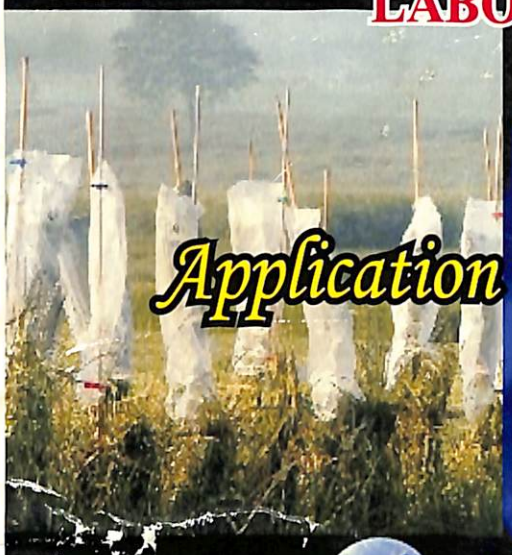
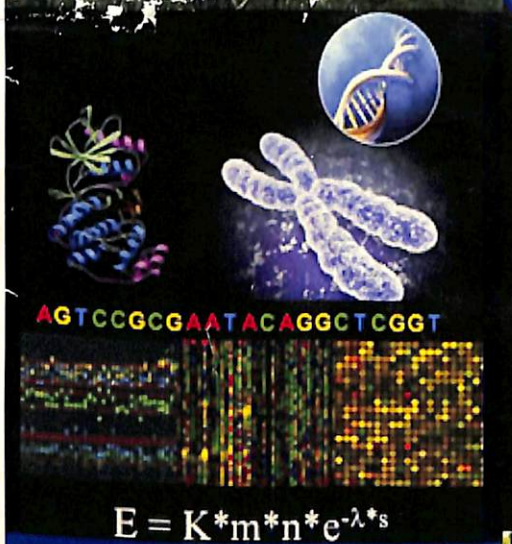
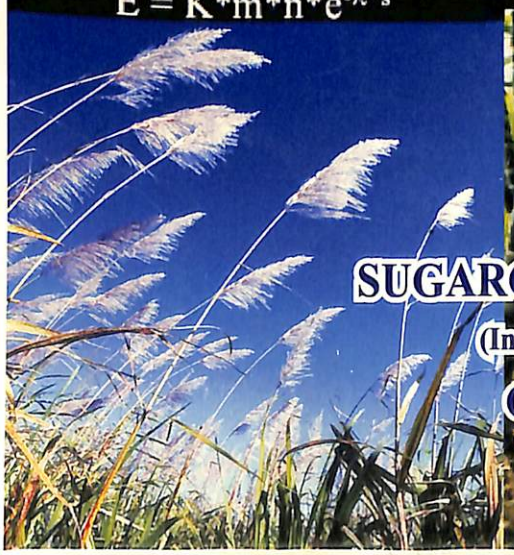
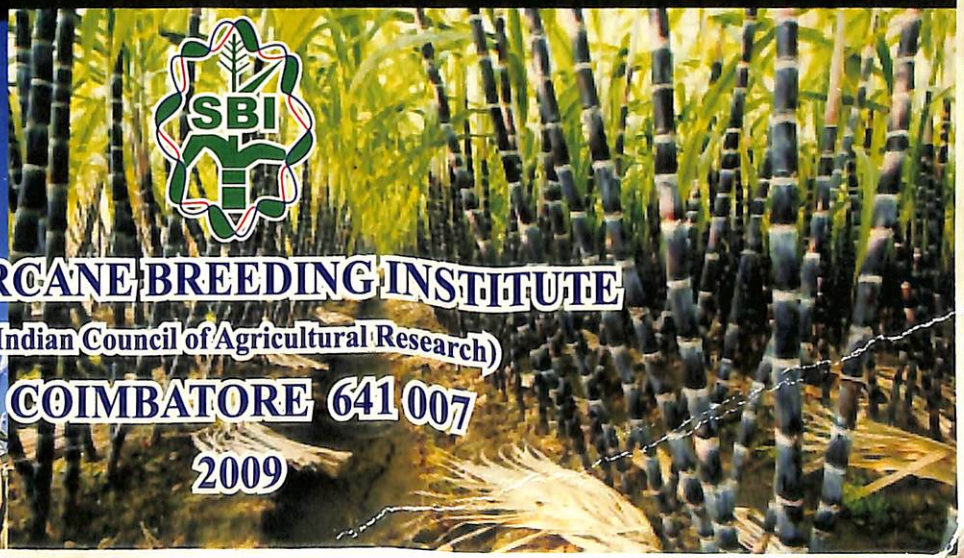




LABORATORY MANUAL ON

Application of Molecular Tools for Crop Improvement





SUGARCANE BREEDING INSTITUTE
(Indian Council of Agricultural Research)
COIMBATORE 641 007

2009

WINTER SCHOOL ON

Application of Molecular Tools for Crop Improvement

LABORATORY MANUAL

Compiled and Edited by

A.Selvi
and
N. Vijayan Nair

ICAR

SUGARCANE BREEDING INSTITUTE
(Indian Council of Agricultural Research)
COIMBATORE – 641 007
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Preface

Varieties sustain crop productivity to a large extent. Major breakthroughs in crop productivity had been achieved through the development and deployment of improved, high yielding and well adapted crop varieties. The productivity of crop varieties has to be continuously improved if the ever-growing demand for agricultural products is to be met, which is a stiff challenge. This is possible through the integration of modern biotechnological tools with the proven, conventional plant breeding techniques.

Varietal development is a complex and long-drawn process involving generation of appropriate genetic variability followed by several stages of evaluation and selection for various traits under different environments. The plant breeding techniques are based on principles of genetics and have undergone changes concurrent with the developments in genetics and related fields. In this context the recent developments in molecular biology have contributed significantly to our understanding of the plant genomes, providing greater opportunities for the improvement of the crop plants through genetic manipulations. Molecular techniques have a variety of application in crop improvement including the study of phylogeny and genetic diversity of crop plants, marker assisted selection, genome mapping, introgression of wild genomes, identification of genes of importance and their manipulations. Molecular markers also have been used for the characterization of pathogens and pests, which is important for their effective management and to reduce crop losses. Mobilization of genes from one source to another also has become possible now through transformation techniques. The Winter School on the "Application of molecular tools for Crop Improvement" is organized at the Sugarcane Breeding Institute, Coimbatore, to provide an exposure to the Scientists of the National Agricultural Research System to molecular techniques as applied to crop improvement programmes.

Sugarcane Breeding Institute has been engaged in research on various aspects of sugarcane biotechnology for the past 15 years. The Institute has developed capabilities for research in the areas of plant tissues culture, molecular markers, gene identification, genetic transformation and molecular diagnostics during the period. The Winter School has been designed to provide hands-on training in these areas to the participants with a view to update their professional skills. This manual is a compilation of protocols for the experiments to be carried out during the course of

this training programme. I thank all the resource persons who have contributed to the preparation of this manual. I also thank Dr. A. Selvi for compiling and editing the manual in its present form. It is hoped that the compilation will serve as a useful reference for the participants.

I am grateful to the Indian Council of Agricultural Research for sanctioning this Winter School and for the financial Support received. I am extremely thankful to Dr. S.P. Tiwari, DDG (Education) and Dr. B.S. Bisht, ADG (HRD) for their support and encouragement in organizing this Winter School.

1.12.2009

N. Vijayan Nair

Course Director

Contents

Plant DNA extraction

A.Selvi and N.V. Nair

DBT Lab

Isolation of DNA of the required purity from plants is a basic requirement for any type of molecular analysis. Historically extraction of usable nucleic acids from plants has been difficult. Of late, many procedures exist for plant genomic DNA extraction and the technique used depends on the type of experimentation proposed. In general, success in DNA extraction is measured by DNA yield, quality, in terms of its molecular weight and their utility for restriction, amplification and ligation etc. All plant DNA isolation involves two steps. The first step involves the removal of cell wall and cell membrane and the second step is the separation of DNA from the cell wall debris, contaminating proteins, lipids and RNA without affecting the integrity of the DNA. Some important considerations while extracting plant DNA are,

1) The technique should involve steps to disrupt the cell and release the nuclear contents and thereafter solubilise the DNA. This is usually done by grinding the plant tissue in a pestle and motar using dry ice or liquid nitrogen or in hot buffer.
2) Disruption of cell membrane is achieved by using detergents like SDS or CTAB in the extraction buffer. CTAB is a cationic detergent, which helps in the lysis of cell membranes and will form complexes with nucleic acids.
3) To prevent the action of indigenous endonucleases, EDTA is used. EDTA is a chelating agent that binds magnesium ions, which is a cofactor for most of the nucleases.
4) Impurities such as RNA, proteins and polysaccharides are removed by treating the extract with RNase, chloroform and phenol.

The following method for the DNA isolation from young immature leaves of sugarcane has been adopted from Walbot (1988) with modifications.

1. Cut 0.5 gm of young sugarcane leaves and transfer to the precooled, sterilized mortar.
2. Grind the leaves quickly with 10-15 ml of grinding buffer.
3. Transfer the ground plant material to a sterilized centrifuge tube and centrifuge at 6500 rpm for 6 min at 4 ° C.

4. After centrifugation discard the supernatant and suspend the pellet in 3ml of suspension buffer.
5. To the suspended pellet add 200 µl of 20% SDS. Mix gently and place on a water bath at 70 ° C for 15 min.
6. After incubation, add 1.5 ml ammonium acetate (7.5 M), mix well and place on ice for half an hour.
7. Centrifuge the sample at 14,000 rpm for 10 min at 4° C.
8. Decant the supernatent without disturbing the pellet, in 6 ml isopropanol in sterilized centrifuge tube to precipitate the DNA.
9. Incubate on ice for 30 min or over night, for complete precipitation of DNA.
10. Centrifuge at 15,000 rpm for 15 min at 4°C to pellet the DNA. Discard the supernatant and resuspend the pellet in 500ul of TE and keep it in a water bath at 65° C for 15 min.

Removal of RNA

11. Transfer the DNA solution into sterilized microfuge tube using sterilized cut tips, and add 15 µl of RNase (10mg/ml)
12. Incubate in a water bath at 37 ° C for 15 min.

Removal of Proteins

13. Add equal volume of phenol: chloroform (1:1) to the DNA solution and mix well.
14. Spin at 10,000 rpm for 10 min at room temperature. Remove the aqueous phase without disturbing the interphase and transfer to fresh microfuge tube.
15. Repeat phenol-chloroform extraction and remove the aqueous phase.
16. Precipitate the DNA by adding 50 µl of sodium acetate ($1/10^{th}$ vol) and 1ml of ice-cold absolute ethanol (twice the volume of DNA solution).
17. Keep at -20 °C for half an hour or overnight.
18. Pellet the DNA by centrifuging at 15,000 rpm for 15 min at 4 °C.
19. To the pellet, add 70 % ice-cold ethanol, unsettle the pellet and spin at 10,000 rpm for 10 min at 4 °C.
20. Discard the supernatant and repeat the 70% ice cold ethanol washing.
21. Discard the supernatant and dry the pellet in air or using vaccum concentrator.
22. Dissolve the pellet in 200 µl TE and store at -20 °C.

DNA quantification

- Take 1 ml TE buffer in a cuvette and calibrate the spectrophotometer at 260 nm as well as 280 nm wavelength.
- Add 5 µl DNA to 995 µl TE, mix properly and record the OD at both 260 nm and 280 nm.
- Estimate the DNA concentration using the formula

$$\text{Amount of DNA (µg/ µl)} = \frac{(OD)_{260} \times 50 \times \text{dilution factor}}{1000}$$

(DNA has maximal absorbance at about 260 nm, An OD of 1.0 corresponds to 50 µg/ml for double stranded DNA).

The quality of the DNA is judged from the ratio of the OD values recorded at 260 and 280 nm. The A260/A280 ratio around 1.8 indicates a good quality of DNA. Ratio lesser than 1.8 indicates protein contamination and ratios of 2.0 or greater indicates RNA contamination.

Agarose gel Electrophoresis:

Electrophoresis of the DNA on 0.8 % agarose gel followed by staining with ethidium bromide and viewing under UV should reveal a single high molecular weight band close to the well.

1. Weigh 800 mg of agarose and transfer to a conical flask containing 100 ml of 0.5X TBE (Tris – Borate- EDTA) buffer.

2. Melt the agarose into a clear solution using a microwave oven. Cool the molten agarose to ~ 50° C and add ethidium bromide to a final concentration of 0.5 µg /ml and mix well.

3. Pour the molten agarose in a pre-set template with a well forming comb taking care that no air bubbles are formed as these will affect the migration of DNA fragments during electrophoresis.

   (The template for casting the gel is set in advance, by cleaning with 70% alcohol and sealing the ends with adhesive tapes. The template is kept horizontally on a leveling table and an appropriate well forming comb in placed. The teeth of the

comb should not touch the bottom of the plate leaving atleast 1 mm between the comb and gel plate.)

4. The molten agarose is allowed to gel for 30 to 45 min after which the comb and sealing tapes are removed carefully.

5. The template is mounted on an appropriate electrophoresis tank and the tank is filled with 0.5X TBE buffer until the gel is immersed upto 1 mm.

6. Load 2 µl of DNA sample mixed with 2µl of 6X loading buffer and 8µl of sterile water.

7. Run the gel at 50 -70 V for one hour.

8. Visualize the DNA on a UV transilluminator.

Reagents and stock solutions

Grinding buffer	**Final conc.**	**For 300 ml**
Tris (1M) pH 8.0	50 mM	15.00 ml
EDTA (0.25 M)	50 mM	60.00 ml
NaCl (4M)	250 mM	18.75 ml
Sucrose	15% (W/V)	45.00 g

Suspension Buffer:	**Final conc.**	**For 200 ml**
Tris (1M) pH 8.0	20 mM	4.0 ml
EDTA (0.25M)	10 mM	8.00 ml

TE buffer	**Final conc.**	**For 100 ml**
Tris (1M) pH 8.0	10 mM	1.0 ml
EDTA (0.25M)	1 mM	0.4 ml

Tris (1M) – 200 ml

Dissolve 31.5g of Tris HCl in 100 ml of distilled water. Adjust the pH to 8. Make up to 200 ml. Autoclave and store in a refrigerator.

EDTA (0.25M) - 200ml

Dissolve 18.0612g of EDTA in distilled water to make a final volume of 200ml. After adjusting the pH to 8 autoclave and store in a refrigerator.

Sodium chloride (4M)

Dissolve 23.376 g of NaCl in distilled water and make up the final volume to 100ml. Autoclave and store in a refrigerator..

Sodium acetate (3M) pH 5.2

Dissolve 24.609 g of sodium acetate in distilled water, adjust the pH to 5.2 with acetic acid and make up the final volume to 100 ml with distilled water. Autoclave and store in a refrigerator.

Ammonium acetate (7.5M)

Dissolve 57.8 g of ammonium acetate in distilled water and make up the volume to 100 ml. Autoclave all the above solutions and store at 4 ° C. Autoclave and store in a refrigerator.

SDS (20%)

Dissolve 20 g of SDS in 100ml of distilled water and store at room temperature.

RNase (10mg/ml)

Dissolve 10 mg of RNase in 1.0 ml of sterile distilled water. Boil for 10 min to destroy the DNase and store at –20 ° C.

Phenol: Chloroform Mixture

a. **Buffering the phenol**

1) Take freshly distilled or white crystals of phenol. Melt at 65 ° C in water bath.

(Phenol is highly corrosive and can cause severe burns. Wear gloves, protective clothing and safety glasses when handling phenol)

2) Add 0.5 g of 8-hydroxyquinoline to 500 ml of liquefied phenol.

3) Add 200ml of 1 M Tris HCl pH = 8.0

4) Shake it well and keep till the two phases separate. Check the pH of the aqueous with the help of the pH paper. (If pH is less than 7.6, a fresh batch of Tris should be prepared and rebuffer till the pH comes to greater than 7.6). Store phenol layer under TE at 4°C in a brown bottle.

b. To 24 parts of chloroform add 1 part of isoamyl alcohol

c. Mix (a) and (b) in equal proportion.

Agarose gel electrophoresis:

5X TBE buffer pH 8.0	-	1lit
Tris base	-	54 g
Boric acid	-	27.5g
EDTA (0.5M) pH 8.0	-	20 ml

Dissolve in distilled water and make up to 1 lit and store at room temperature.

Loading dye (6X) 10ml

Sucrose (40% w/v)	4g
Bromophenol blue (0.25%)	0.025g

Dissolve in distilled water and make up to 10 ml and store under refrigeration.

Ethidium bromide: (10 mg/ml)

Dissolve 250 mg of ethidium bromide in 25 ml of distilled water and store in dark under refrigeration. (Ethidium bromide is a carcinogenic chemical, hence should be handled only with gloves).

Reference

Walbot V. 1998. Preparation of DNA from single rice seedlings. Rice Genet News. 15: 149-151.

Random Amplified Polymorphic DNA analysis

A.Selvi, N.V. Nair, P.Govindaraj, G. Hemaprabha and L. Nivedha
DBT lab and Molecular Breeding Laboratory

Random Amplified Polymorphic DNA popularly known as RAPDs are DNA fragments amplified by the Polymerase Chain Reaction (PCR) using short (generally 10bp) synthetic primers of random sequence. The optimum length for these primers is ten nucleotides. These oligonucleotides serve as both forward and reverse primer and usually are able to amplify fragments from 3-10 genomic sites simultaneously. These primers are random sequences with no prior knowledge of the sequence of the DNA being tested. Therefore with some of the randomly chosen nucleotides no sequences are amplified while in majority of the primers multiple bands are produced. Amplified fragments (within the 0.5 – 5 kb range) are separated by gel electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size. These polymorphisms are considered to be primarily due to variation in the primer annealing sites. RAPDs have been used successfully in elucidating the genome structure of crop plants, clarifying phylogenetic relationships, construction of genetic maps, and detecting major genes. studies at the individual level (e.g. genetic identity) to studies involving closely related species. Due to their very high genomic abundance, RAPDs have also been applied in gene mapping studies.

Advantages:

- No prior sequence knowledge is necessary.
- Small amount of DNA can be used (25ng per reaction).
- The technique is so simple.
- Detection is not radioactive.
- Produces high levels of polymorphism
- Low cost compared to other techniques, such as allozymes and RFLP.

Polymerase chain reaction (PCR)

The polymerase chain reaction is a three-step process, which is repeated for several cycles. The three steps are *denaturation, annealing* and *extension.* In the first of the chain reaction, denaturation, the two DNA strands are separated by heating the DNA

to 94°C. Heat treatment breaks the relatively weak bonds between the DNA bases yielding two long single strands. In the second step, annealing, two primers attach themselves to the single strands. These primers are small, synthetic stretches of single-stranded DNA each about 10 base pairs long. They are selected so that they are complementary to the two single strands. The annealing temperature is usually in the range of 36°C depending on the length and composition of the primer. Once the primers have attached themselves, two short stretches of double-stranded DNA are generated. In the third step, extension, these short stretches serve as starting blocks for the enzyme *Taq polymerase*. Starting of the 3'-end of the primers, the enzyme adds the nucleotides complementary to the template at about 72°C, linking them together. It extends the primers in the direction of the target sequence, thereby making a double strand out of the two single strands. At the end of this first cycle, two new DNA double strands identical with the first one is synthesized. The three-step cycle can now be repeated as often as necessary where only the sequence flanked by the two primers is amplified. The new DNA sequences provide the template in the next cycle for the creation of new strands, resulting in four in the next cycle and then in 8, 16, 32 copies and so on.

Note: All the enzymes used are very expensive! Always keep the enzyme on ice and remove the enzyme from the freezer just before starting the required step of your experiment!

Preparation of cocktails:

When large scale PCR amplification is required, it is preferred that a cocktail is made where all of the components are combined except the template DNA. This is more accurate and more efficient in terms of the components of the PCR reaction. The cocktail is then aliquoted in the reaction vessels in the appropriate volume and then template DNA is added just prior to amplification. Usually extra cocktail for 2 -3 reactions is made in order to account for losses during pipetting steps. It is possible to prepare the cocktail without Taq polymerase and store this frozen. In this way, many cocktail can be prepared in advance of the PCR amplification. The cocktails are thawed when needed and the appropriate amount of Taq polymerase is added before aliquoting. Taq polymerase should not be added before freezing as the enzyme may be damaged due to chilling. For preparing the cocktails, calculated

amount of distilled water is added first in the cocktail tube because of its larger volume. Then PCR buffer, dNTPs, and forward and reverse primers are added. Taq DNA polymerase is added at the end. The cocktail is mixed gently through mild shaking or tapping. Required quantity of cocktail is added in the PCR tubes containing template DNA. The quantity of each constituent to be added in the cocktail is calculated and recorded in the observation note book to avoid mistakes and future reference.

PCR reaction mix:

The total volume of PCR varies between 10 µl to 25 µl depending upon the type of experiment and smaller volume reduces the cost of screening larger populations. Whenever more accurate results are expected, relatively a larger volume of PCR volume is preferred for ease and accuracy of pipeting.

Protocol:

Materials required:

Thermocycler (MJ Research Inc Model: PTC-100), Micropipettes, Thin walled PCR tubes

Reagents used for amplification:

Template DNA (10ng/µl)
RAPD primers (Operon, USA)
dNTPs
$MgCl_2$
10 X PCR buffer
Taq DNA polymerase
MilliQ water

Preparation of cocktail mix

S.No.	Constitutents	Stock	Volume µl	Final Concentration
1.	Taq Buffer with $MgCl_2$	10X	1.5	1X
2.	dNTPs	1.25mM	1.2	100µM
3.	$MgCl_2$	25mM	0.12	0.2 mM
4.	Primer	5ng/µl	1.5	7.5 ng
5.	Taq DNA polymerase	3U/µl	0.3	1U
6.	Sterile milliQ water		9.18	

RAPD reaction mix:

- In a sterile 0.2 ml thin walled PCR tube, add 1.2 µl (from 10ng/ µl stock of DNA) of genomic DNA (12ng).
- Add 13.8µl of the cocktail mix to the corresponding PCR tubes to make a total volume of 15µl of the reaction mix.
- Mix the contents by brief centrifugation.
- Place the tubes in the thermal cycler to carry out the amplification.

The amplifications are carried out under the following conditions

1. Initial denaturation 95°C for 3min.
2. Denaturation 94°C for 1min
3. Annealing 37°C for 1min.
4. Extension 72°C for 2min

Repeat the step-2 to step-4 for 44 times.

5. Final extension 72°C for 7min
6. Store 4°C for infinity.

Agarose Gel Electrophoresis:

- Prepare a 1.5% agarose gel in 0.5 X TBE buffer.
- Mix the samples with 1X loading dye.
- Slowly add 5ul mixture into the slots of the submerged gel using a micropipette.
- Run the gel at 150V for 2 hrs.

- Stain the gel with ethidium bromide solution (0.5µg/ml).
- View the gel under UV light and photograph the gel using a CCD camera.

Materials required:

1.5g of Agarose for 100 ml of the gel solution

5X TBE Buffer pH- 8.0

Tris buffer - 54g
Boric acid - 27.5g
EDTA (0.5M) pH- 8.0 - 20ml
(Dissolved in distilled water and make upto 1lit and store at room temperature).

6X loading dye for 10ml

Sucrose (40% W/V) - 4g
Bromophenol blue (0.25%) - 0.025 g
Dissolve in distilled water and make upto 10ml and store in the refrigerator.

Ethidium Bromide: (10mg/ml)

Dissolve 250 mg of Ethidium bromide 25ml of distilled water and store in dark under refrigeration.

Inter Simple Sequence Repeats (ISSR) analysis

A.Selvi, N.V. Nair and L. Nivedha
DBT lab

Inter Simple Sequence Repeats (ISSR) is a simpler technique compared to other marker systems and they produce highly reproducible results and generates abundant polymorphism in plant system. To amplify Inter simple sequence repeat markers, the primers are designed on microsatellites and they amplify inter-SSR DNA sequences. Here, various microsatellites anchored at the 3 prime end are used for amplifying genomic DNA which increases their specificity. The extra nucleotide will permit amplification only if the primer binds to a 5' end of a microsatellite with a suitable first nucleotide in the flanking sequence. Such extra nucleotides are termed anchors and they ensure that amplification will always start from the 5' end of the microsatellite. An unlimited number of primers can be synthesized for various combinations of di-, tri-, tetra- and pentanucleotides [(4)3 = 64, (4)4 = 256] etc. with an anchor made up of a few bases. The ISSRs are mostly dominant markers, though occasionally a few of them exhibit codominance.

Protocol

Materials required:

Thermocycler (MJ Research Inc Model: PTC-100), micropipettes, thin walled PCR tubes.

Preparation of cocktail mix

S.No	Constitutents	Stock	Volume µl	Final concentration
1.	Taq Buffer with $Mgcl_2$	10X	1.5	1X
2.	dNTPs	1.25mM	2.4	200µM
3.	Primer	30ng	2.0	30ng
4.	Taq DNA polymerase	3U/µl	0.3	1U
5.	Sterile milliQ water		7.3	

ISSR reaction mix:

- Add 1.5 µl (from 10ng/ µl stock of DNA) of genomic DNA (15ng) to the 0.2 ml thin walled PCR tube.
- To the above 13.5 µl of the cocktail mix is added to make a final volume of 15 µl.
- Mix well and briefly spin the tube in a centrifuge.
- The PCR tubes are placed in the thermal cycler.

Amplification is carried out under the following conditions.

1. Initial denaturation 94°C for 3min.
2. Denaturation 94°C for 30 sec
3. Annealing 50°C for 30 sec.
4. Extension 72°C for 45 sec

   Repeat the **step-2 to step- 4 for 44 times.**
5. Final extension 72°C for 10min
6. Store 4°C for infinity.

(Annealing temperature varies from 50-55°C depending on AT and CG content of the primers).

The amplified products are analysed on 1.5% Agarose gel and stained the gel with Ethidium bromide.

Microsatellite marker analysis

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DBT lab and Molecular Breeding Laboratory

In recent years, microsatellite markers or Simple Sequence Repeats (SSR) or Sequence Tagged Microsatellite Sites (STMS) have become the most popular marker system used in many crops for variety identification, genetic mapping, diversity analysis etc. Microsatellites are tandem arrays of short nucleotide sequence ranging from one to six bases in length (Eg. TATATATATATATA (two nucleotide repeats), GTTCGTTCGTTCGTTCGTTC (four nucleotide repeats) and produce polymorphism based on the difference in the number of DNA repeat units at a given locus. Microsatellites can be amplified by PCR using unique sequences that flank the tandem repeats. The number of repeats and therefore, the length of PCR product, may vary between individuals. This size polymorphism is detected by gel electrophoresis of the PCR products. The ability of SSRs to reveal co-dominantly inherited multi-allelic product of loci can be readily mapped, creating an advantage over most PCR methods. A typical protocol consists of sequence of steps consisting of Polymerase chain reaction, gel electrophoresis, staining and documentation.

Materials required

Thermocycler (MJ Research Inc Model: PTC-100), micropipettes, thin walled PCR tubes.

Preparation of cocktail mix:

S.No.	Constituents	Stock	Volume µl	Final Concentration
1.	Taq Buffer with $MgCl_2$	10X	1.5	1X
2.	dNTPS	1.25mM	2.4	200µM
3.	Forward primer	30ng	1.0	30ng
4.	Reverse Primer	30ng	1.0	30ng
5.	Taq DNA polymerase	3U/µl	0.3	1U
6.	Sterile milliQ water		5.8	

Microsatellite amplification reaction mix:

- First add 3 µl (from10ng/ µl stock of DNA) of genomic DNA (30ng) to the 0.2 ml thin walled PCR tube.
- To the above add 12 µl of the cocktail mix to make a final reaction volume of 15 µl.
- Mix gently and briefly centrifuge it.
- The tubes are placed in the thermal cycler and PCR is carried out under the following temperature conditions.

1.	Initial denaturation	94°C	for 5min.
2.	Denaturation	94°C	for 1 min
3.	Annealing	52°C	for 1min
4.	Extension	72°C	for 1 min
	Repeat the **Step-2 to Step- 4 for 29 times.**		
5.	Final extension	72°C	for 10min
6.	Store	4°C	for infinity.

(Annealing temperature varies from 50-65°C depending on AT and CG content of the primers).

Detection of Microsatellite marker polymorphism:

Microsatellite markers are based on size polymorphism that is usually due to the variation in the number of tandem repeats. To detect the polymorphism between individuals, the PCR products are separated by gel electrophoresis. Sometimes the size variation may be due to the level of single nucleotide. Hence to resolve this minor variation, PCR products must be separated using a very high resolution matrix. The standard matrix is polyacrylamide although for some application specially formulated agarose (Metaphore agarose) may be sufficient. For high throughput or automated systems, the amplified products are analysed on the PAGE gels. For our experiment, analysis of the amplification products are done on a 4% PAGE gel and the gels are stained with silver nitrate.

Polyacrylamide Gel Electrophoresis:

Preparation of glass plates:

- The glass plates are soaked in distilled water for half an hour and washed thoroughly with a mild soap solution and rinsed with distilled water.

- Both the plates are wiped with alcohol using a lint free tissue. This may be repeated one or two times so that the detergent attached to the plates will be removed.

Assembling and pouring the gel:

- Plate assembly consists of one small notched plate, one long plate and two spacers. Place the well cleaned 1.2 mm spacers in between the plates perfectly so that there is no gap between the gel plates and the spacers.
- Keep the plates in the casting stand and screw them so that plates are tightly held. The lower portion of the assembled glass plates are sealed with cellophane tape to prevent the leaking of gel in the bottom.

(The gel matrix consists of 30% acrylamide and bis-acrylamide in the ratio of 29:1. Acrylamide: bisacrylamide stock can be prepared in advance and stored in amber bottles under 4°C. Different gel concentrations can be used for the analysis and commonly 4 % to 8 % of the gel in 1X TBE buffer is widely used).

- Just before the preparation of gel prior to casting, required volume (8.3 ml) of 30% acrylamide : bisacrylamide stock is taken. To this 41.5 ml of 1X TBE is added.
- To the above solution 300 µl of 10 % ammonium persulphate and 40 µl of N, N, N', N' - tetramethyl ethylene diamine (TEMED) are added and mixed gently by swirling
- For pouring the acrylamide solution, hold the plate assembly near the top (notched area) with one hand so that the bottom edge makes about a 45 degree angle with the bench top. Tilt the assembly so that one of the bottom corners is raised above the other (off the bench top) and then carefully pour the acrylamide solution into the space between the glass plates starting at the lower corner. A slow, but steady pouring of the acrylamide works best and avoiding trapping of air bubble. As the acrylamide solution fills the space between the gel plates, lower the plate assembly so that bottom corners are on the bench top. If bubbles form during the pouring, try to dislodge them by tapping somewhat forcefully on the glass plates while holding the assembly nearly vertical. Bubbles near the top may be removed by using a hook made by cutting a piece of X ray film.

- When the acrylamide solution nears the top of the gel, stop pouring and position the gel horizontally so that the acrylamide fills in the remaining space. If not, fill the space with remaining solution gently without creating bubbles.
- Insert the comb at the top of the gel and ensure that the comb is evenly placed. Allow the polymerization process complete for 30 min. (The assembly can be used immediately or can be stored either wrapping the assembly in wet paper towel saturated with dH_2O or keeping the assembly in the electrophoresis apparatus with 1 X TBE buffer in the bottom and top chamber after removing the comb).

Polyacrylamide gel electrophoresis (PAGE):

- Remove the adhesive taps fixed in the bottom of the gel assembly carefully and clean outside of the glass plates either with paper towel saturated with d H_2O or in the sink with warm water. Let plates dry in rack or wipe out with dry paper towel.
- Add two and half liters of 1X TBE buffer in the bottom chamber of the electrophoresis unit.
- Remove any excess polyacrylamide from around the comb and top of the glass plates with a razor blade. Clean the plates with paper towels.
- Fit the gel assembly into the apparatus and fill the upper tank with 1lit quantity of 1X TBE buffer. Remove any air bubble from the top of the gel.
- Be certain the well area is completely devoid of air bubbles and small pieces of polyacrylamide.

Loading and running samples:

- Add tracking dye (bromophenol blue and xylene cyanol – see annexure for composition) 3µl of 6X dye for 15µl of PCR product and mix it well.
- Load 3 µl of the sample into the well as per the type of comb used. If the concentration of PCR product is high smaller quantity should be loaded for better resolution of DNA fragments. (Take care to avoid spillage of the sample to adjacent wells. Loading of the sample should be done as quick as possible as the first loaded sample may start dispersing if it is kept in the well for long period. Multichannel pipettes can be also used for quick loading of samples).

- Each gel must be loaded with DNA size markers. 3µl of 20bp ladder (30 ng) is loaded either in the first well or in the last well for sizing of various alleles. This is particularly important when performing surveys to identify markers that may be used for mapping. DNA ladders can also be added on either side of the gel.

Running the gel:

- When the gel assembly is set and samples loaded, electrophoresis is carried out at constant voltage of 140V for 2.30 hrs. Low voltage with longer duration helps in the finer separation of closely sized markers. Voltage to be set and the duration depends upon the size of the gel. In a typical 4 % gel, bromophenol blue migrates at approximately 40 bases and xylene cyanol migrates at approximately 170 bases.

Silver staining and documentation:

- Electrophoresis is stopped when the bromophenol blue reaches the base of the gel. The gel assembly is taken out and the plates are separated with the help of the sharp spatula or knife by just pushing the top plate.
- The gels are placed in fixer solution for 30 mintues in a rotary shaker.
- The solution is drained off and the gel is washed twice with distilled water for 1 or 2 mintues.
- The gel is then placed in the staining solution for 30 mintues with moderate agitation.
- Remove the staining solution and wash immediately with distilled water.
- Keep the gels for developing in developer solution. The gel is gently agitated until the bands become visible. Usually it takes 10-15 min for the bands to appear.
- The developer solution is drained and the gel is washed with distilled water for 1-2 min.
- The gel is photographed using the gel documentation system.

Annexure

I. Instruments needed:

1. Thermal Cycler
2. Gel electrophoresis system with powerpack
3. Gel documentation system

II. Reagents required:

Acrylamide: bisacrylamide solution 30% (29:1)

Acrylamide - 29gm
Bisacrylamide - 1 gm
Dis.water - 100ml
Make up to 100ml and filter it then store in brown container at 4°C

Ammonium per sulfate solution (10 %)

Dissolve 100mg of ammonium per sulphate in 1ml distilled water.

Preparation of 4% Acrylamide gel:

30% acrylamide: bisacrylamide - 8.3ml
1X TBE buffer - 41.5ml
10% Ammonium per sulphate - 300µl (freshly prepared)
TEMED - 40µl

Loading dye 6X:

Sucrose (40% W/V) - 4g
Bromophenol blue (0.25%) - 0.025 g
Xylene cyanol (0.25%) - 0.025 g
Dissolve in distilled water and make upto 10ml and store at refrigerator.

Reagents for silver staining:

Fixer:

60ml of alcohol is made upto 500ml with distilled water and 1ml glacial acetic acid is added.

Siler staining solution:

0.5g silver nitrate is dissolved well in 500ml distilled water and stored at brown bottle.

Developer:

15g of sodium hydroxide is dissolved in 500ml of distilled water with 750µl formaldehyde.

Amplified Fragement Length Polymorphism

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Amplified fragment length polymorphism (AFLP) is a polymerase chain reaction based marker widely used for fingerprinting. AFLP was developed by Keygene BV, Wageningen, The Netherlands. Among the several marker systems available, AFLP is capable of generating hundreds of highly replicable markers from DNA of any organism. The high resolution and replicability of AFLP's make them desirable for fingerprinting of plant genomes. The technique is based on the selective amplification of restriction fragments from a total digest of genomic DNA. The technique involves:

1. Restriction digestion of DNA and ligation to adapters.
2. Selective amplification of a subset of restriction fragments.
3. Analysis of the amplified fragments on high resolution sequencing gels.

The genomic DNA is restricted with the enzyme combination EcoRI / MSeI. EcoRI is a 6 base cutter where as MSeI is a 4 base cutter. A combination of these two enzymes generates fragments that amplify well and are less than 1Kb facilitating the separation on denaturing polyacrylamide gels. The amplification of restricted DNA is accomplished by ligating the restricted fragments to adapters and using primers complimentary to the adapters and restriction site, for amplification. The primers also have selective nucleotides at their 3' end, which extends into the restriction fragments thereby amplifying those fragments that match the selective nucleotides in the primer. The number of selective nucleotides used in the primer may vary based on the complexity of the genomes being amplified. For simple or small genomes one or two selective nucleotides are used and for large and complex genomes three selective nucleotides are used thus generating a readable pattern for both genomes. Generally 50-200 restriction fragments are amplified which requires a high resolution denaturing polyacrylamide sequencing gels for separating the fragments. The fragments can be viewed by silver staining or by autoradiography where the primers are labeled with (γ33P)ATP before amplification. Automated sequencers are also used to generate AFLP fingerprints by using fluorescently labelled primers for amplification.

Materials Required

1. AFLP Analysis System I - Invitrogen Life Technologies
2. Taq DNA polymerase.

Restriction digestion of genomic DNA

Add the following in a 1.5ml micro centrifuge tube.

5X reaction buffer	5 µl
DNA	10 µl (250ng)
EcoRI / MSeI	2 µl
Water	8 µl
Total Volume	25 µl

- Mix the contents by brief centrifugation.
- Incubate at 37°C for 2 hrs followed by incubation at 70°C for 15 min.
- Place on ice.

Ligation of adapters:

Add the following to the digested DNA:

Adapter ligation solution	24.0 µl
T4 DNA ligase	1 µl
	25 µl

- Mix gently and centrifuge briefly.
- Incubate at 20°C for 2 hrs.
- Dilute the ligation mixture 10 times (1:10 dilution) by taking 10µl ligation mixture and adding 90 µl of TE.
- Mix well and use 5 µl of the diluted ligation mixture for amplification. (Store the remaining undiluted ligation mixture at -20°C).

Pre amplification reactions:

- Prepare the pre amplification mixture in a thin walled PCR tube.

Diluted template DNA (Ligation mixture)	5 µl
Pre-amp primer mix	40 µl
10X PCR buffer + Mg (15mM)	5 µl
Taq DNA polymerase	0.33 µl (Bangalore Genei 3u/µl)
	50.33 µl

- Mix gently and centrifuge briefly.
- Place on a thermal cycler and program 20 cycles at

  94°C for 30 sec

  56°C for 60 sec

  72°C for 60 sec

And store at 4°C for infinity

- Make 1:50 dilution of the pre-amplified product by adding 3µl of the amplified product to 147µl of TE buffer.
- Mix gently and centrifuge.

Selective amplification:

- Dilute 18µl of EcoRI primer with 32 µl of distilled water.
- Prepare the selective amplification mixture in a thin walled PCR tube.

Diluted pre amplified DNA	-	5.0µl
MSeI primer (dNTPs mixed)	-	4.5µl
EcoRI primer	-	0.5µl
10X PCR buffer + Mg (15mM)	-	2.0µl
Taq DNA polymerase	-	0.33 µl
Water	-	7.67 µl
		20 µl

- Mix gently and centrifuge briefly.
- Perform selective amplification as follows.

A. Perform one cycle at

94°C for 30 s
65°C for 30 s
72°C for 60 s

B. Lower the annealing temperature of each subsequent cycle by 0.7°C during 12 cycles. This gives a touch down phase of 13 cycles.

C. Then perform 23 cycles at:

94°C for 30 s
56°C for 30 s
72°C for 60 s

The amplified products are analysed on denaturing polyacrylamide gels and viewed by silver staining.

Denaturing polyacrylamide gel electrophoresis

Preparation of glass plates

- Soak the gel plates in 2% NaOH for half an hour and wash thoroughly with a mild soap solution and rinse with distilled water.
- Wipe both the plates using lint free tissue.
- Apply repel silane (dichloro dimethyl silane, BDH) to the large plate and spread evenly using a lint free tissue. Rinse with distilled water followed by absolute ethanol.
- Apply 5 ml of bind silane solution (Electran / BDH) to the smaller plate and spread evenly with a lint free tissue. Let the solution dry and wipe with a fresh kim -wipe soaked in water. Dry with paper towels and rinse with ethanol and dry with paper towels.
- The two plates are separated by 0.4mm thick spacers and secured by large clips on the sides and mounted on the casting unit.

Gel casting

- Prepare 75ml of 6% denaturing polyacrylamide gel by mixing appropriate quantities of 40% (19:1) acrylamide-bisacrylamide solution with 7M urea in 1X TBE.
- Just prior to casting the gel add 300µl of 10% APS and 35µl TEMED.
- Mix thoroughly and pour into the glass plates using a 50 ml syringe.
- Place the well forming comb between the glass plates and secure tightly using large clips.
- Allow the gel for polymerization for 1 hour.

Electrophoresis

- Mount the gel sandwich on the electrophoresis unit. Gently add 1X TBE to the upper and lower buffer tanks.
- Remove the comb and flush the wells with 1X TBE.
- Subject the gel for a pre-run for 20 min at 50W.

- After the pre-run flush the well thoroughly before loading the samples.
- Add equal amount of denaturing dye to the sample.
- Denature the sample at 95°C for 5 min and immediately cool on ice.
- Load 5 µl of the sample on the gel.
- Electrophorese at constant power (50W) until the xylene cyanol (slower dye) is two thirds down the length of the gel (1-1 1/2 hrs approx.).
- After the run is over, drain the buffer from the upper tank and remove the gel sandwich and lay it on the workbench with small plate facing upwards.
- With the help of a spatula gently separate the glass plates. The gel adheres to the small plate and the gel along with the plate is used for silver staining.

Silver staining

- Place the plate with the gel attached in a tray with 10% acetic acid. Place it on a shaker with moderate agitation for 15min or until loading dye bands disappear.
- Place the gel in a tray with distilled water for 10 min with moderate shaking.
- Remove and place the gel in a tray with the staining solution for 15 min with moderate agitation.
- Remove the gel from staining solution and rinse in distilled water for 10 sec (more than 10 sec destaining may occur. So care should be taken not to exceed 10 sec.)
- Place the gel in chilled developing solution and gently agitate until bands are visible (approx 3 min).
- When bands are clearly visible remove the gel and place in acetic acid solution for 2-3 min to stop the reaction.
- Rinse the gel in distilled water for 2 min and repeat rinsing for one more time (rinsing is critical to prevent discoloration of the gel).

Reagents and stock solutions:

Bind silane solution

The solution is prepared in the ratio of 1:10,000:2,000,000 of bind saline: glacial acetic acid: absolute ethanol

Bind saline	0.5 µl
Acetic acid	5.0 ml
Ethanol	955 ml

Acrylamide stock (40%)

Acrylamide	38 g
Bis acrylamide	2 g

Dissolved in 100ml of distilled water and stored refrigerated in dark.

*(Filter the Acrylamide stock and store)

Gel solution for 6% denaturing polyacrylamide gels

Acrylamide stock (40%)	15ml
5X TBE	20ml
Urea	45g

Dissolved and made upto 100ml with distilled water

APS 10%

Dissolve 100mg in 1ml water and add 400 µl for 100ml gel solution.

TEMED:

Add 45 µl for 100ml gel solution.

Loading dye

Xylene cyanol	-	0.005g (0.025%)
Bromophenol blue	-	0.005g (0.025%)
10mM EDTA	-	400 µl (0.5M)
Formamide	-	18 ml (98%)
Water	-	1.6 ml
Total volume		20 ml

SOLUTIONS NEEDED FOR SILVER STAINING

Fixer:

10% glacial acetic acid – Add 200 ml of glacial acetic acid in 1800ml double distilled water.

Stain:

Dissolve 2g of silver nitrate and 3 ml 37% formaldehyde in 2 ltrs of double distilled H_2O. Store in brown bottle.

Developer:

Dissolve 60g sodium carbonate in 2 l double distilled water. Place developer on ice; add 400µl Sodium thiosulphate (10 mg/ml) and 3 ml 37% formaldehyde just before developing.

2% NaoH – dissolve 40g NaOH in 2 l double distilled water.

Total RNA isolation from plant tissue

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RNA is unique among biological macromolecules in that it can encode genetic information, serve as an abundant structural component of cells, and also possesses catalytic activity (ribozymes). We can now imagine that there was a primitive form of life based entirely on RNA. It is now realized that RNA which at first glance appears to be very similar to DNA has its own distinctive structural features. It is principally found as single stranded molecule. It functions as the intermediate, the mRNA between the gene and the protein –synthesizing machinery. Yet another role of RNA is as regulatory molecule, which through sequence complementarity binds to and interferes with the translation of certain mRNAs. RNA content can vary widely between tissues, cell-types, physiological state, etc. Three major kinds of RNA are produced i.e. messengerRNA(mRNA) that encodes the aminoacid sequences of one or more polypeptides specified by a gene or set of genes, transfer RNA(tRNA) reads information encoded in the mRNA and transfers the appropriate aminoacid to the growing polypeptide chain during protein synthesis. Ribosomal rRNAs are constituents of ribosomes, the intricate cellular machines where proteins are synthesized. Total RNA consists of all these types, majority being tRNA and rRNA while mRNA makes up between 1-5% of total cellular RNA. Only 1-3% of the human genome is translated into proteins. Since all genes are first transcribed into RNA, if we could just capture the RNA and sequence that, we would have the genes. Because sequence technology prefers DNA, the RNA is extracted from the cytoplasm and then copied back into DNA using an enzyme called reverse transcriptase.

Extraction methods

Obtaining high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments, including Northern analysis, nuclease protection assays, RT-PCR, RNA mapping, in vitro translation and cDNA library construction. One of the major limitations in these technologies is the isolation of large quantities of highly pure RNA from plant tissues rich in complex polysaccharides, polyphenolics and waxes. Any contamination of the isolated RNA

affects the downstream applications and requires extra cleaning procedures that result in a reduced RNA yield, especially the low molecular weight molecules. Reliable isolation techniques must yield intact high quality RNA that is free of RNases, proteins and genomic DNA. Ongoing research into optimizing RNA analysis has identified two steps in the RNA isolation process that can be improved; treatment and handling of tissue or cells prior to RNA isolation and storage of the isolated RNA. Since most of the actual RNA isolation procedure takes place in a strong denaturant (e.g. GITC, LiCl, SDS, phenol) that renders RNases inactive, it is typically prior to, and after the isolation, when RNA integrity is at risk. Many factors can affect reproducibility and relevance of gene expression profiling results. These include the source of the RNA (tissue or organism) and the sampling and isolation techniques. Methods for isolating intact RNA from plant tissues include lengthy procedures such as ultra centrifugation, toxic or expensive chemicals, and chemicals such as SDS and phenol in order to inhibit RNases. Many commercially available kits for RNA isolation are available that decrease time and cost. Previous descriptions of rapid cell lysis using heat and detergents followed by repeated organic extractions with acid phenol containing longer incubation times, more complex buffers, heat treatment etc are no longer necessary as commercial reagents are available for every specific need.

Thorough cellular disruption is critical for high RNA quality and yield. The extraction method used may affect RNA quality. DNA is exceptionally stable and can be isolated from ancient environmental samples. RNA is sensitive to degradation with inadequate sample handling or storage. The quality and quantity of purified RNA is also variable and after extraction RNA is thought to be unstable during long term storage.

RNA isolation

Materials

Buffers and Solutions

Chloroform

Ethanol

Isopropanol

Liquid Nitrogen

Monophasic lysis reagent- (Trizol (Sigma), Tri reagent (Ambion) etc)

RNA precipitation solution

Equipment

•Pre-cooled pestle and mortar (This is important to avoid the thawing of frozen tissues).

•High speed centrifuge

•Spectrophotometer

•Agorose gel electrophoresis equipment

Monophasic Lysis Reagents

Homogenous solutions for the isolation of total RNA are phenol-based reagents containing a unique combination of denaturants and RNase inhibitors. They are used in a convenient, single-step disruption/separation procedure. A large number of commercial reagents are available. These reagents are all monophasic solutions containing phenol, guanidine, or ammonium thiocyanate and solubilizing agents. E.g. Trizol Reagent (Life Technologies), TRI Reagent (Ambion), etc. When using commercial reagents for the simultaneous isolation of RNA, DNA, and protein, the manufacturer's instructions must be followed. In most cases, these differ little from one another.

The tissue or cell sample is homogenized or disrupted in the reagent, chloroform is mixed with the lysate, and the mixture is separated into three phases by centrifugation. The RNA is then precipitated from the aqueous phase with isopropanol. The entire procedure can be completed in one hour to produce high yields of intact RNA for use in Northerns, nuclease protection assays, RT-PCR and in vitro translation.

RNA isolation using monophasic solutions

This is one of the most effective methods for isolating total RNA and can be completed in one hour starting with fresh tissue or cells. The procedure is very effective for isolating RNA molecules of all types from 0.1–15 kb in length. The resulting RNA is intact with little or no contaminating DNA and protein. This RNA can be used for Northern blots, mRNA isolation, *in vitro* translation, RNase protection assay, cloning and polymerase chain reaction (PCR). Here procedure using TRI reagent is described below.

Sample Preparation

1. Homogenize tissue samples in TRI Reagent (1 ml per 50–100 mg of tissue) in an appropriate homogenizer. The volume of the tissue should not exceed 10% of the volume of the TRI Reagent. For suspension cells, isolate cells by centrifugation and then lyse in TRI Reagent by repeated pipetting.

a. If samples have a high content of fat, protein, polysaccharides, or extracellular material such as tuberous parts of plants an additional step may be needed. After homogenization, centrifuge the homogenate at 12,000 *g* for 10 minutes at 2–8 °C to remove the insoluble material (extracellular membranes, polysaccharides, and high molecular mass DNA). The supernatant contains RNA and protein. If the sample had a high fat content, there will be a layer of fatty material on the surface of the aqueous phase that should be removed. Transfer the clear supernatant to a fresh tube and proceed with step 2.

b. After the cells have been homogenized or lysed in TRI Reagent, samples can be stored at –70° C for up to 1 month.

2. To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Add 0.2 ml of chloroform per ml of TRI Reagent used. Cover the sample tightly, shake vigorously for 15 seconds, and allow to stand for 2–15 minutes at room temperature. Centrifuge the resulting mixture at 12,000 *g* for 15 minutes at 2–8 °C.

3. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). Transfer the aqueous phase to a fresh tube and add 0.5 ml

of 2-propanol per ml of TRI Reagent used in sample preparation, step 1 and mix. Allow the sample to stand for 5–10 minutes at room temperature.

4. Centrifuge at 12,000 *g* for 10 minutes at 2–8 °C. The RNA precipitate will form a pellet on the side and bottom of the tube. Store the interphase and organic phase at 2–8 ° C for subsequent isolation of the DNA and proteins.

5. Remove the supernatant and wash the RNA pellet by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent used in step 1.

6. Vortex the sample and then centrifuge at 7,500 *g* for 5 minutes at 2–8° C.

7. Briefly dry the RNA pellet for 5–10 minutes by airdrying or under a vacuum. Do not let the RNA pellet dry completely, as this will greatly decrease its solubility.

8. Add an appropriate volume of formamide, water, or a 0.5% SDS solution to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55–60° C for 10–15 minutes. Final preparation of RNA should be free of DNA and proteins. It should have a A 260 / A 280 ratio of 2.

Purification of RNA from Cells and Tissues by Acid Phenol-Guanidinium Thiocyanate Chloroform Extraction

In this single-step technique, cells are homogenized in guanidinium thiocyanate and the RNA is purified from the lysate by extraction with phenol:chloroform at reduced pH. Many samples can be processed simultaneously and speedily.

Materials

Buffers and Solutions

Chloroform:isoamyl alcohol (49:1, v/v)

Ethanol

Formamide (Optional)

Deionized formamide is used for the storage of RNA.

Isopropanol

Liquid nitrogen

Phenol

Sodium acetate (2 M, pH 4.0)

Solution D (denaturing solution)

Cells and Tissues

Solution D

4 M guanidinium thiocyanate

25 mM sodium citrate•$2H_2O$

0.5% (w/v) sodium lauryl sarcosinate

0.1 M β-mercaptoethanol

To prepare denaturing solution: Dissolve 250 g of guanidinium thiocyanate in 293 ml of H_2O, 17.6 ml of 0.75 M sodium citrate (pH 7.0), and 26.4 ml of 10% (w/v) sodium lauryl sarcosinate. Add a magnetic bar and stir the solution on a combination heater-stirrer at 65°C until all ingredients are dissolved. Store Solution D at room temperature, and add 0.36 ml of 14.4 M stock -β- mercaptoethanol per 50 ml of Solution D just before use. Solution D may be stored for months at room temperature but is sensitive to light.

Protocol

1. Isolate the desired tissues by dissection and place them immediately in liquid nitrogen.
2. Transfer approx. 100 mg of the frozen tissue to a mortar containing liquid nitrogen and pulverize the tissue using a pestle. The tissue can be kept frozen during pulverization by the addition of liquid nitrogen.
3. Transfer the powdered tissue to a polypropylene snap-cap tube containing 3 ml of Solution D. Homogenize the tissue for 15-30 seconds at room temperature.
4. Transfer the homogenate to a fresh polypropylene tube and sequentially add 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of phenol, and 0.2 ml of chloroform-isoamyl alcohol per ml of Solution D. After addition of each reagent, cap the tube and mix the contents thoroughly by inversion.
5. Vortex the homogenate vigorously for 10 seconds. Incubate the tube for 15 minutes on ice to permit complete dissociation of nucleoprotein complexes.

6. Centrifuge the tube at 10,000*g* for 20 minutes at 4°C, and then transfer the upper aqueous phase containing the extracted RNA to a fresh tube.
7. Add an equal volume of isopropanol to the extracted RNA. Mix the solution well and allow the RNA to precipitate for 1 hour or more at -20°C.
8. Collect the precipitated RNA by centrifugation at 10,000*g* for 30 minutes at 4°C.
9. Carefully decant the isopropanol and dissolve the RNA pellet in 0.3 ml of Solution D for every 1 ml of this solution used in Step 1. Decant the supernatant into a fresh tube. Do not discard it until the pellet has been checked.
10. Transfer the solution to a microfuge tube, vortex it well, and precipitate the RNA with 1 volume of isopropanol for 1 hour or more at -20°C.
11. Collect the precipitated RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the pellet twice with 75% ethanol, centrifuge again, and remove any remaining ethanol with a disposable pipette tip. Store the open tube on the bench for a few minutes to allow the ethanol to evaporate. Do not allow the pellet to dry completely.
12. Add 50-100 µl of DEPC-treated water. Store the RNA solution at -70°C. Addition of SDS to 0.5% followed by heating to 65°C may assist dissolution of the pellet.
13. Estimate the concentration of the RNA by measuring the absorbance at 260 nm of an aliquot of the final preparation.

KCl

To prepare a 4M solution: Dissolve an appropriate amount of solid KCl in water, autoclave for 20 minutes on liquid cycle and store at room temperature. Ideally, this 4 M solution should be divided into small (approx. 100 µl) aliquots in sterile tubes and each aliquot thereafter used one time.

NaCl

To prepare a 5 M solution: Dissolve 292 g of NaCl in 800 ml of water. Adjust the volume to 1 liter with water. Dispense into aliquots and sterilize by autoclaving. Store the NaCl solution at room temperature.

Sodium acetate

To prepare a 2 M solution: Dissolve 272.2 g of sodium acetate•$3H_2O$ in 800 ml of water. Adjust the pH to 4 with glacial acetic acid. Adjust the volume to 1 liter with water. Dispense into aliquots and sterilize by autoclaving.

Preparation of RNase-free water

Measure water into RNase-free glass bottles. Add 1 ml of 0.1% (v/v) diethylpyrocarbonate (DEPC) to 1000 ml water. Let stand overnight. Autoclave.

Troubleshooting Guide

A. Low yield may be due to:

a. incomplete homogenization or lysis of samples.

b. the final RNA pellet may not have been completely dissolved.

B. If the A260/A280 ratio is <1.65:

a. the amount of sample used for homogenization may have been too small.

b. samples may not have been allowed to stand at room temperature for 5 minutes after
homogenization.

c. there may have been contamination of the aqueous phase with the phenol phase.

d. the final RNA pellet may not have been completely dissolved.

C. If there is degradation of the RNA:

a. the tissues may not have been immediately processed or frozen after removing from the animal.

b. aqueous solutions or tubes used for procedure may not have been RNAse-free.

Working with difficult tissues

1. Lipid rich tissues

Plant tissues are rich in lipids can complicate the RNA extraction process, making it difficult to get a clean separation of RNA. Once the plant homogenate has been extracted with phenol:chloroform:IAA., white flocculent material will make up most of the volume of the aqueous phase after centrifugation. This white material likely

contains lipids and does not form a tight interface. To remedy the situation, add one-tenth volume chloroform:IAA, mix well, and recentrifuge.

2. Polysaccharides

An option for plant tissue involves the use of polyvinylpyrrolidone (PVP) in the lysis step prior to the organic extractions. PVP complexes with polysaccharide and polyphenol compounds commonly found in plants. The complexed material is centrifuged out of the lysate, and the lysate is then processed according to protocol. This step prevents carryover of contaminants that may inhibit downstream applications.

3. Viscous tissues

Extraction of viscous homogenates sometimes results in incomplete phase separation. Adding more lysis solution and/or re-extracting with phenol:chloroform:IAA will help alleviate this problem. Also, multiple phenol:chloroform:IAA extractions can be performed to ensure the partitioning of DNA into the organic phase during the acid phenol extractions of the RNA isolation procedure. High yields of intact RNA can be achieved once abundant DNA and nucleases are brought under control.

Integrity of RNA

The most common method used to assess the integrity of total RNA is to run an aliquot of the RNA sample on a denaturing agarose gel stained with ethidium bromide (EtBr). Intact total RNA run on a denaturing gel will have sharp, clear 28S and 18S rRNA bands (eukaryotic samples). The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is completely intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit the 2:1 ratio of high quality RNA. Completely degraded RNA will appear as a very low molecular weight smear. (Poly (A) selected samples will not contain strong rRNA bands and will appear as a smear. The UV absorbance wavelengths of the preparation are investigated at 260 and 280 nm using spectrophotometry. For pure RNA,

OD_{260}/OD_{280} = 2.0). If the ratio is less than 2, the preparation is likely to be contaminated with proteins, polysaccharides and genomic DNA.

Tips to get intact RNA

i. Homogenize samples immediately after harvesting.

ii. Flash freeze samples in liquid nitrogen. In order to inactivate RNase by flash freezing, it is important that tissue pieces be small enough to freeze almost immediately upon immersion in liquid nitrogen.

iii. When samples have been flash frozen they must be stored at -80°C and never allowed to thaw. Even brief thawing prior to homogenization in a guanidinium-based lysis solution can result in RNA degradation and loss.

DNase treatment

When the RNA will be used for RT-PCR, DNase treatment to remove residual contaminating DNA is very essential .High quality DNase enzyme must be used, with a quick and easy way to remove the DNase after the treatment without using organic solvents or heat treatment.

RNase contamination

Since RNases are found almost everywhere, it is essential that any item that could contact the purified RNA is RNase-free. All surfaces, including pipettors, benchtops, glassware, and gel equipment, should be decontaminated with a surface decontamination solution like RNaseZap™ (Ambion) or RNaseZap Wipes(Ambion) RNase-free tips, tubes, and solutions should always be used and gloves should be changed frequently.

DEPC (Diethyl pyrocarbanate) treatment

DEPC removes RNase contamination.The working material such as tips, tubes,tip boxes,centrifuge tubes, measuring cylinders etc should be treated with 0.1% DEPC solution for 2 hours at room temperature and then dried and autoclaved. Electrophoresis tank should be cleaned with detergent solution, washed with sterile water and wiped with ethanol before use for checking RNA.

Precipitation

The purified RNA may need to be concentrated by precipitation for downstream applications. An ammonium acetate (NH_4OAc) precipitation (0.1 volumes of 5 M NH_4OAc, and 2-2.5 volumes 100% ethanol, at -20°C for >25 min) gives good recovery of RNA. After precipitation, avoid complete drying of the RNA pellet because it can make RNA difficult to resuspend.

Resuspension

The final step in many RNA isolation procedures is to suspend the purified RNA pellet. Resuspension solution should be RNase-free with a low pH (pH 6-7), to protect against RNA degradation by introduced RNases or simple DEPC treated water can be used for resuspension.

Storage

For short-term storage, resuspended RNA should be stored at -20°C; for long-term storage, it should be stored at -80°C. Although RNA resuspended in water or buffer can be stored at -80°C, RNA is most stable in an NH_4OAc/ethanol precipitation mixture at -80°C. Aliquotting RNA solutions into several tubes will both prevent damage to the RNA from successive freeze-thaw events, and help to prevent accidental RNase contamination.

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Reverse transcription polymerase chain reaction (RT-PCR)

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DBT lab

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantitation currently available. Compared to other commonly used techniques for quantifying mRNA levels such as northern analysis, RT-PCR can be used to quantify mRNA levels from much smaller samples. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell.

RT–PCR can be used for cloning, cDNA library construction and probe synthesis. Typically 1 to 5 µg of total RNA in a 20 µl RT reaction is used. In case of rare or less abundant mRNA messages, it may be advantageous to use greater RNA amounts in order to generate sufficient cDNA for PCR amplification. The technique consists of two parts– the synthesis of DNA from RNA by reverse transcription (RT) and the subsequent amplification of a specific DNA molecule by polymerase chain reaction (PCR). The RT reaction uses an RNA template (typically total RNA or polyA + RNA), a primer (random or oligo dT primers), dNTPs, buffer and a reverse transcriptase enzyme to generate a single-stranded DNA molecule complementary to the RNA (cDNA). The cDNA then serves as a template in the PCR reaction.

Purification of mRNA

The presence of a polyA tail is unique to mRNA, and provides a mechanism of distinguishing and isolating mRNA from the more abundant rRNA and tRNA molecules. mRNA can be physically isolated from its more abundant relatives by passing total RNA over a column to which polymers of deoxythymidine (oligo-dT) are bound. RNA molecules that do not contain multiple adenine residues will be unable to adhere to such a column and will flow straight through the column. mRNA molecules, on the other hand, will bind through complementary base pairing to the column and will be eluted only when the concentration of salt flowing through the column is lowered. mRNA makes up between 1-5% of total cellular RNA and is most frequently used for 1) detection and quantitation of extremely rare mRNAs, 2)

synthesis of probes for array analysis, and 3) the construction of random-primed cDNA libraries, where the use of total RNA would generate rRNA templates that would significantly dilute out cDNAs of interest. Removal of ribosomal and transfer RNA results in up to a 30-fold enrichment of a specific message.

Quality of RNA

Intact mRNA must be used with RT-PCR, to accurately quantify mRNA levels; use of low-quality RNA compromises the derived expression results, which occurs through cleavage with RNases during improper handling or through storing the RNA in sub-optimal conditions. RNA that will be used in RT-PCR should be completely free of genomic DNA contamination. The presence of even trace amounts of genomic DNA can generate false positive products amplified during the PCR. The most effective method of removing DNA from RNA preparations is to digest with DNase I.

Reverse transcriptase (RT) enzymes

The commercially available reverse transcriptase (RT) enzymes used in RT-PCR reactions construction are derived either from Moloney murine leukemia virus (MMLV-RT) or from Avian myeloblastosis virus (AMV-RT). Both enzymes have the same fundamental activities, but differ in a number of characteristics, including temperature and pH optima. MMLV-RT is a single polypeptide of 71 kDa in size, while AMV-RT is composed of two polypeptide chains 64 kDa and 96 kDa in size. Most importantly, MMLV-RT has a very weak RNaseH activity compared to AMV-RT, which gives it an obvious advantage when being used to synthesize DNA from long RNA molecules. M-MLV Reverse Transcriptase will extend an oligonucleotide primer hybridized to a single-stranded RNA or DNA template in the presence of deoxynucleotides, producing a complementary strand. M-MLV Reverse Transcriptase is commonly used in the first step of RT-PCR reactions as well as for primer extension experiments.

RNaseH activity: RNaseH is a ribonuclease that degrades the RNA from RNA–DNA hybrids, such as those formed during reverse transcription of an RNA template. RNaseH functions as both an endonuclease and exonuclease to hydrolyse its target molecules.

Internal controls

The housekeeping genes like Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) which is a key enzyme in glycolysis, is constitutively expressed in many tissues and has been reported to be an useful internal control.

Procedure

First strand cDNA synthesis

The procedure for first-strand reverse transcription using RevertAid H- cDNA synthesis kit (Fermentas Inc, USA). is described below followed by gene specific primers for PCR amplification.

Components of the kit

M-MuLV Reverse Transcriptase
RiboLock™ RNase Inhibitor
5X Reaction Buffer
10mM dNTP Mix
Oligo(dT)18 Primer
Random Hexamer Primer
Forward GAPDH Primer, Reverse GAPDH Primer,
Control GAPDH RNA
DEPC-treated Water

Description

The First Strand cDNA Synthesis Kit is a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates. The kit uses M-MuLV Reverse Transcriptase, which has lower RNase H activity compared to AMV reverse transcriptase. The enzyme maintains activity at 37°C and is suitable for synthesis of cDNA up to 9 kb. The recombinant RiboLock™ RNase Inhibitor, supplied with the kit, effectively protects RNA from degradation. The kit is supplied with both oligo(dT)18 and random hexamer primers. Random hexamer primers bind non-specifically to the RNA template and are used to synthesize cDNA from all RNAs in the total RNA population. The oligo(dT)18 primer selectively anneals to the 3'-end of poly(A) RNA, synthesizing cDNA only from poly(A) tailed mRNA. Gene-specific primer may also be

used with the kit to prime synthesis from a specified sequence. First strand cDNA synthesized with this system can be directly used as a template in PCR or realtime PCR. It is also ideal for second strand cDNA synthesis or linear RNA amplification

RNA quantity

• Use 10 ng - 5 µg of total RNA or 1ng - 500 ng of poly(A) mRNA to generate first strand cDNA as
the initial step of a two-step RT-PCR protocol.

• Use 1 µg of isolated mRNA to generate first strand cDNA for second-strand synthesis and
subsequent cloning reactions.

Primers

1. Synthesis of first strand cDNA can be primed with either oligo(dT)18 primer, random primers or gene specific primers.
2. Oligo(dT)18 primes cDNA synthesis from the poly(A) tail present at the 3'-end of eukaryotic mRNA.
3. Random Primers initiate cDNA synthesis from the total RNA population (rRNA and mRNA).
4. Gene-specific primers are used to synthesize specific cDNA from a pool of total RNA or mRNA and must be obtained by the user.

First Strand cDNA synthesis procedure

The first strand cDNA reaction can be performed as an individual reaction or as a series of parallel
reactions with different RNA templates. Therefore, the reaction mixture can be prepared by combining reagents individually or a master mix containing all of the components except template RNA can be prepared.

1. After thawing, mix and briefly centrifuge the components of the kit. Store on ice.
2. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Template RNA

Total RNA	- 0.1-5 µg
or poly(A) mRNA	- 10 ng - 0.5 µg
or specific RNA	- 0.01 pg - 0.5 µg

Primer

oligo (dT)18 primer	-1 µl
or random hexamer primer	-1 µl
or gene-specific primer	- 15-20 pmol
DEPC-treated water to	- 11 µl
Total volume-	- 11 µl

3. Add the following components in the indicated order:

5X reaction buffer	- 4 µl
RiboLock™ RNase Inhibitor (20 U/µl)	- 1 µl
10 mM dNTP mix	- 2 µl
M-MuLV Reverse Transcriptase (20 u/µl)	- 2 µl
Total volume	- 20 µl

4. Mix gently and centrifuge.

5. For oligo(dT)18 or gene-specific primed cDNA synthesis, incubate for 60 min at 37°C.

6. For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 37°C.

For GC-rich RNA templates the reaction temperature can be increased up to 45°C.

7. Terminate the reaction by heating at 70°C for 5 min.

8. The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

II. PCR Amplification of First Strand cDNA

1. The product of the first strand cDNA synthesis can be used directly in PCR or qPCR. The volume of first strand cDNA synthesis reaction mixture should not comprise more than 1/10 of the total PCR reaction volume.
2. Normally, 2 µl of the first strand cDNA synthesis reaction mixture is used as template for subsequent PCR in 50 µl total volume. Taq DNA polymerase can be used to amplify fragments less than 3 kb.

Control reactions

1. Positive and negative control reactions should be used to verify the results of the first strand cDNA synthesis steps.
2. Reverse transcriptase minus (RT-) negative control is important in RT-PCR reactions to assess for genomic DNA contamination of the RNA sample. The control RT- reaction contains every reagent for the reverse transcription reaction except for the RT enzyme.
3. No template negative control (NTC) is important to assess for reagent contamination. The NTC reaction contains every reagent for the reverse transcription reaction except for RNA template.
4. Positive control RNA template and gene-specific primers are supplied with the kit. The GAPDH-specific control PCR primers are designed to be complementary to human, mouse and rat GAPDH genes and generate 496 bp RT-PCR product.

Trouble shooting

Low yield or no RT-PCR product

Degraded RNA template

RNA purity and integrity is essential for synthesis of full-length cDNA.

Always assess the integrity of RNA prior to cDNA synthesis. Sharp 18S and 28S RNA bands should be visible after denaturing agarose gel electrophoresis of total eukaryotic RNA. Follow general recommendations to avoid RNase contamination

Low template purity

Trace amounts of agents used in RNA purification protocols may remain in solution and inhibit first strand synthesis, e.g. SDS, EDTA, guanidine salts, phosphate, pyrophosphate, polyamines, spermidine. To remove trace contaminants, re-precipitate the RNA with ethanol and wash the pellet with 75% ethanol.

Incorrect primer choice

Use the correct primer for the RNA template. Use the random hexamer primer instead of the oligo(dT)18 primer with bacterial RNA or RNA without a poly(A) tail. Ensure sequence-specific primers are complementary to 3'-end of the template RNA.

RT-PCR product longer than expected

RNA template is contaminated with DNA.

Amplification of genomic DNA containing introns. Perform DNase I digestion prior reverse transcription (see protocol on p.3). To avoid amplification of genomic DNA, design PCR primers on exon-intron boundaries.

RT-PCR product in negative control

RNA template is contaminated with DNA. PCR product in the negative control (RT-) indicates the reaction is contaminated with DNA. Perform DNase I digestion prior reverse transcription

GEL ELUTION

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Cleanup of DNA is often a prerequisite for efficient downstream applications such as cloning, sequencing, microarray analysis, or amplification. A range of kits are available for gel elution of DNA. Apart from purification of DNA fragments from agarose gels, removal of enzymes after PCR and restriction digestion, removal of unincorporated nucleotides, primers and primer-dimers, removal of residual salts, phenol, chloroform or ethidium bromide and for purification of RNA-free plasmid DNA. DNA elution using the GenElute™ PCR DNA Purification Kit (Sigma) is described below.

Principle

The GenElute Gel Extraction Kit (Sigma) is designed for the rapid purification of linear and plasmid DNA fragments from standard or low-melting agarose gels. This kit can also be used to purify DNA from polyacrylamide gels. Each column can bind up to 10 µg of DNA from up to a 3.5 g agarose slice. The GenElute Gel Extraction Kit combines silica- binding technology with the convenience of a spin or vacuum column format. DNA fragments of interest are extracted from slices of an agarose gel by solubilizing the gel. The Gel Solubilization Solution can dissolve an agarose gel slice from gels run in either TBE or TAE buffer. Extracted DNA fragments selectively adsorb onto the silica membrane in the presence of the Gel Solubilization Solution. Contaminants are removed by a simple spin or vacuum wash. Finally, the bound DNA is eluted in Tris buffer. The isolated DNA is suitable for a variety of downstream applications, such as automated DNA sequencing, PCR, restriction digestion, cloning, and labeling.

Components of the kit

Column Preparation Solution

Gel Solubilization Solution

Wash Solution Concentrate

Elution Solution (10 mM Tris-HCl, pH 9.0)

GenElute Binding Column

Collection Tubes

Equipment and Reagents Required

Cutting tools for gel (scalpel and blades) or razor blades

Pipettors and tips

Water bath or heating block at 50-60 °C

Ethanol, 95-100%

Isopropanol, 99-100%

Microcentrifuge and tubes

Water, Molecular Biology grade

3 M Sodium Acetate Buffer, pH 5.2

Preparation Instructions

1. **Wash Solution:** Dilute the entire 12 ml of the Wash Solution Concentrate G with 48 ml of 95-100% ethanol prior to initial use.
2. **Agarose Gel Electrophoresis:** Use fresh electrophoresis running buffer. Electrophoresis buffer, which has been used repeatedly, will reduce the DNA recovery efficiency. Minimize examination of ethidium bromide-stained gels with an UV transilluminator. If possible, use a transilluminator equipped with a long-wavelength (302 nm) UV light source, as this will minimize the damaging effects of UV light on nucleic acids.
3. **Elution Solution:** If purifying plasmid DNA or large linear DNA fragments (>3 Kb) preheat the Elution Solution to 65 °C prior to use.
4. All centrifugations (spins) are performed at 12,000 to 16,000 g

Procedure

1. Excise the DNA fragment of interest from the agarose gel with a clean, sharp scalpel or razor blade. Trim away excess gel to minimize the amount of agarose.
2. Weigh the gel slice in an eppendorf tube.
3. Add 3 gel volumes of the Gel Solubilization Solution to the gel slice. i.e. for every 100 mg of agarose gel, add 300 µl of Gel Solubilization Solution.
4. Incubate the gel mixture at 50-60 °C for 10 minutes, or until the gel slice is completely dissolved.
5. Vortex briefly every 2-3 minutes during incubation to help dissolve the gel.

To adequately dissolve a gel with an agarose concentration greater than 2%, it is necessary to increase the ratio of the Gel Solubilization Solution volume to the gel weight to 6:1.

6. **Preparation of the binding column**

Preparation of the binding column can be completed while the agarose is being solubilized in step 3.

a. Place the GenElute Binding Column G into one of the provided 2 ml collection tubes.

b. Add 500 µl of the Column Preparation Solution to each binding column.

c. Centrifuge for 1 minute. Discard flowthrough liquid.

d. The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields.

8. Once the gel slice is completely dissolved (step 3) make sure the color of the mixture is yellow (similar to fresh Gel Solubilization Solution with no gel slice) prior to proceeding to the following step.

a. If the color of the mixture is red, add 10 µl of the 3 M Sodium Acetate Buffer, pH 5.2, and mix.

b. The color should now be yellow. If not, add the 3 M Sodium Acetate Buffer, pH 5.2, in 10 µl increments until the mixture is yellow.

9. Add 1 gel volume of 100% isopropanol and mix until homogenous. For a gel with an agarose concentration greater than 2%, use 2 gel volumes of 100% isopropanol.

10. Load the solubilized gel solution mixture from step 6 into the binding column that is assembled in a 2 ml collection tube.

a. It is normal to see an occasional color change from yellow to red once the sample is applied to the binding column.

b. If the volume of the gel mixture is >700 µl, load the sample onto the column in 700 µl portions. c. Centrifuge for 1 minute after loading the column each time. Discard the flow-through liquid.

11. Add 700 µl of Wash Solution (diluted from Wash Solution Concentrate G as described under Preparation Instructions) to the binding column. Centrifuge for 1 minute.

a. Remove the binding column from the collection tube and discard the flow-through liquid.

b. Place the binding column back into the collection tube and centrifuge again for 1 minute without any additional wash solution to remove excess ethanol.

c. Residual Wash Solution will not be completely removed unless the flow-through is discarded before the final centrifugation.

12. Transfer the binding column to a fresh collection tube. Add 50 µl of Elution Solution to the center of the membrane and incubate for 1 minute. Centrifuge for 1 minute.

Yields of large linear DNA fragments (>3 Kb) can also be increased by up to 20% by preheating the elution solution to 65 °C.

13. To increase the concentration of the eluted DNA, the volume of Elution Solution may be reduced to 25 µl.

T/A cloning of PCR fragment

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The ability to construct recombinant DNA molecules and maintain them in cells is called DNA cloning. This process typically involves a vector that provides the information necessary to propagate the cloned DNA in the cell and an insert DNA that is inserted within the vector and includes the DNA of interest. By creating recombinant DNA molecules that can be propagated in a host organism, a particular DNA fragment can be both purified from other DNAs and amplified to produce large quantities. Once the desired DNA fragment is isolated, it must be introduced into a vector for replicating in the host organism. The most common host used to propagate DNA is the bacterium E.coli.

Vector DNAs typically have three characteristics.

(i). they contain an origin of replication that allows them to replicate independently of the chromosome of the host

(ii). they contain a selectable marker that allows cells that contain the vector (and insert) to be readily identified.

(iii). they have unique sites for one or more restriction enzymes. This allows DNA fragments to be inserted at a defined point within the vector.

The most common vectors are small (approx. 3 kb) circular DNA molecules called plasmids. These molecules were originally derived from circular DNA molecules that are naturally found in bacteria

Ligation

Inserting a fragment of DNA into a vector is a relatively simple process. The vector is prepared by digesting with an enzyme that has unique site inorder to linearize it. In commercially available ligation kits, the vector is kept linearized. Suppose a plasmid vector has unique restriction site for EcoRI, the vector is prepared by digesting with EcoRI which linearizes the plasmid. Because EcoRI generates protruding 5' ends that are complementary to each other, the sticky ends are capable of reannealing to reform a circle. Treatment of the circle with the enzyme DNA ligase and ATP would seal the nicks to reform a covalently closed circle. The target DNA is prepared by cleaving it with a restriction enzyme in this case, with EcoRI to generate potential

insert DNAs. Vector DNA is mixed with an excess of insert DNAs cleaved by EcoRI under conditions that allow sticky ends to hybridize. DNA ligase is then used to link the compatible ends of the two DNAs. Adding an excess of the insert DNA relative to the plasmid DNA ensures that the majority of vectors will reseal with insert DNA incorporated.

In principle, cloning in plasmid vectors is very straightforward. Closed circular plasmid DNA is cleaved with one or more restriction enzymes and ligated in vitro to foreign DNA bearing compatible termini. The products of the ligation reaction are then used to transform an appropriate strain of E. *coli.* The resulting transformed colonies are screened by hybridization, by PCR, or by digestion with restriction enzymes to identify those that carry the desired DNA sequences. One of the simple procedure using T/A cloning principle is described below.

T/A Cloning of PCR fragment

Description

The InsTAclone™ PCR Cloning Kit (Fermentas Inc, USA) is a T/A system for direct one-step cloning of PCR products with 3'-dA overhangs (1) generated by *Taq* DNA polymerase and other thermostable DNA polymerases which lack proofreading activity. The kit includes a ready-to-use cloning vector pTZ57R/T. The 3'-ddT overhangs at both ends of the cloning site prevent recircularization of the vector during ligation, resulting in high cloning yields and low background.

Cloning Principle

The InsTAclone™ PCR Cloning Kit takes advantage of the terminal transferase activity of *Taq* DNA polymerase and other non-proofreading thermostable DNA polymerases. Such enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favors direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Such overhangs at the vector cloning site not only facilitate cloning, but also prevent the recircularization of the vector. As a result, more than 90% of recombinant clones contain the vector with an insert. Recombinant clones are selected based on blue/white screening.

Procedure

1. Ligation reaction:

Vector pTZ57R/T- 3 µl

5X Ligation Buffer- 6 µl

PCR product –variable (1:3 ratio of vector insert)

Water, nuclease-free to 29 µl

T4 DNA Ligase- 1 µl

Total volume- 30 µl

If using PCR product directly from PCR mixture, do not add more than 4 µl to avoid inhibition of T4 DNA Ligase by salts. The vector to insert ratio should always be at least 1:3.

2. Vortex briefly and centrifuge for 3-5 seconds.

3. Incubate the ligation mixture at room temperature (22°C) for 1 hour or at 16°C overnight. If maximal number of transformants is required, incubate overnight at 4°C.

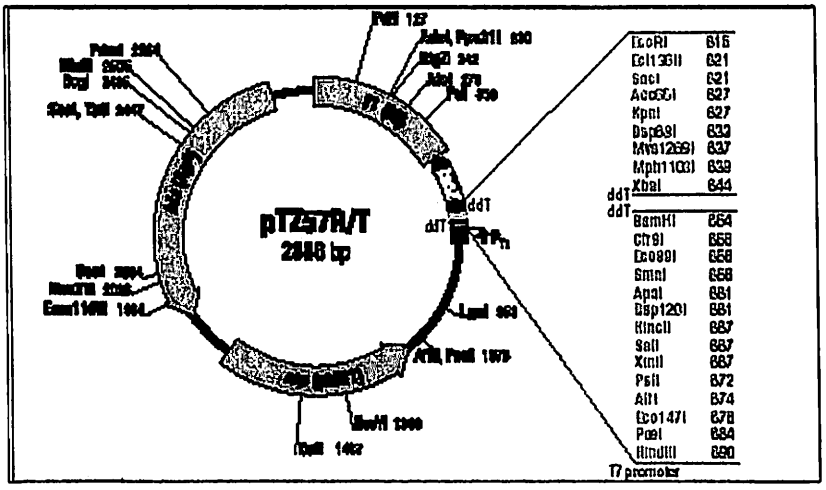
4. Use 2.5µl of the ligation mixture directly for bacterial transformation.

Procedure - Determining DNA concentrations for ligations

For any given ligation, use the following formula:

Amount of insert = insert size / vector size x (molar ratio of insert / vector) x amount of vector to be used.

Vector used for ligation in the TA cloning kit



Two Dimensional Gel Electrophoresis

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Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a method of protein separation, by which proteins in a mixture are separated according to their isoelectric point (pI) in the horizontal direction (isoelectric focusing [IEF]) and molecular weight in the vertical direction (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]). 2D-PAGE is used for the isolation/separation/purification of proteins and further characterization with mass spectrometry and identification of specific proteins. The isoforms of a protein can easily be isolated with 2D-PAGE.

1. Sample Preparation

Appropriate sample preparation is absolutely essential for excellent 2-D results. In general, it is advisable to keep sample preparation as simple as possible. A sample with low protein and high salt concentration, for example, could be diluted normally and analyzed or desalted, then concentrated by lyophilization or precipitated with TCA and ice-cold acetone, then re-solubilized with rehydration solution. The composition of sample solution is particularly critical for 2-D because solubilization treatments for the first-dimension separation must not affect the protein pI, nor leave the sample in a highly conductive solution.

Sample preparation includes the following steps:

1. Take one gram of fresh tissue, grind in liquid nitrogen to a fine powder.
2. Resuspend the powder in an ice-cold solution of 10% w/v trichloroacetic acid (TCA) in acetone with 0.07% w/v Dithiotrietol (DTT) for at least 1 h at -20 °C.
3. Centrifuge it for 30 min at 12,000 rpm and discard the supernatant.
4. Rinse the pellet thrice with acetone containing 0.07% w/v DTT for 1 h at -20 °C.
5. Lyophilize the pellet for two hours to remove any traces of acetone.
6. Solubilize the resulting lyophilized powder in lysis buffer (7 mM urea, 4% CHAPS, 14 mM DTT, and 0.2% Ampholyte) for 1 h at 37°C.

7. Centrifuge at 12,000 rpm for 15 min.
8. Collect the supernatant in a fresh tube.
9. Quantify the protein concentration using the Bradford method (Bradford, 1976).

Note:

- The samples must be stored at -80° C, if stopped at any step during the sample preparation.
- All the reagents and buffers should be prepared with ultra pure chemicals and use ddH_2O in all the steps.
- DTT should be added freshly wherever applicable.

2. Rehydration

Dissolve 100µg of protein in rehydration buffer containing 8 M urea, 2% w/v CHAPS, 18 mM DTT, 0.5% w/v IPG buffer pH 4–7 and a trace of bromophenol blue. The steps mentioned below are to be carried out.

1. Clean the Immobiline strip tray (Ettan IPGphor) and wipe out with paper towels and Kimwipes.
2. Take the strips from - 80 °C and remove the plastic cover carefully.
3. Apply the sample on the strip tray and carefully place the strip over the sample, ensuring that the entire length of the strip touches the sample.
4. Cover the strip tray with coverfluid and close the tray.
5. Leave the sample tray at room temperature for 18 h.

Note: Care should be taken to wear gloves while handling the strips and ensure that the gel side of the strip faces down.

3. First Dimension – Isoelectric focusing (IEF)

1. After rehydration, wet the pre-made IEF strips with HPLC-grade water.
2. Dry the strips slightly between two pieces of Whatman paper to remove water.

3. Make sure that the square end of each strip is at the cathode (Black/-) and the '+'pointed end at the anode (Red/+). Also note that the anode and cathode ridges are in the correct orientation.
4. Electrophores for 24-36 hr or 45000 Vhrs, using the following sequence of settings:

Voltage	Amps	Wattage	Time
500 V	100mA	33V	1 hr
1000 V	110mA	70V	1 hr
2950 V	140mA	32V	24 hr

5. Bromophenol blue migrates towards the anode within 1 hr from the start of the electrophoresis.

Note: If by the next day the bromophenol blue has not disappeared (strip becomes colorless), continue running until the dye disappear.

4. Second Dimension

4.1. Equilibration

Equilibrate the focused strips twice for 15 min in 20 ml equilibration solution as follows:

1. First equilibration in solution containing 6 M urea, 30% glycerol, 2% SDS, 2% DTT, 50 mM TRIS-HCl buffer (pH 8.8).
2. Second equilibration in solution containing 2.5% iodoacetamide in place of DTT.

4.2. Second-Dimension SDS-PAGE:

Perform second dimensional electrophoresis with 1 mm thick, 12 % SDS-polyacrylamide gel in BioRad Protean xi - vertical slab gel electrophoresis unit.

Casting of acrylamide gels (70 ml) and Reagents Preparation:

Acrylamide gel %	12 %	15 %
40 % Acrylamide	21 ml	26.5 ml
1.5 M Tris HCl pH 8.8	17.5 ml	17.5 ml
ddH_2O	31.5 ml	26.25 ml
10% (w/v) APS*	0.35 ml	0.35ml
TEMED	17.5 µl	17.5 µl

*Ammonium persulphate (APS): 0.1 g per ml of ddH_2O. Prepare freshly each time.

- Electrophoresis buffer (25 mM Tris, 190 mM glycine and 0.1% SDS)
- 1% agarose in electrophoresis buffer.

After casting the gel, perform the following steps:

1. Add 1x running buffer powder and 2 liter ddH_2O to the electrophoresis tank. Allow the buffer to mix and cool at least 3 hr before running the gels.
2. Rinse top of gel with 1x electrophoresis buffer.
3. Align the acidic end of the strip with the left end of the gel and lower the strip carefully using a pair of forcep. Make sure that the strip lies flat on the surface of the gel by gently pressing down with two rulers. Eliminate bubbles between the gel surface and the strip.
4. Overlay 1-2ml of agarose and allow it to solidify (10 min).
5. Place the 2-D gels into the electrophoresis tank by sliding gel plate sets between the rubber gaskets. Lubricate the gasket with the running buffer prior to inserting the plates. Make sure gaskets are not folded and that they form a smooth seal along the entire length of each set of gel plates.
6. Place the lid over the gel tank, ensuring the electrodes make good connection. Plug leads into power supply.
7. Run the gel at a constant current of 8 mA for 18-20 hr.

8. Turn off the power supply and remove gels from unit
9. Gels can be stained by either Coomassie brilliant blue (CBB) staining or silver staining methods.

Note: Do not let the agarose to solidify for more than 15 min as the proteins will begin to diffuse. If agarose solution fails to polymerize fully within the first 10 min, place the gel plate at 4 °C for approximately 2 to 4 min.

5. Gel Staining

I. Coomassie brilliant blue staining

Reagents:

Fixing solution : 7% acetic in 50% methanol prepared with ddH_2O

Dye solution : 0.1% Coomassie brilliant blue R 250 in fixing solution

Destaining solution : 5% acetic acid in 20% methanol prepared with ddH_2O

1. After SDS-PAGE, transfer the gel to a plate containing the fixing solution and shake for at least 1 h.
2. Pour out the fixing solution, and replace with the dye solution and incubate for 20 min.
3. Destain the gel with destaining solution and continue with fresh solution until the background is clear.
4. Wash the gel thrice with ddH_2O for 5 min.
5. Acquire the image of the gel in a densitometer (Bio-Rad or GE health care).
6. Gels can be stored in ddH_2O at 4°C for several months.

II. Silver Staining

Reagents

Fixing solution - 10% acetic acid in 40% ethanol prepared with ddH_2O.

Sensitizing solution* - 30% ethanol, 0.2% sodium thiosulphate and 6.8% sodium (oxidizer) acetate in ddH_2O.

Silver nitrate solution * - 0.25% silver nitrate in ddH_2O.

Developing solution* - Add 0.015% formaldehyde to 2.5% sodium carbonate

prepared in ddH_2O just before use.

Stop solution - 5% acetic acid in ddH_2O.

Note: * - To be prepared fresh.

Staining procedure (with gentle shaking all through)

1. Place the gel in a tray containing fixing solution and agitate on a shaker for at least 1 hr. Ensure that the fixer solution covers the gel completely.
2. Drain the fixer solution from the tray.
3. Add sensitizing solution and agitate for 30 min.
4. Drain the sensitizing solution from the tray.
5. Wash the gel thrice in ddH_2O for 5 min.
6. Add silver nitrate solution and agitate for 20 min.
7. After 20 min, drain the silver nitrate solution into an appropriate waste beaker.
8. Wash the gel twice in ddH_2O for 1 min.
9. Add developing solution to the gel, and agitate until yellow or until brown "smokey" precipitate appears. Then pour off developer, add fresh developer as needed and continue in this manner until desired intensity of spots is achieved.
10. Drain the developing solution, add stopper solution and agitate for 10 min.
11. Wash the gel thrice in ddH_2O for 5 min.
12. Acquire the image of the gel with a densitometer.
13. Store the gels in ddH_2O at 4° C.

Note: Gels can be stored at 4° C in ziplock bags for up to two years.

6. Image Analysis

Analyze gel image using IMAGE MASTER Software (GE Health care) and mark protein spots for excision.

7. Excision of Protein Spots (for sequencing by Mass spectrometry)

1. Assign the spot(s) in the gel that are to be sequenced.

2. Cut out the protein spot with a pipette tip.
3. Transfer the gel piece to a microfuge tube.
4. Chop up the gel piece with pipette tip.
5. Add a solution of 50% methanol/10% acetic acid to the gel pieces.
6. Incubate for 30 min.
7. Spin down and discard the supernatant.
8. The sample is ready for Mass Spectrometry Sequencing.

8. Useful Links

Commercial 2-D Electrophoresis and Proteomics Sites

Amersham Biosciences

http://www5.amershambiosciences.com/APTRIX/upp00919.nsf/Content/proteomics_HomePage

BioRad Proteomics Workstation:

http://www.proteomeworks.bio-rad.com/

Genomic Solutions:

http://www.genomicsolutions.com/

2-D Analysis Software Sites

Nonlinear Dynamics:

http://www.phoretix.com/

PDQuest:

http://proteomeworks.bio-rad.com/html/tech5.html

Flicker for 2D gel analysis:

http://www-lecb.ncifcrf.gov/flicker/

NCI/NCRDC LMMB Image Processing Section (GELLAB software):

http://www-lecb.ncifcrf.gov/lemkin/gellab.html

Compugen (Z3 software):

http://www.2dgels.com/

Expasy Index to 2D PAGE databases and services:

http://www.expasy.ch/ch2d/2d-index.html

HSC 2DE Gel Protein Databases list:

http://www.harefield.nthames.nhs.uk/nhli/protein.html

Phosphoprotein Database:

http://www-lecb.ncifcrf.gov/phosphoDB/

Cambridge Proteomics Facility:

http://www.bio.cam.ac.uk/proteomics/index.html

Rice 2D Database:

http://semele.anu.edu.au/2d/2d.html

COMPLUYEAST-2D PAGE Database:

http://babbage.csc.ucm.es/2d/2d.html

Danish Centre for Human Genome Research 2D PAGE Databases (Aarhus):

http://proteomics.cancer.dk/

Siena-2DPAGE:

http://www.bio-mol.unisi.it/2d/2d.html

PMMA-2D Page at Purkyne Military Medical Academy, Czech:

http://www.pmma.pmfhk.cz/

HP-2D PAGE (Max Delbruck Center, Berlin):

http://www.mdc-berlin.de/~emu/heart

MitoDat—Mendelian Inheritance and the Mitochondrion:

http://www-lmmb.ncifcrf.gov/mitoDat/

SWISS-2DPAGE at Geneva University Hospital:

http://www.expasy.ch/ch2d/ch2d-top.html

Proteome BioKnowledge® Library:

http://www.proteome.com/YPDhome.html

Yeast Proteome Map:

http://www.ibgc.u-bordeaux2.fr/YPM/

Melanie:

http://us.expasy.org/melanie

Sources of Information and Methods on 2-D Electrophoresis and Proteomics

Australian Proteome Analysis Facility:

http://www.proteome.org.au/

The Tubingen Proteome Project:

http://www.uni-tuebingen.de/uni/kxm/Proteome/

University of Aberdeen Protein Lab and Proteomics Facility:

http://www.abdn.ac.uk/~mmb023/proteome/index.htm

The EXPASY Swiss 2D-PAGE http://www.expasy.ch/

The Harefield Hospital in London provides links to worldwide databases,upcoming meetings, 2-D gel analysis software, and more:

http://www.harefield.nthames.nhs.uk/nhli/protein/

The laboratory of Dr. James R. Jefferies, parasitology Group, Institute of Biological Sciences, University of Wales at Aberystwyth, Ceredigion, Wales, UK:

http://www.aber.ac.uk/~mpgwww/Proteome/Tut_2D.html#Section_1

Proteomics tools for mining sequence databases in conjunction with Mass Spectrometry experiments: http://prospector.ucsf.edu/

Websites for theoreticaland technical procedures on 2-D gel electrophoresis

- http://www5.amershambiosciences.com/applic/upp00738.nsf/vLookupDoc/172581038-R140/$file/80-6429-60AB_Version_May_2002.pdf
- http://www.bio-rad.com/LifeScience/pdf/Bulletin_2651.pdf
- http://proteomics.cancer.dk/procedures/procedure.html
- http://www.aber.ac.uk/parasitology/Proteome/Tut_2D.html
- http://ca.expasy.org/ch2d/protocols

Genetic Transformation

N. Subramonian and Anna Philip

Genetic Transformation lab

Laminar Air Flow Chamber - Principle, Preparation and Use

A laminar airflow chamber has a small motor that draws in air through a coarse pre-filter, where it loses large dust particles. The air is then blown out through a fine high efficiency particulate air (HEPA) filter that removes particles larger than 0.3µm. The ultra clean air devoid of fungal and bacterial contaminations flows through the work area as long as the chamber is on. The velocity of air flow through the HEPA filter is maintained at about 27 ± 3 m/min preventing contamination of the work area by the technician working at the chamber. The airflow does not hamper use of burner or spirit lamp.

Aseptic operations: Aseptic operations include personal hygiene and clean practices in the laminar flow chamber to avoid contamination from the outside. For good results the level of cleanliness and care to be taken during aseptic operations is the same as doctors observe while operating on patients. The nails on fingers to be trimmed close. Avoid use of jewelry during operations.

Preparation of LFC

1. The technician should wash hands in soap & disinfectant till the elbows & remove footwear before entry in to culture room.
2. Switch on the LFC and run for 15 minutes.
3. Swab the hands with 70% alcohol and sit at LFC.
4. With a cotton swab dipped in 70% alcohol swab roof (front and back), filter (left to right) & floor (front & back) of LFC, leaving no area unclean. Change cotton swabs in between.
5. Swab instruments & place in 70 % alcohol; swab autoclaved sterile vials or media vessels in LFC for use.
6. Swab the outer surface of the LFC.
7. Switch off tube light & switch on the UV lamp for 25 minutes.
8. Avoid exposure of self to UV light.

9. Switch off UV lamp & use chamber after 5 – 10 minutes.

Use of LFC

1. Swab hands & arms up to elbow with 70 % alcohol before sitting at LFC.
2. Rest your elbows at the edge of sterile worktable & avoid leaning in to chamber.
3. Dip instruments in 70 % ethanol, flame sterilize, cool & place instruments on a stand.
4. Cultures and fresh media for subcultures are to be placed at arms length near LFC.
5. Place minimum number of culture vials in LFC while working after swabbing in 70 % alcohol.

Please note the following

- Hands should work away from exposed culture and vials, never pass hands over exposed culture or open sterile media vials.

- When pouring sterile liquids from a flask hold the base of the flask in the palm. Flame the mouth of the flask and tilt the flask with neck pointing towards the filter just above the container to receive the liquid. After transfer flame and cap both the flask and the container.

- When opening a sterile Petri dish, hold the lid with the thumb and middle finger on opposite sides of the lid and open, never permit the hand to pass over the sterile bottom of the dish.

- At the end of each step remove all unnecessary glassware, instruments, culture vials, waste material and material for wash. The material for disposal to be discarded into the dustbin and removed from the culture room at the end of the session. The material for washes to be placed in the wash area. The waste contents of culture vials to be suitably disposed, containers rinsed in tap water and left in wash area.

- At the end of the session at LFC, remove all the instruments, culture vials, etc and swab the LFC with alcohol before shutting off the airflow. If gas burner was in use switch off the cylinder. All electrical gadgets that were in use to be switched off before leaving the culture room.

Plasmid DNA Isolation (Mini Prep)

1. Transfer a single bacterial colony into 2 ml of LB medium containing appropriate antibiotic in a loosely capped 15 ml tube. Incubate the culture overnight at 37°C with a vigorous shaking.

LB (Luria-Bertani) Medium.

Per liter:

To 950 ml of deionized water, add

Bacto-tryptone -10g

Bacto-yeast extract -5 g

NaCl -10g

1. Shake until the solutes have dissolved. Adjust pH to 7.0 with 5N NaOH (0.2ml). Adjust the volume of the solution to 1 liter with deionized water. Sterilize by autoclaving for 20 min at 15lb/sq.in. on liquid cycle.
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at 10,000 rpm for 30 seconds at 4° C.
3. Discard the supernatant and resuspend the bacterial pellet in 100 µl of ice cold solution I by vigorous shaking.
4. Add 200 µl of freshly prepared Solution II. Close the tube tightly and mix the contents by inverting the tube rapidly five times. Do not vortex. Store the tube on ice.
5. Add 150 µl of ice cold Solution III. Close the tube and invert it gently it disperse Solution III through the viscous bacterial lysate
6. Centrifuge at 10,000 rpm for 5 Min at 4° C in a microfuge. Transfer the supernatant to a fresh tube.
7. Precipitate the double stranded DNA with 2 volumes of 95 % ethanol at room temperature. Mix by inverting the tube five times.
8. Centrifuge at 10,000 rpm for 5 min at 4° C.
9. Remove the supernatant using a micropipette. Stand the tube in a inverted position on a paper towel to allow all of the fluid to drain away.

10. Rinse the pellet of double stranded DNA with 1 ml of 70 % ethanol at 4° C. Remove the supernatant as described in step 9 and allow the pellet of nucleic acid to dry in the air for 10 min.

11. Re-suspend the nucleic acids in 50 µl of TE (pH 8.0) and store at -20° C.

Optional:

This step can be included after step 4 to remove RNA. Add pancreatic RNase (Sigma) at the concentration of 50 µl/ml from a stock of 10 mg/ml and incubated at 37 ° C for 30 min. Add equal volume of phenol: chloroform. Mix gently by inverting the tube. After centrifugation at 10,000 rpm for 2 min at 4° C in a microfuge, transfer the supernatant to a fresh tube.

RNase A stock

Prepare a 10 mg/ml stock of RNase in the following:

10 mM Tris-HCl, pH 7.5

15 mM NaCl

Solution I

50 mM Glucose

25 mM Tris (pH 8.0)

10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of 100 ml and stored in 4° C.

Solution II

0.2 N NaOH

1 % SDS

Solution III

Potassium acetate - 60 ml

Glacial acetic acid - 11.5 ml

Water - 28.5 ml

Purification of Plasmid DNA

- The plasmid DNA isolated from 1.5 ml LB is resuspended in 30 µl of TE (pH 8.0) (Tris 10 mM, EDTA 1 mM) and 1 µl of DNase free RNase I (10 µg/ml) and incubated at 37°C for 45 minutes.
- Equal volume of phenol and chloroform and isoamyl alcohol (24: 12: 1) is added and centrifuged at 10,000 rpm for 15 minutes.
- The aqueous phase is transferred to a new tube and equal volume of phenol and chloroform and isoamyl alcohol was added and centrifuged at 10,000 rpm for 15 minutes.
- The aqueous phase was transferred to a fresh eppendorf tube and 1/10th volume of 3M sodium acetate was added and the plasmid was precipitated with two volumes of chilled absolute ethanol incubated at –20°C for 1 hour. The precipitated DNA was centrifuged at 12,000 rpm for 15 minutes at 4°C.
- The DNA pellet was rinsed with 70% ethanol, dried briefly and dissolved in 100 µl of TE. The DNA was quantified by spectrophotometric method and agarose gel electrophoresis.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying and purifying 0.5 to 25 kb DNA fragments. Electrophoresis is the separation of DNA fragments of different size by application of a constant electric field on the fragments placed in a matrix of polymerized agarose. DNA being negatively charged travels towards the anode. The speed of migration, which has an inverse relation with the size of DNA, differs for different sizes of fragments leading to separation. The separated fragments may be visualized by staining the gels with an intercalating dye (ethidium bromide) which fluoresces in UV light. The separated DNA fragments may be once again purified from gel (if necessary) and used for further analysis/manipulation.

The protocol can be divided into three stages:

1. A gel is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated.
2. The DNA samples are loaded into the sample wells and the gel is run at a voltage and for a time period that will achieve optimal separation.
3. The gel is stained or, if ethidium bromide has been incorporated into the gel, visualized directly upon illumination with UV light.

Resolution of DNA fragments on standard agarose gels

Materials:

Electrophoresis buffer (TAE or TBE)
Ethidium bromide solution
Electrophoresis grade agarose
10 X loading buffer
DNA molecular weight markers
Microwave oven
55° C water bath.
Horizontal gel electrophoresis apparatus
Gel casting platform
Gel combs
DC power supply

Preparing the Gel

1. Prepare an adequate volume of electrophoresis buffer (TAE or TBE) to fill the electrophoresis tank and to prepare the gel.

TAE (Tris – acetate)

Working Solution 1X

Concentrated Stock (50 X) for 1 Litre

242 g Tris base

57.1 ml Glacial acetic acid

100 ml of 0.5M EDTA (pH 8.0)

TBE (Tris – borate)

Concentrated Stock (5X)

54 g Tris base

27.5 g Boric acid

20 ml 0.5M EDTA (pH 8.0)

2. Add the desired amount of electrophoresis grade agarose to a volume of electrophoresis buffer sufficient for constructing a gel. Melt the agarose in a microwave oven and swirl to ensure even mixing. Gels typically contain 0.8 to 1.5 % of agarose.

3. Melted agarose should be cooled to 55° C before pouring onto a gel platform. To facilitate visualization of DNA fragments during the run, ethidium bromide solution can be added to the melted agarose to a final concentration of 0.5 mg/ml. Gels are typically poured in 0.1 to 1 cm thickness. It is important to keep in mind that the volume of the sample wells will be determined by both the thickness of the gel and the size of the gel comb.

Appropriate agarose concentrations for separating DNA fragments of various sizes

Agarose (%)	Effective range of resolution of linear DNA fragments(kb)
0.5	30 to 1.0
0.7	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

4. Seal the casting platform if it is open at the ends. Pour in the melted agarose and insert the gel comb, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the agarose are removed before the gel sets. Most gel platforms are sealed by taping the open ends with adhesive tape.

Loading and running the gel

5. After the gel has hardened, remove the tape from the open ends of the gel platform. This is usually sufficient to ensure that the sample wells are completely sealed and to prevent tearing of the agarose upon removal of the comb. Low percentage gels and gels made from low gelling/melting temperature agarose should be cooled at 4° C to gain extra rigidity and prevent tearing.

6. Place the gel casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm (or just until the tops of the wells are submerged). Make sure no air pockets are trapped within the wells.

7. DNA samples should be prepared in a volume that will not overflow the gel wells by addition of the appropriate amount of 10 X loading buffer. Samples are typically loaded into wells with a pipette or micropipette. Care should be taken to prevent mixing of the samples between wells. Be sure to include appropriate DNA molecular weight markers.

10 X Loading Buffer (storage temperature 4°C)

0.25 % bromophenol blue

0.25 % xylene cyanol FF

30 % glycerol in water.

8. Be sure that the leads are attached so that DNA migrates into the gel toward the anode or positive lead. Set the voltage to the desired level, typically 1 to 10 V/cm of the gel, begin electrophoresis. The progress of the separation can be monitored by the migration of the dyes in the loading buffer.

Staining the gel

9. Turn off the power supply when the bromophenol blue dye from the loading buffer had migrated a distance judged sufficient for separation of the DNA fragments. If ethidium bromide has been incorporated into gel, the DNA can be visualized by placing on a UV light source and can be photographed directly.

Preparation and Transformation of Competent *E. coli*

Fresh competent *E.coli* prepared using calcium chloride

The following procedure is frequently used to prepare batches of competent bacteria that yield 5 x 10^6 to 2 x 10^7 transformed colonies per µg of super coiled plasmid DNA. This procedure works well for most strains of E.coli.

1. Pick a single colony (2 – 3 mm in diameter) from a freshly grown plate and incubate in 10ml LB broth for 16 – 20 hours at 37°C and inoculate 1 ml of the grown culture into 25 ml of LB broth. Incubate the culture for 3 hours at 37°C with vigorous shaking (300 cycles/minute in a rotary shaker). For efficient transformation, it is essential that the number of viable cells should not exceed 10^8 cells /ml. To monitor the growth of the culture, determine OD 600 every 20 – 30 minutes. The culture can be kept on ice once the OD reaches 0.375.

2. Aseptically transfer the cells to sterile centrifuge tubes and cool the cultures to 0°C by keeping the tubes on ice for 10 minutes. All subsequent steps in this procedure should be carried out aseptically.

3. Recover the cells by centrifugation at 6000 rpm for 10 min at 4°C.

4. Decant the media from the cell pellet and resuspend each pellet in 12.5 ml ice cold 0.1 M Calcium chloride and store on ice. (Calcium chloride can be stored as a 1M stock solution in 10 ml aliquots at -20°C. When preparing competent cells, thaw an aliquot and dilute it to 100 ml with pure water. Filter sterilize the solution and then chill it to 0°C).

5. Recover cells by centrifugation at 6000 rpm for 10 minutes at 4°C.

6. Decant the supernatant from the cell pellets and resuspend each pellet in 2 ml of ice cold 0.1 M calcium chloride.

7. At this point the cells can be dispensed into aliquots that can be frozen at -70°C. The cells maintain competency under these conditions, although the transformation efficiency may drop slightly during prolonged storage.

Transformation

8. Using a chilled sterile pipette tip, transfer 200 µl of each suspension of competent cells to a sterile microfuge tube. Add DNA (no more than 50 ng in a volume of 10µl or less) to each tube. Mix the contents of the tubes by swirling gently. Store the tubes on ice for 30 minutes.

Be sure to include the following controls in your experiment.

a. Competent bacteria that receive a known amount of the standard preparation of super coiled plasmid DNA
b. Competent bacteria that receive no plasmid DNA at all.

9. Transfer the tubes to a rack placed in a circulating water bath that has been preheated to 42°C. Leave the tubes in the rack for exactly 90 seconds. Do not shake the tubes.

10. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1 – 2 minutes.

11. Add 800 µl LB to each tube. Incubate the cultures for 45 minutes in a shaker incubator (225 cycles/minute or less) at 37°C to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid.

12. Transfer the appropriate volume (up to 200 µl per 90 mm plate) of transformed competent cells onto LB agar medium containing the appropriate antibiotic. Add additional broth if small volumes of culture (<10 µl) are transferred. Using sterile bent glass rod gently spread the transformed cells over the surface of the agar plate.

13. Leave the plate at room temperature until the liquid has been absorbed.

14. Incubate the plates at 37°C in inverted position. Colonies should be appearing by 12 – 16 hours. When selecting for resistance to ampicillin, transformed cells should be plated at low density (< 10^4 colonies per 90 mm plate) and the plates should not be incubated for more than 20 hours at 37°C. β-Lactase, secreted into the medium from ampicillin – resistant transformants, rapidly inactivates the antibiotic in regions surrounding the colonies. Thus plating cells in media with ampicillin at high density or incubating for long period results in the appearance of ampicillin-sensitive satellite colonies.

Agrobacterium mediated transformation

Preparation of explants

- Healthy shoots (main shoots or offshoots) with intact apical meristem and young leaves are collected from the field-grown sugarcane plants.
- Cylinders of young leaf rolls (5–8 cm height) are cut-removed just above the shoot tip.
- These cylinders are treated with 0.1% (w/v) mercuric chloride for 3 min. After each treatment, they were washed thoroughly in sterilized distilled water for 3 times.
- Subsequently, the outer leaves/sheaths are pealed one by one until the cylinder reaches approximately 0.5 cm in diameter.
- The cylinders are chopped into thin rolls (0.5–1.0 mm thick) and used as starting materials for infection.
- The explants are allowed to dry on a sterile filter paper for 10 min.

Culture preparation

- The *Arobacterium* culture containing the gene of interest was raised at 28°C in YEP medium with appropriate antibiotic for bacterial selection for 36hours in a shaker.
- The culture after reaching 0.8-1 OD is taken and spun down at 5000rpm for 10min in a centrifuge.
- To the culture 20 ml of infection medium containing 200µM acetosyringone is added and suspended.
- This is incubated at 28°C in a rotary shaker for 1 hour.

Co-Cultivation

- The dried explants are now placed in the liquid infection medium and incubated for 15mins in a shaker at 28°C.
- A brief vacuum infiltration is given and then the explants are dried on sterile filter paper and placed on MS solid media supplemented with 200µM of acetosyringone.

- This is incubated at 28°C in dark for two days.

Cefotaxime wash

- After 2 days the explants are transferred to a sterile conical flask and washed with sterile water three times.
- Now to the explants 20ml water + 500mg/l cefotaxime is added and washed by shaking for 10 minutes.
- The explants are dried and placed on MS medium supplemented with Cefotaxime 500mg/l with the plant selection antibiotics and incubated at 28°C in the dark for callusing.

Material required:

1. **Infection medium** – MS liquid media + 1.5% sucrose+1.5% glucose+2, 4-D 1mg/l + 100µM Acetosyringone. pH- 5.3.
2. **YEP Medium** – For 100ml

Yeast Extract – 0.1g
Beef Extract - 0.5g
Peptone - 0.5g
Sucrose - 0.5g
$MgSo_4$ $7H_2O$ – 1.5g

Biolistic bombardment

Preparation of gold particle

- 6 microgram of 1 micron (µm) and 6 mg of 1.5 – 3 µm gold particles (Bio-rad) are weighed in a microfuge tube, suspended in 200 µl of absolute alcohol and vortexed for 1-2 minutes.
- The gold suspension is centrifuged for 30 sec at 10,000 rpm. The alcohol is decanted.
- To the gold pellet, 200 µl of sterile Milli-Q water is added and after vortexing for 1 minute, centrifuge for 30 seconds at 10,000 rpm. The water is decanted. The water wash is repeated two times and the final resuspension is done in 200 µl of Milli-Q water and stored at 10°C for further use.

Coating of DNA onto gold particles

- 50 µl of gold suspension is mixed with 5 µg of plasmid DNA, vortex mixed and 20 µl of 0.1 M Spermidine is added with constant vortexing.
- 50µl of 2.5 M Calcium chloride solution is added drop by drop to this mixture with constant vortexing.
- The cocktail is set aside for 10 minutes at room temperature and later centrifuged at 10,000 rpm for 10 seconds and the soup decanted.
- To the DNA coated gold particles 200 µl of absolute alcohol is added and 10 µl is spotted on each macro carrier film.

Explant Preparation

- One week before transformation the explants (embryogenic calli) are placed on a fresh MS callusing medium.
- The explant that is the target tissue is arranged in concentric circles on MS osmotic medium in the center of the petri plate.
- The explants are kept on the osmotic medium for four hours before used for bombardment.

Apparatus setup and bombardment

- The Biolistic gun is placed in the laminar air flow and the inner chamber is

wiped with alcohol.

- The rupture disk, macrocarriers, stopping screen and other accessories are sterilized with 70% alcohol and allowed to dry inside the laminar air flow and kept under UV for half an hour.
- Rupture disk, macrocarrier spotted with gold coated DNA and stopping screen are placed sequentially in the appropriate holders.

Biolistic bombardment

- After setting up the apparatus the explants in the Petri plate are placed in the chamber directly below the stopping screen.
- The chamber is closed and the bombardment is carried out at a pressure of 1100 psi of Helium.
- The explants are bombarded twice; first one at a distance of 4cm and subsequently at 8cm from the stopping screen.
- The bombarded explants are incubated in the dark at 25°C and transferred to primary selection medium after 16 hours.

Transient GUS expression studies

- 24 hours after bombardment the explants are incubated in a microfuge tube containing X-gluc.
- The microfuge tube containing explants are placed overnight at 37°C.
- The X-gluc is decanned and the explants are washed with 70% alcohol.
- Again 70% alcohol is added and the explants are incubated at room temperature for 2-3 hours for destaining.
- The blue spots are observed under stereo microscope.

Materials required:

Preparation of X-gluc: 2mM Stock:

Dissolve X-gluc 5.2mg in 50µl of DMSO and make up to 5ml using a mixture containing 50mM Sodium phosphate buffer pH 7.0, 0.1mM Potassium Ferro cyanide and 0.1mM potassium Ferricyanide.

X-Gluc Staining Solution

2.5 ml of 2mM X-gluc stock was taken and mixed with 1ml methanol and 1.5ml of sodium phosphate buffer.

Estimation of proteins through HPLC

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Solutions:

1. 10 mM Tris buffer, pH.7.5,
2. 50 mM Tris buffer, pH 8.0
3. Acetonitrile
4. Water (HPLC grade)

Sample separation:

Grind 1.0 gram leaf material in 5 ml of 50 mM Tris buffer (pH 8.0) using a mortar and pestle and centrifuge the extract at 10,000 rpm for 15 minutes at 4 °C. The supernatant may be used for further analysis.

Solvent clarification:

Prior to use, filter the solvent through membrane filter using solvent filtration kit to remove the particulates quickly and effectively. Degass the solvent by passing through membrane degasser (for ensuring detector stability), as air bubbles may cause spikes and noise in detector.

Sample filtration:

Before injection, pass the sample through sample filtration syringe (0.5 mm nylon membrane) to assure complete particulate removal so as to prevent undesirable contaminants from clogging within the columns.

Conditions for instrumentation:

Run the reverse phase HPLC with 50 mM Tris buffer (pH 8.0) using acetonitrile as mobile phase in the ratio of 75: 25 at a flow rate of 1.0 ml per minute, maintained by a binary pump. The separation may be carried out in a C-18 column (Particle size 3mm, dimension 83x 46 mm), and detect the separated peaks at 280 nm using a band width of 5nm. Calibrate the retention time and area percent of the sample peaks using the Class VP series software (Shimadzu).

Separation:

1. Equilibrate the column with 10 mM Tris buffer (pH 7.5) for 1-2 hours at a flow rate of 1 ml min^{-1}.
2. Inject 20 µl of samples using a syringe with blunt tipped needle.

3. Run the sample with the mobile phase (50 mM Tris buffer, pH 7.5 and acetonitrile or water and acetonitrile)
4. The retention time, height, area, area percent, height percent and width of the peak obtained for the sample may be detected at 280 nm

Quantification:

Run the known concentration of standard protein, and using the peak area and retention time the protein concentration in the sample is calculated.

Annexure

Good laboratory Practices

Good laboratory practices demand certain basic level of cleanliness, systematic work, proper labeling and safe practices. Under these requirements, all glassware/vessels to be used for preparation of solutions need to be clean. Vessels have to be cleaned in a mild acid, rinsed in water, soaped well and thoroughly rinsed in running tap water. Finally they have to be rinsed in distilled water, drained and air – dried.

Rules under GLP:

- Know where to find exits, fire extinguishers, eyewash station, first aid kit, and cleanup materials for spills.
- Do not eat, drink, smoke, store food or apply cosmetics in the lab.
- Restrain long hair and dangling jewelry. Wear a lab coat.
- Use safety glasses in all experiment in which solutions or chemicals are heated. Never leave heat source unattended.
- Disinfect work surfaces at the beginning and at the end of every lab period.
- Keep liquids away from the edge of lab benches.
- Properly label glass ware/vials/containers (medium code, date of inoculations and clone code).
- Use mechanical pipetting device, avoid mouth pipetting.
- Place contaminated glassware, plastic ware, or disposable materials for autoclaving or directly into 10 % bleach solution in the kitchen before decontamination and reuse or disposal.
- Wear disposable gloves when handling toxic material.

- Report all spills or accidents to the instructor/group leader.

- Wash your hands and remove protective clothing before leaving the lab.