm of agarose in 100ml 1x TAE	Checking PCR product of size <1000bp

Procedure:

1. Mix required amount of Agarose with 1x TAE buffer melt in a microwave oven
2. The mixture cooled to 60ºC (Luke warm temperature)
3. Add Etbr at 0.5 µg/ml & mix gently to avoid bubble formation
4. Now pour the mixture into gel mould containing comb
5. Allow the Agarose to solidify. (15-20min)

Operation of electrophoresis unit:

Agarose gels of 0.5 per cent and 0.8 to 1.2 per cent are used to analyze the DNA samples for plasmid profile and quantification respectively. 1X TAE buffer is poured into the gel buffer reservoir in the electrophoresis apparatus to about 2 mm above gel surface. DNA samples are mixed with loading dye in 3: 1 ratio and loaded into respective gel slots. Electrophoresis is carried out at **10V for 20h to analyze plasmid profile** or **to quantify DNA at 60V for 1-2h.** The gel is examined in the trans illuminator using UV light and documented using Gel documentation system.

Polymerase Chain Reaction (PCR)

Calculation of annealing temperature:

Calculate annealing temperature for both forward and reverse primers using formula

Annealing temp = 2°C les than least calculated Tm

Tm=4(G+C) +2(A+T)

Example:

Forward primer: ATGAACACCGTGCTCAACAAC

Tm=4(10) +2(11) = 40+22=62°C

Reverse primer: TGGTACTTGAAGAGGGACCAG

Tm=4(11) +2(10) = 44+20=64°C

Annealing temp = 62-2 = 60°C

PCR cocktail preparation:

For 1X reaction:

Ingredients	**Working concentration**	**Volume(µl)**
Forward primer	50 µg	1 µl
Reverse primer	50 µg	1 µl
25mM dNTP mix	1mM	0.75 µl
10X Taq buffer	1X	2.5 µl
Sterile water	--	4.25µl
Taq polymerase	1U	0.5 µl
Total		10 µl

PCR programme:

Steps	**Temperature**	**Time**
Initial denaturation	94 ºC	2min
Denaturation	94 ºC	40sec
Annealing	Calculated Tm for respective primers	45sec
Extension	72 ºC	1min/2min based on size of the product. <1000bp-1min,1000-2000bp-2min, Rule: increase 1 min for every 1000 bp
Cycles 29		
Final extension	72 ºC	7min

Materials required

DNA ladders
Loading Dye

Loading of PCR product= 5 µl of PCR product+3 µl loading dye (2x) + 4 µl water

Loading of GENOMIC/PLASMID DNA/eluted product

2 µl of DNA + 3 µl loading dye (2x) +4 µl water

Note: Final concentration of dye in the well should be 1X. Based on that add the required amount of DYE.

Preparation of competent cells of *E. coli*: calcium chloride (CaCl2) method

Materials required:

LB broth

100 mM $CaCl_2$

85% glycerol

Procedure:

- Inoculate single colony of *E.coli* into 5ml of LB broth and keep it in shaker overnight (16hrs-18hrs).
- Reinoculate 1% of overnight grown culture (250µl) into 25ml LB broth in 250ml flask and grow with (200rpm) shaking at 37 ^oC for about 3hrs (OD 0.4 to 0.6 at 600 nm)
- Transfer the culture to 50ml polypropylene (centrifuge) tubes and keep on ice for 20min.
- Pellet the cell suspension by centrifugation at 5000rpm for 10min at 4^o C.
- Discard the supernatant, resuspend the pellet with 10ml of ice cold CaCl2 and keep it in ice for 20min.
- Again centrifuge at 5000rpm for 10 min at 4^{o}C.
- Discard the supernatant; suspend the pellet with 1.7ml ice-cold CaCl2+300µl of ice cold 85% glycerol.
- Make aliquots of 100µl and store immediately in -80^o C

Transformation of *E. coli* with recombinant plasmid by heat shock method

Transformation Procure

- Take aliquot of 100 µl of competent cells, keep it in ice till it thawed to liquid.
- Add 5-10 µl of plasmid DNA/ligated mixture, mix gently and keep on ice for 30min.
- Subject the cells to heat shock at 42º C for 1 min twice with interval of 10 sec in ice
- Transfer immediately to ice. Keep it in ice for 5min without any disturbance.
- Add 900 µl of LB/SOC broth and incubate at 37 º C with shaking for 2-3hrs.
- (For control use competent cells without plasmid or Ligated product others are same above)
- Spread plate 50, 100 and 150 µl of cells on LB agar medium containing Appropriate antibiotics

Plating:

	LB agar plates with Antibiotics	LB agar plates without Antibiotics
Competent cell with ligated mixture	Positive	Positive
Competent cell without ligated mixture	Negative	Positive

Alpha complementation test

Alpha complementation test:

To select transformed colonies of *E.coli* carrying the insert and to eliminate the colonies with self ligated

Materials required:

X-Gal preparation: 20mg/ml

0.1M IPTG

Procedure:

- Spread 40µl of 0.1M IPTG and 40µl of x-gal simultaneously on agar plates containing Appropriate antibiotics
- Wrap the plates with aluminium foil and incubate at 37°C for one – two hours.
- Select recombinant colonies of E.col randomly from the LB+antibiotics plates and short straek with sterile tooth pick or yellow tips on the x-gal IPTG plates.
- Again wrap the plates with aluminium foil and incubate at 37°C for 12-16hrs.
- Then the plates stored at 4°C for 4hrsto allow further color development.

Screening of recombinant colonies by colony PCR:

Divide LB amp plates at the back with marker into small boxes and short streak white colonies. Incubate over night at 37 °C for 12-16hr. well grown culture used as template for PCR.

Expression of genes in *E. coli* (BL21)

Expression genes in *E. coli* (BL21)

1. Inoculate single colony of the recombinant *E. coli* harbouring the pET2A plasmid into 5ml LB broth containing kanamycin 50 ug/ µl and allow to grow at 37^0C for overnight.
2. Inoculate 1% of the overnight grown culture (250 µl) to the 25 ml LB broth containing kanamycin 50 ug/ µl in 250 ml conical flask and allow to grow at 37^0C until absorbance of 0.6 at 600 nm.(3hrs)
3. Add Isopropyl b-D-thiogalactopyranoside (IPTG) to each flask at a final concentration of 1 mM.
4. Allow the Cells to grow further at 30^0C for another 6 hrs till it reaches an optical density of 1.3 at 600 nm.
5. Centrifuge the culture at 12000 rpm for 10 min at 4ºC.
6. Resuspend the cells in 20ml of TE buffer containing 2 mM PMSF and sonicate them until more than 90% cells are broken.
7. **Sonication:** (4 times with 1 min interval in buffer tubes)

   Amplitude - 20

   Pulser - off

   Tune for minimum - 20

   Timer - 60 seconds
8. Centrifuge the sonicated cells at 7000 rpm for 15 min at 4ºC.
9. Resuspend the pellet in the TE buffer containing 0.1% Triton X-100 and ish twice in the same buffer.
10. Dissolve the pellet in 200µl of sterile double distilled water and stored it in -40ºC for further use.
11. Carry out SDS-PAGE analysis with 8% protein gel (load 5 µl from 200 µl stock in single well).

Preparation of Antibiotics

Preparation of Antibiotics

Name of the antibiotic	Concentration used mg/ml	Solvent	Mode of sterilization	Storage
Ampicillin	100mg/ml	Sterile water	Filter sterilization	-20°C
Erythromycin	50mg/ml	Ethanol (100%)	-	-20°C
Kanamycin	100mg/ml	Sterile water	Filter sterilization	-20°C
Rifampicin	10mg/ml	Methanol	-	-20°C
Cefotoxime	250mg/ml	Sterile water	Filter sterilization	-20°C
Hygromycin		Sterile water	Filter sterilization	-20°C
Tetracycline	10mg/ml	75% ethanol	-	-20°C
Streptomycin	250mg/ml	Sterile water	Filter sterilization	-20°C

Preparation of Plant growth hormones

Preparation of plant growth hormone

Hormones	Solubility	Sterilization	Storage	Function
6-Benzylaminopurine (6-BAP)	1N NaOH	Autoclave/ filter	Powder – Room Temperature (RT) Liquid – 2-8^{0}C	Callus formation and growth
2,4Dichlorophenoxyacetic acid (2,4 D)	Ethanol / 1N NaOH	Filtration	Powder – RT liquid – 0-5^o C	Induction of somatic embryo
Gibberellic acid (GA_3) / Gibberellic acid 4+7	Ethanol	Filtration	Powder - RT Liquid – 2-8^{0}C	Shoot elongation/Breakdormancy. Promote degradation of reserve in seeds.
Indole-3-acetic acid (IAA)	Ethanol / 1N NaOH	A/F	Powder – 2-8^{0}C Liquid - 2-8^{0}C	Root inducing
Indole-3-butyric acid (IBA)	Ethanol / 1N NaOH	A/F	Powder – 2-8^{0}C Liquid - 2-8^{0}C	Adventitious root formation. Inhibition of axillary buds
Kinetin (6-furfurylaminopurine)	1N NaOH	A/F	Powder – 2-8^{0}C Liquid - 2-8^{0}C	Adventitious shoot formation. Inhibition of root formation. Callus formation and growth. Stimulation of axillary bud.
α-Naphthalene acetic acid (NAA)	1N NaOH	Autoclave	Liquid at 2-8^{0}C	Adventitious shoot and root formation.
Zeatin	1N NaOH	Filtration	Powder – 0^{o}C Liquid - 0^{o}C	Callus growth. Adventitious shoot formation
6-Benzylaminopurine riboside	1N NaOH	Filtration	Powder – 2-8^{0}C Liquid - 2-8^{0}C	Adventitious shoot formation. Inhibition of root formation
N-Benzyl-9-(Tetrahydropyranyl)-adenine (BPA)	Ethanol	A/F	Powder – 2-8^{0}C Liquid - 2-8^{0}C	Increase branching, haploid plant regeneration from cultured anthers
DL-dihydrozeatin (DHZ)	Ethanol	Filtration	Powder – 2-8^{0}C Liquid - 2-8^{0}C	Maintain cytokinine activity level in an oxidative environment
6- (γ- γ-Dimethylamino) purine riboside 2 ip-	Water	Filtration	2-8^{0}C	Cytokinin growth regulator

riboside				
6- (γ- γ- Dimethylamino) purine 2 ip	1N NaOH	A/F	Powder – 0^{o}C Liquid - 0^{o}C	Cytokinin growth regulator
L- ornithine hydrochloride	Water	filtration	Room temp (RT)	Polyamine growth regulator. Promotion of shoot and root formation and somatic embryogenesis
Phenylacetic acid (PAA)	Ethanol	A/F	Powder – RT Liquid - 2-8^{0}C	Auxin like growth regulator
Picloram	1N NaOH	A/F	Powder RT Liquid - 0^{o}C	Auxin source in plant tissue culture
Putrescine hydrochloride	Water	filtration	Room temp	Polyamine growth regulator
Zeatin riboside	Water	filtration	Powder – 0^{o}C Liquid - 0^{o}C	Plant regeneration. Direct and efficient regeneration from leaf explant. Produce max. no. of shoots per explant.
Thioduzuron (TDZ)	DMSO	filtration	Powder – 2-8^{o}C Liquid - 2-8^{o}C	Plant regeneration. Direct and efficient regeneration from leaf explant. Produce max. no. of shoots per explant.
Acetosyringone (ACS)	DMSO	filtration	Powder – Room temp Liquid - 2-8^{o}C	Induce vir genes in A.t mediated plant transformation.

Genetic transformation of tobacco

Genetic transformation of tobacco

1. Preparation of explants

From the leaves of *in vitro*-grown tobacco plants, leaf bits of about 5 mm^2 are made using a sterile scalpel blade and inoculated onto pre-culture medium (4.2g/L MS powder;) in petri plates. Fifteen to twenty leaf discs are inoculated per plate with their dorsal surface touching the medium. Pre-culturing of tobacco leaf bits is done for two days at 25 ± 2ºC, 16 hrs photoperiod and 2000 lux light intensity provided by the cool white fluorescent lamps.

2. Co-cultivation and transformation

The *A. tumefaciens* strain, harboring plant expression cassette is grown in YEP broth containing 100 mg/L kanamycin and 10 mg/L rifampicin at 28oC in a shaker at 175 rpm. The *Agrobacterium* culture is grown to late log phase (O.D. of 1.0 at 600 nm) is used for infection of explants. *Agrobacterium* cells are harvested at 5000 rpm for 10 min and resuspended in 30ml MS broth. Precultured explants are treated with the *Agrobacterium* suspension. The leaf discs are immersed in *Agrobacterium* suspension under gentle agitation for 15 minutes, then blotted on sterile filter paper and placed on shoot regeneration medium for two days under the culture conditions already described. In control experiment, explants are placed on regeneration medium for two days without *Agrobacterium* infection.

3. Selection and plant regeneration

The explants, after co-cultivation are transferred to selection medium containing 250 mg/L Cefotaxim and 50-100 mg/L kanamycin. The explants showing shoot regeneration are sub cultured at 3 weeks interval. The regenerated shoots that are longer than 2 cm are transferred to rooting medium containing 250 mg/L Cefotaxim and 30 mg/L kanamycin.

4. Media composition

Table 1 Media composition

Shoot regeneration medium (1000 ml) (pH 5.8)	**Rooting medium (1000 ml)**
MS medium powder 4.20 g Calcium chloride 0.44 g BAP (1mg/ml stock) 1.0 ml	MS medium powder 4.20 g Calcium chloride 0.44 g IBA (1mg/ml stock) 1.0 ml

NAA (1mg/ml stock)	0.1 ml	Sucrose	30.0 g
Sucrose	30.0 g	Agar	8.0 g
Agar	8.0 g	pH	5.8 (adjusted using KOH)

Note:

1. Regeneration media without antibiotic is used as preculture media
2. Regeneration media without antibiotic and with acetosyringone 100µM is used as co-cultivation media.

Maintenance of Tobacco plants at Green house

- **Plant tissue culture generated**
- **Germinated through seeds**

Southern Blotting

Restriction digestion of genomic DNA

Equal quantity of (~10 µg) of intact high molecular weight genomic DNA of are digested overnight with respective restriction enzyme at 37°C. Reaction mixture accomplwashed with 1X of reaction buffer and appropriate concentration of Genomic DNA along with desired units of restriction enzyme is performed. The respective recombinant plasmid digested with appropriate enzyme is used as a positive control at concentrations of 50pg and 250pg.

Gel electrophoresis of restriction digested genomic DNA

The restriction digested genomic DNA samples and plasmid DNA are resolved in 0.8 % agarose gel at 5 volts/cm for 5 h in 1X TAE buffer, visualized upon ethidium bromide staining (1µg/ml), and documented in gel documentation system (Bio-Rad, USA) also in transilluminator.

Note: Expose the gel for a shorter time. The gel border, lanes and size of the visible bands in ladder should be marked using marker pen on the polythene sheet placing on the gel. Wear proper accessories to avoid UV exposure.

10X TAE: Tris - 121 g; Glacial Acetic acid - 5.71 ml; EDTA - 50 ml from 0.5 M stock; made up to 500 ml with water

Southern transfer

The restriction digested and electroporetically separated genomic DNA is denatured by submerging the gels in two volumes of denaturing solution (1.0 M NaCl / 0.5 N NaOH) for 45 min. with gentle agitation. The gels are washed briefly in sterile de-ionized water followed by neutralizing the gels by submerging in two volumes of neutralizing solutions (1.5 M NaCl / 0.5 M Tris pH 7.0) for 45 min with gentle agitation. The gels are washed briefly in sterile de-ionized water and the DNA is transferred to positively charged nylon membrane (Zeta Probe, Catalog # 11209299001; Bio-Rad, USA) through upward capillary transfer in 20X SSC buffer for 16 h by following the standard protocol (Capillary transfer protocol). After complete transfer of genomic DNA, the nylon membrane is washed briefly in 2X SSC buffer and air dried for 5 min. The DNA is cross-linked by exposing the membrane in UV-cross linker (Stratalinker® 1800 Stratagene, CA,

USA) at 1200 µJ for 2 min. The cross-linked membranes are sealed in plastic bags and kept at 4ºC until used for Southern blot hybridization.

Note: Cut the gel to remove marker lanes before depurination. Care should be taken in gel handling during transfer process as the denaturation solution is slightly viscous. After the transfer the gel can be visualized in transilluminator to cross check.

Denaturation solution (500 ml) : 1M NaCl; 0.5 N NaOH

Weigh NaCl - 29.22g; NaOH – 10.00g and dissolve in 400ml of double distilled water (Millipore) and make up to 500ml. autoclave and store at room temperature.

Neutralization solution (500 ml) : 1.5 M NaCl; 0.5 M Tris; pH 7.0

Weigh NaCl - 43.83g; Tris – 30.22g and dissolve in 300ml of double distilled water and adjust the pH to 7.0 with conc. HCl (initial pH will be 10.5, it requires more volume of HCl) and make up to 500ml with double distilled water. Autoclave and store at room temperature.

20X SSC (1000 ml): NaCl; Sodium citrate; adjust pH to 7.0 with conc. HCl

Dissolve 175.3g of NaCl and 88.2g of Sodium citrate in 800ml of double distilled (Millipore) water. Adjust the pH to 7.0 with conc. HCl and make up to 1000ml. Autoclave and store at room temperature.

Capillary transfer protocol

Keep the gel casting tray upside down with a Whatman No. 3 paper wick on a platform and the edges of the wick are folded down the wall towards centre. Wet the paper wick using 20X SSC and remove the air bubble by rolling the glass rod. Place two Whatman No. 3 filter paper (cut appropriate to gel size) on the wick and wet using SSC and remove the air bubble by rolling the glass rod. Lay the pre processed gel on the wick such that the bottom even side of the gel should face up. Remove the air bubble by rolling the glass rod on the gel. Cut the edge of the nylon membrane to remember the marker side and also note the side which contacts the gel. Place the nylon membrane on the gel and remove the air bubble. Place one Whatman No 3 filter paper and remove air bubble. Then place a bunch of ordinary filter paper and a weight on it. Allow it for 16 hours.

Mega prime oligo labeling kit

About 100ng of gel eluted PCR fragment of codon optimized *cry2Ai* gene amplified from recombinant binary vector is used as probe by radiolabelling with [α- ^{32}P] dCTP. Two micro liters of electroeluted template DNA is mixed with 5 µl of random primer (Mega prime oligo labeling kit, Amersham, USA) in a microfuge tube; the volume is made upto 33 µl with sterile distilled water and denatured by heating for 5 minutes on boiling water bath, cooled on ice. To the denatured DNA, 10 µl of labeling mix containing dATP, GTP, TTP, 5µl of [α- ^{32}P] dCTP (50 µCi) and 2.5µl of Klenow fragment of DNA polymerase I are added and incubated at 37ºC for 20 min in a water bath. The reaction is stopped by adding 5µl of nick translation stop solution containing 0.5M EDTA. The radiolabelled probe is purified by passing through Sephadex G-50 column to exclude un-incorporated [α- ^{32}P] dCTP.

Labeling mix : 50 ng Denatured DNA template; 10µl dNTP mix; 50 µCi α p32 dCTP; 2 units Klenow enzyme

Stop buffer : 6mg/ml Blue Dextron; 1mg/ml Orange G; 0.5M EDTA (pH 8.0)

Column buffer : 1M NaCl; 2M Tris (pH 7.0); 0.5MEDTA (pH 7.0)

Sephadex G-50 : 7.3 g of SephadeX G50 in 100 ml column buffer

Pre-hybridization

The membrane should be rolled gently into the cylindrical hybridization bottles and 25 ml of pre-hybridization buffer (pre warmed at 65°C) is added (air bubble between membrane and bottle should be avoided). Tightly close the bottle and place in a pre-warmed hybridization oven at 65°C. Pre-hybridization of the membrane is done for 30 min at 65°C.

Pre-hybridization solution : 0.5M Na_2HPO_4 (pH 7.2); 7% (w/v) SDS; 1mM EDTA (pH 7.2)

Stock solutions	**25ml Working**	**200ml Working**	**Final conc**
2M Na_2HPO_4	6.25ml	50ml	0.5M
10% SDS	17.5ml	140ml	7%
0.5M EDTA	50 µl	400 µl	1mM
Sterile distilled water	1.2ml	9.6ml	

Hybridization with probe

The radio labeled probe is denatured by taking required amount in a microfuge tube along with 50 µl of water and it is placed in a boiling water bath for 5 min. Then the microfuge tube with probe is immediately placed in ice for 2 min. The denatured probe is added into pre-warmed hybridization solution. The pre-hybridization buffer is poured off from the hybridization bottle and replaced with 20 ml of fresh hybridization solution (maintained at 65°C) containing denatured [α- ^{32}P] dCTP labeled probe. The bottle with hybridization solution is closed tightly and replaced in hybridization oven. Hybridization is performed for 20 hours at 65°C.

Washing of nylon membrane and Exposure

The hybridization buffer is poured off from the bottle. About 40 ml of 1X SSC and 0.1% SDS is added and the bottle is agitated gently on a slowly rotating platform at 65ºC for 15 min. After the first ish solution is poured off and fresh 40 ml of 0.1X SSC and 0.1 % SDS is added to the bottle and incubated for 15 min at 65°C with gentle agitation. Ish solution is removed and the membrane is dried on Whatmann No.1 filter paper for 5 min. at room temperature and exposed to X-ray film (Kodak XAR) in dark room, in signal intensifier screen (Hyper cassette ® from Amersham, USA) for 2 days or appropriated duration at -70oC freezer.

Wash-I : 1X SSC; 0.1 % SDS

Add 5.0ml of 20X SSC and 1.0ml of 10% SDS then make up to 100ml with distilled water.

Wash-II : 0.1X SSC; 0.1% SDS

Add 0.5ml of 20X SSC and 1.0ml of 10% SDS then make up to 100ml with distilled water.

X-ray film development

After the required time of exposure (generally varies with the nature of sample and radioactive count on the membrane), X-ray film is taken out in dark room and immersed in developer solution for 2 min. followed by immersion in water for 1 min. Finally the X-ray film is immersed in fixer solution for 2 min. followed by rinsing in water for 1-2 min. and then thoroughly washed in running tap water, air dried.

Developer solution:

13.2 g of Pack A content is dissolved in 800 ml distilled water, after complete dissolving 89g of Pack B component is added and dissolved by slow stirring. After complete dissolving volume is made up to 1000 ml and stored in amber bottle.

Fixer solution:

268g of fixer is dissolved in 800 ml of distilled water by slow stirring, after complete dissolving volume is made up to 1000 ml, filtered through country filter paper and stored in amber bottle.

ANNEXURE

Quantification of Protein by Lowry's method

Reagent A : 2% Sodium Carbonate in 0.1 N Sodium Hydroxide.
0.1N Sodium Hydroxide (NaOH): 40mg of NaOH in 100ml of water
2%Sodium Carbonate: 2 g of Na_2CO_3 in 100 ml of 0.1N NaOH

Reagent B : 0.5% $CuSO_4 . 5H_2O$ (copper sulfate) in % potassium sodium tartrate.
% Potassium Sodium Tartorate:
0.5% CuSO4.5H2O: 0.5g of CuSO4.5H2O in 100 ml of % Potassium Sodium Tartorate

Reagent C : Alkaline copper solution: 50 ml of A and 1 ml of B prior to use.

Reagent D : Folin-Ciocalteau reagent –1X from 2X.
1X Folin-Ciocalteau: Dilute 5ml 0f Folin-Ciocalteau reagent with 5 ml of distilled water.

TAE buffer preparation: (50x)/1000ml (STOCK)

2M Tris base	- 242 gm
0.5M EDTA	-186.1gm
Glacial Acetic Acid	-57.1ml
P^H	8.5

Calculation: 2M tris base

Molarity = weight/mol.wt×1000/volume

2M = W/121×1000/1000

W =242gm.

0.5M EDTA

Molarity = weight/mol.wt×1000/volume

0.5M = W/372.2×1000/1000

=186.1gm

Preparation:

First weigh 242 g of tris base and dissolve in 750ml of distilled water and carefully add 57.1 ml glacial acid. To this add 186.1 g EDTA. Dissolve all the contents using magnetic stirrer. Adjust the pH to 8.5. Autoclave and store at 4ºC

Note: As EDTA will not go completely into solution until the pH is adjusted to about 8.0, it is better to prepare a stock solution of 0.5M EDTA ahead of time.

1X TAE buffer preparation: 1000ml (working)

Take 20ml of TAE from 50X stock solution and make up to 1000ml with double distilled water. Store at room temperature

Cal: V1N1=V2N2

V1×50x=1000ml×1x

V1=1000×1x/50x = 20ml

EtBr Stock solution:

- Take 10mg Ethidium bromide (Etbr) and dissolve in 1ml of distilled water. Store @4ºc.

Note: Etbr is highly carcinogenic so handle with at most care.

dNTP mix preparation

Concentration of dNTP used for PCR is 2.5 mM.

dATP-100mM; dCTP-100mM; dTTP-100mM; dGTP-100mM

40µl 25mM dNTP mix = 10µl dATP100mM + 10µldCTP 100mM + 10µl dTTP100mM + 10µl

dGTP100mM

Calculation: concentration of dNTP/total volume

Ex: 100mM dNTP/40 µl=25mM

Concentration of each dNTP will be 25mM.

Working dilution:

5 µl of 25mM dNTP mix +45 µl of sterile autoclaved water= 50 µl 2.5mM dNTP

LOADING DYE PREPARATION

Manual method:

Sucrose -40%

Bromo phenol blue-0.25%

Procedure: 0.25 gm bromo phenol blue dissolved in 100ml of 40% sucrose solution, filter sterilize and store at-20 ^{o}C

LB broth:

Tryptone : 10gm
Sodium chloride: 10gm
Yeast extract: 5gm
pH -6.8 to 7.0

100 mM $Cacl_2$: Mol.wt-147.2

100mM=w/147.2×1000/100

w =1.472gm

Weigh 1.472gm of cacl2 dissolve in 70ml water and makeup to 100ml.Autoclave and store @ 4^{o} C.

Media compositions

LB (100ml)

Tryptone	1.0g
NaCl	1.0g
Yeast extract	0.5g
pH	6.0-7.0
Agar	2.0g

YEP (1000 ml)

Peptone	10gm
NaCl	5gm
Yeast	10gm
Agar	15gm
pH	7.0

Autoclave and store @room temperature

40% Glycererol

Take 40ml of glycerol and make upto 100ml with water. Autoclave and store @ 4º temperature

10% Glycererol

Take 10ml of glycerol and make upto 100ml with water. Autoclave and store @ 4º temperature