"True wisdom lies in gathering the precious things out of each day as it goes by.” - E.S. Bouton
APPENDIX-I

1. Agarose Gel preparation

Weighed appropriate amount of agarose and dissolved in appropriate volume of 1X TAE buffer (For 1% agarose gel, 1 g of agarose is dissolved in 100 ml of 1X TAE buffer). Heated in a microwave oven to dissolve the agarose and cool to 60°C. Add 0.5 µg of ethidium bromide per ml of agarose solution. Poured into gel tray, adjusted the comb and allowed it to solidify for 30-40 min.

2. Competent cell preparation

Competent bacterial cells are more efficient than normal bacterial cells to uptake DNA. Competent cells prepared by freeze thaw CaCl₂ method which is given as below:

1. Competent cells were prepared and stored at -70°C.
2. For transformation experiments single colony of appropriate E. coli (DH5α) strain of bacteria was streaked on LB agar plate without any antibiotics. This was incubated at 37°C for 14 – 16 hours.
3. Starter culture was prepared by inoculating the fresh colony in 5 ml of LB broth.
4. 250 ml of LB was inoculated with 1 ml of overnight grown starter culture of E. coli and allowed to grow at 37°C on a shaker till the OD at 600 nm reached to 0.5.
5. Culture was cooled immediately on ice and cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C. Supernatant was removed carefully and traces of it were removed carefully by inverting the tube on paper towels. However the tube was never removed from ice for long. The bacterial pellet was resuspended in 50 – 70 ml of ice cold 0.1 M CaCl₂ (sterilized by autoclaving) and incubated on ice for 30 minutes. Cells were recovered by centrifugation as above. Resuspend in 50–70 ml of ice cold 0.1 M MgCl₂ and incubated on ice for 30 minutes. Cells were finally recovered and suspended in 10 ml of 0.1 M CaCl₂ containing 10% glycerol. Aliquots of 200 µl each were prepared and immediately stored at -70°C.
Appendix I

3. Denaturing Formaldehyde Agarose gel preparation

Weighed 1 g of agarose and dissolved in 22 ml of 5X MOPS buffer and 70 ml of DEPC treated water. Melted agarose in a microwave oven and cooled the flask to 50°C. Added 10 ml of formaldehyde solution to it and 5 µl of Etbr (10 mg/ml) were added. Poured into gel tray and allowed it to solidify for 30-40 min.

4. Glycerol stock preparation

For long term storage of the cloned cDNA fragments of all the genes, the bacterial colony with the insert were grown in LB medium supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml) or rifampicin (25 µg/ml) for overnight at 37°C. Equal volume of overnight culture and autoclaved glycerol (30%) were mixed in cryovial, frozen in liquid nitrogen and stored at -80°C.

5. Polyacrylamide Gel Preparation (10%)

Prepared gel casting assembly (Atto Corporation, Japan) with glass plates as specifies by manufacturer.

5.1 Preparation of Resolving Gel

Added 0.95 ml of Tris HCl (1.5M; pH 8.8), 2.5 ml of acrylamide/bis-acrylamide solution (30% stock) and 3.6 ml of distilled water in conical flask. Mixed the solution and kept under vacuum for 30 min to remove trapped air.

1. Added 75 µl SDS (10%), 375 µl of APS (10%) and 250 µl of TEMED into the above solution.
2. Swirled the solution and casted the gel into gel casting assembly.
3. Overlaid the gel with water saturated iso-butanol.
4. Removed iso-butanol after the gel polymerised

5.2 Preparation of Stacking Gel

Added 0.625 ml of Tris HCl (0.5 M; pH 6.8), 0.375 ml of acrylamide/bis-acrylamide solution (30% stock) and 1.225 ml of distilled water in conical flask. Mixed the solution and kept under vacuum for 30 min to remove trapped air.

1. Added 25 µl SDS (10%), 125 µl of APS (10%) and 125 µl of TEMED into the above solution.
2. Swirled the solution and casted the gel into gel casting assembly.
3. Inserted comb in stacking gel and kept the gel undisturbed until polymerized.
4. After polymerization, fixed the gel into running assembly.
5. Filled the assembly with 1X electrode running buffer (Tris-glycine, pH 8.3) and ran the gel under specified conditions.

5.3 Staining of Protein Gel

Placed the gel in a tray containing Comassie brilliant blue stain (40% methanol, 10% glacial acetic acid, 0.1% comassie brilliant blue R250). Kept it for 2h on gel rocker with gentle shaking. Destained the gel in destaining solution (10% glacial acetic acid, 20% methanol) until protein bands appear on gel. Stored the gel in 50% methanol.
APPENDIX II

1. 50X TAE

Dissolved 121 g Tris HCl, 28.5 ml glacial acetic acid and 50 ml EDTA (0.5 M) in 500 ml of distilled water.

2. Ampicillin

Added 1 g ampicillin in 10 ml distilled water and shaked well. Filter sterilized and stored in small aliquots. Working concentration of the antibiotic used was 100 μg/ml.

3. Bradford Reagent

Added 50 mg of Comassie Blue G250 in 50 ml of methanol and 100 ml of 85% H₃PO₄ (Phosphoric acid) to the solution and made up the volume up to 500 ml distilled water. Filtered it to remove precipitates. Added an additional 350 ml of distilled water and stored at 4°C. The solution is light sensitive and was therefore prepared and stored in dark. The solution should be used within one month as it degrades.

4. Bromophenol Blue dye (6X)

Added xylene-cyanol (0.25%), bromophenol blue (0.25%) and glycerol (60%) in distilled water.

5. DEPC water

Added DEPC (0.1 mg per 100 ml) to autoclaved distilled water; mixed well and kept undisturbed for overnight.

6. DNA Isolation Extraction Buffer

Added Tris-HCl (1 M) (1 ml), 5 M NaCl (2.8 ml), 10 % CTAB (2 ml), 0.5 M EDTA (400 μl) and β-Mercaptoethanol (20 μl) in distilled water to make a final volume of 10 ml. Used 500 μl per extraction.

7. dNTPs

Prepared mixture of 10 mM of dATP, dCTP, dGTP and dTTP. Mixed well and stored at -20°C.
8. Ethidium Bromide

Dissolved 100 mg of ethidium bromide in 10 ml of distilled water. Shook it for some time. Divided into small aliquots and covered with aluminum foil before storing at 4°C. Working stock concentration was 0.5μg/ml solution.

9. Formaldehyde Gel Running Buffer for RNA (5X FA)

Dissolved 20.6 g of MOPS in 800 ml of DEPC treated 15 mM sodium acetate. Adjusted pH 7 with 2 N NaOH and added 10 ml of DEPC treated EDTA (0.5M; pH 8.0). Made final volume to 1 L with RNase free water.

10. Infiltration Buffer

Took half strength Murashiege and Skoog salts (2.2 g), 1 X B5 vitamins, sucrose (5 %), 0.5 g MES. Set pH at 5.7 % with KOH. Add 0.044 μM benzyl amino purine and 200 μL/L of Silwet-L-77. Use freshly prepared solution for transformations.

11. IPTG (0.1M)

Add 1.2 g IPTG to 50 ml distilled water. Filter-sterilized, and stored at 4°C in small aliquots.

12. Kanamycin

Added 1 g kanamycin in 10 ml distilled water and shake well. Filter sterilized and stored in small aliquots. Working concentration of the antibiotic used was 50 μg/ml.

13. Luria Broth Liquid Media and Agar Plates

Dissolved 10 g of tryptone, 5 g of yeast extract, 10g of NaCl, and 15 g of Agar (if preparing agar plates) in 1 L of distilled water and autoclaved. Added 0.8 % agar before autoclaving if preparing solid medium.

14. Mg⁺⁺ Stock (2M)

Added 20.33 g MgCl₂.6H₂O and 24.65 g MgSO₄.7H₂O to 100 ml distilled water. Filter sterilized and stored in small aliquots.

15. Murashiege Skoog Solution
15.1 Micronutrient Stock Solution

Added 70 mM of Boric acid (H₃BO₃), 14 mM Manganese Chloride (MnCl₂), 0.5 M copper sulphate (CuSO₄), 1mM zinc sulphate (ZnSO₄), 10 mM (NaCl), 0.2 mM sodium molybdate (Na₂MoO₄) and 0.01mM cobaltous chloride (CoCl₂). Dissolved all the chemicals in distilled water one by one. Made final volume upto 100 ml.

15.2 Macronutrient Stock Solution

1 ml of CaCl₂ (1M), 0.375 ml of MgSO₄ (1M), 4.7 ml of KNO₃ (1M), 5.15 ml of NH₄NO₃ (1M), 0.312 ml KH₂PO₄ (1M), 0.62 ml FeEDTA (1M) in 1L of solution.

16. Phosphate Buffer

For 0.1M; pH 7.0, 71.7 ml of K₂HPO₄ (1M) was titrated against 28.3 ml of KH₂PO₄ (1M) and the final volume was made upto 1L. For 0.1M; pH 7.8, 90.8 ml of K₂HPO₄ (1M) was titrated against 9.2 ml of KH₂PO₄ (1M) and the final volume was made upto 1L.

17. Plasmid Isolation Solutions

17.1 P1 solution:

The solution contained TrisHCl pH-8.0 (50 mM), EDTA (10 mM) and 100 μg/ml RNase was added before using.

17.2 P2 solution:

The solution contained NaOH (4M) and SDS (10%).

17.3 P3 solution:

The solution is prepared by dissolving potassium acetate (3M) in distilled water.

18. Rifampicin

Added 1g rifamicin in 10 ml DMSO and shake well. Filter sterilized and store in small aliquots. Working concentration of the antibiotic used was 25 μg/ml.
19. RNase A

Dissolved 10 mg RNase A in 1 ml of double distilled water containing 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl. Heated for 15 min at 100°C. Cooled to RT and stored in aliquots at -20°C.

20. Sodium Azide

Dissolved 30 mg of NaN₃ and 5 ml 10 mM phosphate buffer pH 7.8 at 45 ml of distilled water. Stored at 4°C.

21. Solutions for SDS PAGE and 2DE

21.1 Acrylamide/Bis-acrylamide Solution (30%):

Added 30 g of acrylamide to 0.8 g of bis-acrylamide in distilled water to a final volume of 100 ml and stored at 4°C in dark.

21.2 Agarose Sealing Solution:

Added SDS electrophoresis buffer, agarose 0.5% and bromophenol blue 0.002% (w/v) into a 500 ml erlenmeyer flask. Swirled to disperse. Heated in a microwave oven on low or a heating stirrer until the agarose was completely dissolved. The solution was not allowed to boil over. Dispensed 2 ml aliquots into screw-cap tubes and stored at room temperature.

21.3 Ammonium Persulfate (10%):

Dissolved 0.1 g ammonium persulfate (FW 228.20) in 1 ml distilled water. Fresh ammonium persulfate "crackles" when water is added. If it does not, replace it with fresh stock. Prepare just prior to use.

21.4 Gel Storage Solution:

For gel storage solution 7% acetic acid was used (7ml acetic acid in 100 ml of solution).

21.5 Rehydration Buffer:

It consisted of lysis buffer, 0.002% bromophenol blue, 18 mM DTT* (added prior to use) and 0.5% (v/v) IPG buffer (added prior to use). Stored in aliquots at -20°C. DTT was added just prior to use (2.8 mg DTT per 1 ml aliquot of rehydration stock solution). For rehydration loading, sample was also added to the 1ml aliquot of rehydration solution just prior to use.
21.6 Resolving Gel Buffer (4X):

Tris base 181.7 g was dissolved in 750 ml of double distilled water and the pH was adjusted to 8.8 with HCl. Volume was made up to 1L using double distilled water. Filtered the solution through a 0.45 μm filter and stored at 4 °C.

21.7 Sample Buffer (5X):

Tris-HCl (0.225M; pH6.8), sucrose (10%), 2% SDS, bromophenol blue (0.05%), β-mercaptoethanol (2.5 ml).

21.8 SDS (10%):

5.0 g of SDS (FW 288.38) was dissolved in 40 ml of distilled water and the volume was made up to 50 ml.

21.9 SDS Equilibration Buffer:

Dissolved Tris-HCl (pH 8.8; 50 mM), urea (6 M), glycerol (30% v/v), SDS (2% w/v) and bromophenol blue (0.002% w/v) to make the solution 1L with distilled water. 1% DTT and 4% iodoacetamide were added just prior to use.

21.10 Urea/ thiourea Lysis Solution (pH 7.4):

The solution consisted of urea (7 M), thiourea (2 M), CHAPS (4% w/v), Tris HCl (18 mM), trizma base (14 mM), triton X-100 (0.2% v/v), Pharmalyte (1% v/v; pH 3-10 [Use Pharmalyte 3–10 for Immobiline Dry Strip 3–10 or 3–10 NL, Pharmalyte 5–8 for Immobiline Dry Strip 4–7]) and protease inhibitor in distilled water.

22. T4 DNA Rapid Ligation Buffer (2X)

Added 60 mM Tris-HCl (pH 7.8), 20 mM MgCl2, 20 mM DTT, 2 mM ATP, and 10% polyethylene glycol (MW8000, ACS Grade). Stored in single-use aliquots at –20°C. Avoided multiple freeze thaw cycles.

23. X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside)

Added 100 mg X-Gal in 2 ml N,N‘-dimethyl-formamide. Covered with aluminum foil and stored at ~20°C in small aliquots.
24. Yeast Extract Mannitol

Added 1 g yeast extract, 10 g Mannitol, 0.5 g dipotassium phosphate, 0.2 g magnesium sulphate, 0.1 g sodium chloride and 1 g calcium carbonate to 500 ml distilled water, mixed well and made the final volume 1 L and autoclaved.