# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Strains

**2.1.1.1 Bacterial:** *E. coli (DH5α)* (gifted by Dr. G. Naresh Kumar, M. S. University of Baroda), *Klebsiella aerogenes* (gifted by Dr. V. Nanjundaiya, II Sc., Bangalore).

**2.1.1.2 *Dictyostelium discoideum*:** Ax2 (Axenic derivative of Raper's wild type NC4) (gifted by Dr. V. Nanjundaiya, II Sc., Bangalore).

### 2.1.2 Sequence of primers

Sequence of primers used for the sequencing of vectors constructed for the expression of recombinant proteins. Sequences were synthesized by MWG Biotech AG, Germany

MR01 GATATGAAATTTCAACATACTTTTATTG

MR04 GGGAGCTCTGAAGATCGCAGCCTTCAACATCC

MR05 CCCTTACTTCAGCATCACCTCCACTGG

MR06 GGGAGCTCCTGCTCAAACCAATGCTCCATG

MR07 GGGAGCTCTTAAGCTTGATAATTATTATAAGCTA

MR09 GGGAGCTCTGAGTAAAGGAGAAGAACTTTTCACTG

MR10 GGGAGCTCTTATTTGTATAGTTCATCCATGCC

MR11 CTGCACAATGTAGCTGACGAGGG

### 2.1.3 Enzymes

Enzymes used for digestion and modification of DNA/RNA were procured from Roche, New England Labs and Bangalore Genei)

- AMV reverse transcriptase
- Bacteriophage T4 polynucleotide kinase
- Calf intestinal alkaline phosphate (CIP)
- DNaseI
- DNA ligase (*E. coli*)
- DNA polymerase I (*E. coli*)
- *E. coli* DNA polymerase I Klenow fragment
- Proteinase K

- Restriction enzymes - *ClaI, XhoI, EcoRV, BssHII, BstXI ,SalI, SacI, SacII, NotI, KpnI, XbaI, HindIII, NarI, EcoRI, NdeI.*
- RNase
- *Taq* DNA polymerase
- Bacteriophage T4 DNA ligase

### 2.1.4 Fine chemicals

Fine chemicals were procured from Sigma, USA.

- Geneticin disulfate(G148)
- Ethylenediamine-tetraacetic acid, disodium dihydrate (EDTA),
- Ethylene glycol-bis(β-amminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)
- Adenosie-5'-triphosphate, disodium trihydrate (ATP)
- Isopropylthio-β- D-galactoside (IPTG)
- 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)
- Dithiothreitol (DTT)
- Ammonium persulfate (APS)
- N,N,N',N'-Tetramethylenediamine (TEMED)

### 2.1.5 Restriction map of plasmids

Restriction maps of plasmids used for the construction of the secretory vector and expression of recombinant proteins.

**2.1.5.1** pBR322 (gifted by Dr. G. Naresh Kumar, M. S. University of Baroda)

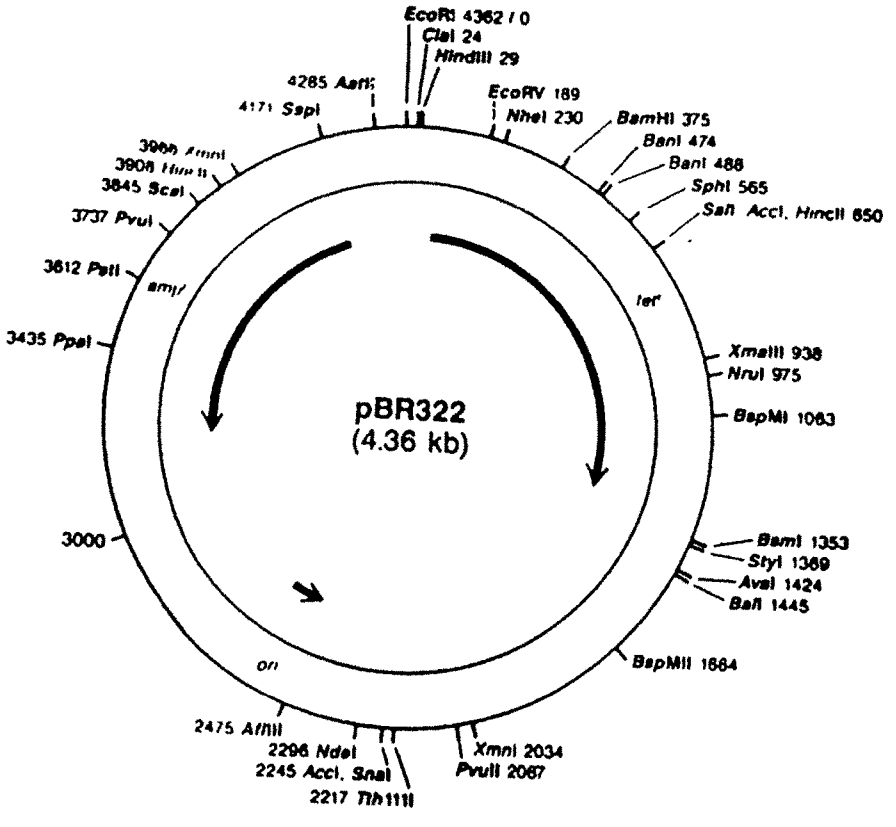
**2.1.5.2** pBluescript II (KS+/-) (gifted by Dr. G. Naresh Kumar, M. S. University of Baroda)

**2.1.5.3** pBluescript II (SK+/-) (gifted by Dr. G. Naresh Kumar, M. S. University of Baroda)

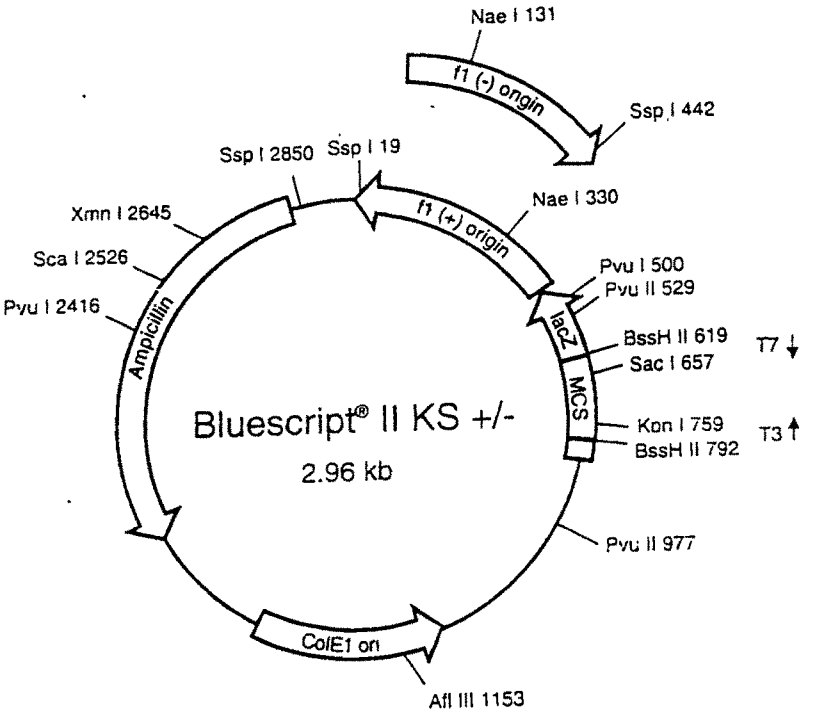
**2.1.5.4** pA15GFP (gifted by Dr. V. Nanjundaiya, II Sc., Bangalore).

**2.1.5.5** PERIII (gifted by Prof. T. Dingermann, Germany).

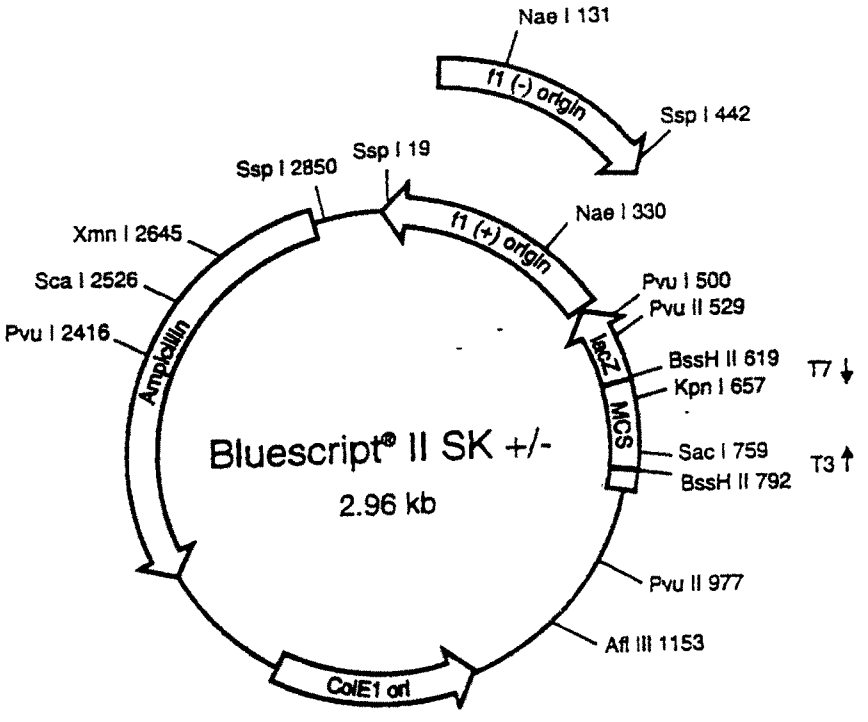
**2.1.5.6** pGEM-T (Promega)



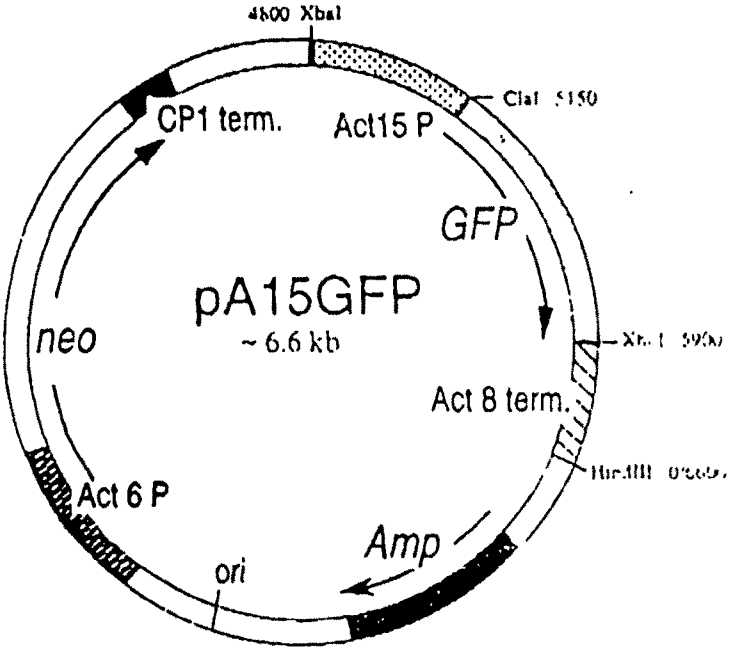

**2.1.5.1 : Restriction map of pBR322**

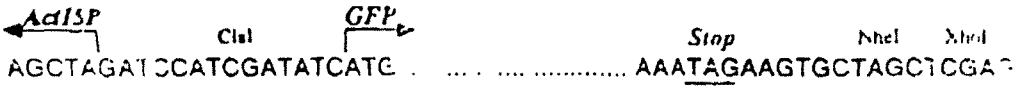



**2.1.5.2 : Restriction map of pBluescript II - KS +/-**

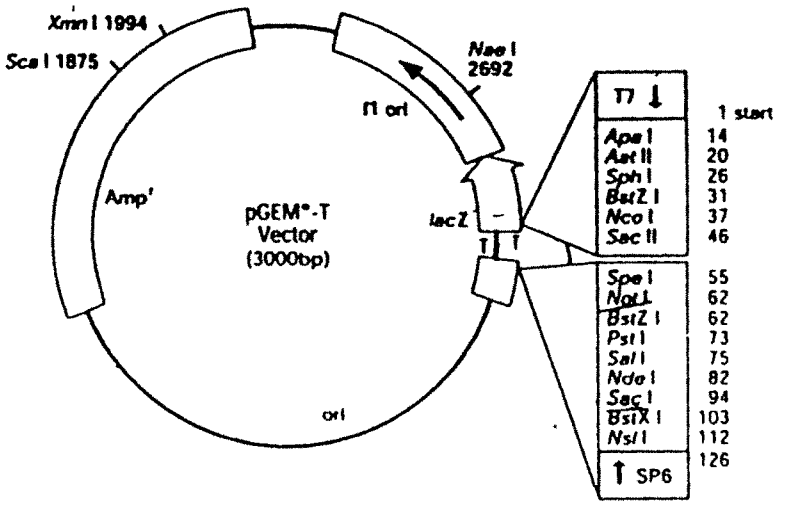



**2.1.5.3 : Restriction map of pBluescript II - SK +/-**



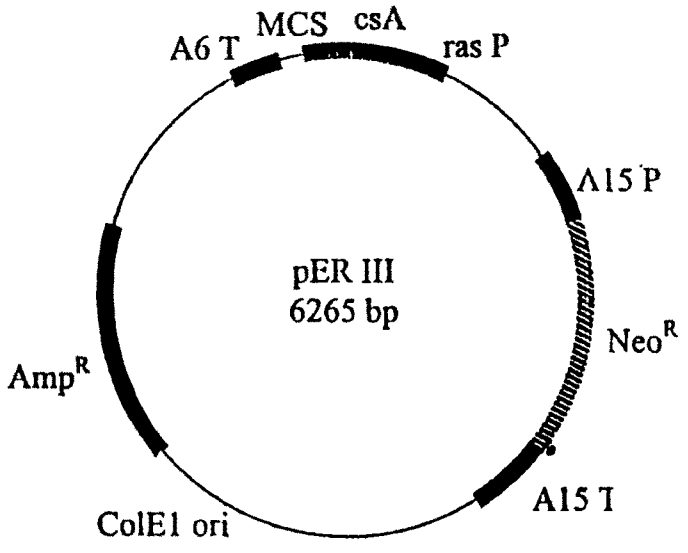

**2.1.5.4 : Restriction map of pA15GFP**




**pGEM®-T Vector Sequence reference points:**

| | |
|---|---|
| T7 RNA Polymerase transcription initiation site | 1 |
| SP6 RNA Polymerase transcription initiation site | 126 |
| T7 RNA Polymerase promoter (–17 to +3) | 2984–3 |
| SP6 RNA Polymerase promoter (–17 to +3) | 124–143 |
| multiple cloning region | 10–113 |
| *lacZ* start codon | 165 |
| *lac* operon sequences | 2821–2981, 151–380 |
| *lac* operator | 185–201 |
| β-lactamase coding region | 1322–2182 |
| phage f1 region | 2365–2820 |
| binding site of pUC/M13 Forward Sequencing Primer | 2941–2957 |
| binding site of pUC/M13 Reverse Sequencing Primer | 161–177 |

## 2.1.5.5 : Restriction map of pGEM-T




*Xba* I

ras-Promotor 5' -ATG TCT AGA TTT TTA GTA TTG ATA ATA TTA TAT AAT ATT TTA AAT AGT
**Met Ser Arg Phe Leu Val Leu Ile Ile Leu Tyr Asn Ile Leu Asn Ser**

*BamH* I *Kpn* I

GCA CAT TCA GCT CCA ACC CAG GAT CCC CGG GTA CCG AGC TCG AAT TCA
**Ala His Ser**

*Xho* I

TCG ATA TCT AGA TCT CGA GCT CGG TCG- 3'

**2.1.5.6 : Restriction map of pER III**

## 2.2 General culturing and preservation *D. discoideum*

(Bonner, 1967; Loomis, 1982)

### 2.2.1 Axenic Growth of *Dictyostelium*

*Dictyostelium* grows with a doubling time of roughly 8 to 12 hours depending on temperature, medium and most likely several unknown factors. To ensure good development it is important that the strains be properly maintained. The first thing to do is to be sure that adequate stocks of frozen cultures and spores are available. It is prudent to freeze several vials of a fresh and young culture and to use these to renew the stocks once every 2 months. Antibiotics may be added to the axenic medium to avoid bacterial contamination.

When cells are grown it is important that they be kept growing in exponential phase rather than allowing them to reach stationary phase. Therefore it is important to establish and maintain a reasonable schedule for splitting the cultures. This routine helps insure an adequate supply of cells that develop well. Cells should never be allowed to grow beyond 2-5 X $10^6$ per ml. On the other hand, it is generally not a good idea to split them so that the cell density becomes less than $1X10^4$ cells per ml.

In our laboratory, the cells were grown in HL-5 medium [ampicillin (60 µl/ml) and streptomycin (100 µl/ml)] by incubating at 21°C with shaking at 180 rpm. For starting a culture to be used for experiment following procedure was followed:

1. Doubling time of the cells was calculated since they were last split.
2. Concentration of cells needed to start with in order to have a certain cell density in given time was calculated.
3. Finally, the volume of the current culture needed to add to the new culture was calculated.
4. Appropriate amount of the current cell culture to the new culture was added, taking care to maintain sterility and incubated at 21°C with shaking at 180 rpm.

- Composition of HL-5 Medium

  Maltose (18 gm)
  Yeast Extract (7 gm) (**Oxoid**)
  Peptone (14 gm) (**Oxoid**)
  $Na_2HPO_4$ (0.51 gm)
  $KH_2PO_4$ (0.48 gm)
  dd. $H_2O$ to 1 liter
  pH 6.4
  autoclaved at 15 lb for 15 min.

### 2.2.2 Growth of *Dictyostelium* on bacteria

*Dictyostelium* can also be grown on bacteria. The two most commonly used bacteria are *Aerobacter aerogenes* (also known as *Klebsiella aerogenes*) and *E. coli* B/R.

Growth on autoclaved bacteria:

1. Bacterial culture was grown to stationary phase in SM broth.
2. The grown culture was autoclaved.
3. *Dictyostelium* cells were inoculated at 1 x $10^4$ cells/ml concentration.
4. Incubated at 21°C with shaking at 180 rpm.
5. *Dictyostelium* cells were washed relatively free of bacterial cells by three sequential centrifugations at 1500 rpm for 4 min. each.

Growth in association with bacteria on agar plates:

1. Desired number (50-100 cells) of *Dictyostelium* cells were mixed with 0.2 ml of overnight culture of bacteria and plated on SM agar plate (for a 90 mm plate).
2. Incubated at 21°C for 24 hours.

- Composition of SM broth

  Glucose (10 gm)

  Yeast Extract (1 gm) (**Oxoid**)

  Peptone (10 gm) (**Oxoid**)

  $MgSO_4.7H_2O$ (1 gm)

  $KH_2PO_4$ (2 gm)

  $K_2HPO_4$ (1 gm)

  dd. $H_2O$ to 1 liter

  pH 6.4

- Composition of SM Agar

  SM broth

  Agar (20 gm)

### 2.2.3 Development of *Dictyostelium*

1. An aliquot of cells (at a density of 1 to 2 x $10^6$ cells/ ml) was taken and cell density was determined by counting them on a hemocytometer.
2. 5 x $10^8$ cells were collected by centrifugation at 1500 rpm for 4 minutes.
3. Pellet was resuspended in 0.5 volumes of sterile KK2 buffer, and spun again. The pellet was washed two times more - for a total of 3 washes.
4. Pellet was resuspended following the final wash in 700 µl of KK2 buffer and it was spread on the KK2 agar petri plate (90 mmdiameter).
5. Plate was dried in the laminar flow.
6. Incubated at 21°C till the fruiting bodies appearred (48-72 hours).

- Composition of KK2 buffer

  $KH_2PO_4$ (2.25 gm)

  $K_2HPO_4$ (0.67 gm)

  dd. $H_2O$ to 1 liter

  pH 6.2

- Composition of KK2 agar
  KK2 buffer
  Agar (20 gm)

### 2.2.4 Preparation and use of *Dictyostelium* stocks

#### 2.2.4.1 Preparing glycerol stocks

1. About 5 X $10^7$ cells harvested during exponential growth (a cell density of about 2 X $10^6$ per ml is generally preferred) were pelleted and chilled on ice.
2. 1 ml of 25% glycerol in $H_2O$ was added. Cells were resuspended by pipetting and transferred to storage vials.
3. Vials were closed and placed in -70°C freezer.

Thawing *Dictyostelium* Cells

1. Vials were removed from -70°C freezer and thawed quickly in a 37°C waterbath (careful to prevent the cells from ever warming up significantly).
2. Cells were transferred to a plate containing 10 ml of HL5 media.
3. Cells were incubated overnight at 21°C. Media was changed next day to remove the glycerol.

Reagents needed

- Glycerol (25% in $H_2O$)
- HL-5 Medium

#### 2.2.4.2 Preparing silica stocks

1. Screw-cap glass vials were filled about half full with silica gel (6-12 mesh, grade 40). Baked at 180°C for 90 minutes with the caps off, but covered with foil.
2. 5% solution of milk powder was prepared in distilled water and autoclaved at 15 lbs for 15 minutes (This solution can be stored at 4°C for several weeks).

3. Milk and silica gel vials were cooled on ice for 30 minutes (Considerable heat is produced when the gel is hydrated which can kill spores. Killing of spores can be minimized by this precooling).

4. Spores were harvested from plates with mature fruiting bodies by tapping the inverted plates on the bench (several plates into the same lid). Spores were resuspended in about 0.4 ml per silica. (The plates should be used as soon as the spores have matured. The plates should not be more than a week old. After this time the viability of the spores decreases rapidly).

5. Spore-milk suspension was pipetted into the cold silica gel. Immediately the vials were capped and shaken vigorously for 5-10 seconds. Vials were placed back on ice for additional 5 minutes.

6. Vials were stored with dissicant at 4°C.

Starting cultures from silica stocks.

1. A few granules of silica stock were transferred to 1 ml of cold sterile SM broth.

2. 0.1-0.2 ml aliquots of this solution was mixed with 0.4 ml diluted bacteria (made by mixing 1 loopful of cells with 5 ml SM broth) and plated on SM plate.

3. Incubated at 21°C for 2-4 days

Reagents needed

- Silica gel
- 5% Milk

**2.3 Construction and confirmation of vectors**

**2.3.1Bacterial transformation**

**2.3.1.1 $CaCl_2$ method**

(Sambrook *et al.*, 1989, vol. 1)

1. A single colony of *E. coli* was aseptically inoculated in 2 ml of LB and incubated overnight (o/n) at 37°C with shaking at 180 rpm.

2. 100 µl of the o/n grown culture was transferred to 10 ml of LB in a flask and grown at 37°C with shaking till the cells reached to the log phase.
3. Aseptically 1.5 ml of culture ($10^7$-$10^8$ cells) was transferred to a microfuge tube and cooled down to 0°C by storing the tube on ice for 10 minutes (all subsequent steps were carried out aseptically).
4. Cells were centrifuged at 5000 rpm for 5 minutes at 4°C. Media was removed and the cell pellet was resuspended in 500 µl of 0.1 M $MgCl_2$ and incubated on ice for 15 minutes.
5. Cells were centrifuged at 5000 rpm for 5 minutes at 4°C. Discarded the supernatant and the pellet was resuspended in 500 µl of 0.1 M $CaCl_2$. Incubated on ice for 15 minutes.
6. Again the cells were centrifuged at 5000 rpm for 5 minutes at 4°C. Removed the supernatant and resuspended the pellet in 125 µl of 0.1 M $CaCl_2$. Incubated on ice for 45 minutes.
7. DNA (not more than 10 µl volume) was added to the competent cells, mixed well, and incubated on ice for 30 minutes.
8. Cells were heat shocked at 42°C for 2 minutes in a water bath and rapidly transferred to ice to chill the cells for 2 minutes.
9. 900 µl of LB was added to the tube and incubated at 37°C for 45 minutes.
10. Appropriate volume (50-200 µl) of transformed competent cells were plated on LA+antibiotic plates (for ligated samples-plated total volume) and incubated overnight at 37°C.

Reagents needed

- 0.1 M $MgCl_2$
- 0.1 M $CaCl_2$
- Luria Broth (LB)
- Luria Agar (LA)
- Antibiotic- Ampicillin (100 µl/ml), Tetracyclin (50 µl/ml)

#### 2.3.1.2 Electroporation

(Sambrook *et al.*, 1989, vol. 1)

1. Electrocompetent cells ($10^7$-$10^8$ cells in 50 µl volume) were thawed on ice.
2. DNA (100-500 ng) was added to the cells, mixed well and transferred to precooled cuvett on ice.
3. Electroporated the competent cells at 2.5 V (Biorad multiporator).
4. 1 ml of SOC medium was added to the cells and transferred the cells to a microfuge tube. Incubated at 37°C for 30 minutes.
5. Appropriate volume (50-200 µl) of transformed cells were plated on LA + antibiotic plate (for ligated samples- total volume was plated) and incubated overnight at 37°C.

Reagents Needed

- SOC Medium
- Luria Agar (LA)

### 2.3.2 Extraction of plasmid (lysis by alkali)

(Sambrook *et al.*, 1989, vol. 1)

1. Single bacterial colony was inoculated into 2 ml of LB medium containing the appropriate antibiotic in a tube and incubated overnight at 37°C with vigorous shaking (180 rpm).
2. 1.5 ml of culture was transferred to a microfuge tube and centrifuged at 5000 rpm for 5 min.
3. Medium was removed and the bacterial pellet was resuspended in 100 µl of ice-cold solution I by vigorous vortexing.
4. 200 µl of freshly prepared solution II was added, mixed the contents by inverting the tube rapidly ten times and stored on ice for 5 minutes (no vortexing).
5. 150 µl of solution III was added, vortexd gently for 10 sec. and stored on ice for 5 minutes.

6. Centrifuged at 8000 rpm for 15 minutes and the supernatant was transferred to a fresh tube. Equal volume of phenol: chloroform was added and mixed by vortexing.
7. Centrifuged at 8000 rpm for 5 minutes, the supernatant was transferred to a fresh tube.
8. 5 µl of DNase free pancreatic RNase (20 µg/ml) was added to the supernatant and incubated at 37°C for 30 minutes.
9. DNA was precipitated with 2 volumes of ethanol, incubated at RT for 15 minutes, and centrifuged at 10000 rpm for 20 minutes.
10. Supernatant was removed and the DNA pellet was washed with 300 µl of 70% ethanol.
11. Ethanol was removed by gentle aspiration after centrifuging at 10000 rpm for 5 minutes.
12. DNA pellet was dried in air/ speed vac for 10 minutes and finally dissolved in appropriate volume of TE (pH 8.0).

Reagents needed

- LB Medium
- Solution I
- Solution II
- Solution III
- TE-Saturated Phenol
- Chloroform:Isoamyl Alcohol (24:1)
- Ethanol (100% and 70%)
- TE buffer

### 2.3.3 Agarose gel electrophoresis

(Sambrook *et al.*, 1989, vol. 1)

1. An agarose mix of required concentration in 1X TAE buffer was prepared, melted and cooled to RT.
2. 10 mg/ml of Ethidium Bromide was added to a final concentration of 1 µg/ml, mixed well and poured the gel into the cast.
3. Comb was inserted and kept at RT for solidification of the gel.

4. DNA sample was mixed in appropriate volume of 5X loading buffer, loaded in the well and subjected to electrophoresis in 1X TAE buffer at 5V/cm for 2-3 hours.
5. Gel was visualized under UV light (302 nm).

Reagents needed

- 50X TAE buffer
- 10 mg/ml Ethidium Bromide
- 5X loading buffer

**2.3.4 Preparation of double-strand oligonucleotides for cloning:**

(Kaufman *et al.*, 1995)

1. Two complementary oligonucleotides of interest were designed (synthesized by a DNA synthesizer).
2. 100 pmol of each of the two complementary oligonucleotides were added in a total volume of 20 µl of sterile water in a microfuge tube.
3. 1 µl of 20X annealing buffer was added to the tube, incubated at 90°C for six minutes, followed by slow cooling to room temprature. (The cooling procedure should take 20 to 30 minutes for complete annealing).
4. Phosphate groups to the 5' ends of the oligonucleotides were added by the kinase reaction.
   a. Kinase reaction was set up as follows in a microfuge tube on ice:
      - The annealed oligonucleotides (15 µl)
      - 10X Polynucleotide kinase buffer (3 µl)
      - 10 mM ATP (1.5 µl)
      - 100 mM DTT (1.5 µl)
      - T4 Polynucleotide kinase (15 units)
      - dd. $H_2O$ to a final volume of 30 µl.
   b. Incubated at 37°C for 40 minutes and heated at 70°C for 10 minutes to Inactivate the enzyme.
   c. Equal volume of TE buffer (pH 7.5) was added and carried out extraction and precipitation.

Reagents needed

- 20X Annealing buffer
  200 mM Tris-HCl, pH 7.9
  40 mM $MgCl_2$
  1M NaCl
  20 mM EDTA, pH 8.0
- T4 Polynucleotide kinase (1 unit/µl)
- 10X Polynucleotide kinase buffer
- 10 mM ATP

### 2.3.5 Restriction enzyme (RE) digestion of DNA

(Sambrook *et al.*, 1989, vol. 1)

1. A standard RE digestion was set up on ice, as follows:
   - Plasmid (0.5-1µg)
   - 10X Appropriate RE buffer (1 µl)
   - 1 mg/ml Acetylated BSA (optional) (1 µl)
   - Appropriate RE (1unit/µg DNA)
   - dd. $H_2O$ to a final volume of 10 µl
2. Incubated at an appropriate temperature depending on the RE used (as recommended by the manufacturer) for 2-3 hours.
3. Digestion efficiency was checked by loading 1µl of the sample on 0.8% agarose gel.
4. RE was inactivated as per the manufacturer's recommendations.
5. Digested sample was ethanol precipitated for cloning experiments.

Reagents needed

- Appropriate RE
- 10X Appropriate RE buffer
- 1 mg/ml Acetylated BSA

### 2.3.6 Phosphorylation of DNA

(Kaufman *et al.*, 1995)

1. Phosphate group was added to the 5' end of the insert DNA by following kinase reaction:
   - Blunt end insert DNA (15 µl)
   - 10 X Polynucleotide kinase buffer (3 µl)
   - 10 mM ATP (1.5 µl)
   - 100 mM DTT (1.5 µl)
   - T4 Polynucleotide kinase (15 units)
   - dd. $H_2O$ to a final volume of 30 µl.
2. Incubated at 37°C for 40 minutes and heated at 70°C for 10 minutes to inactivate the enzyme.
3. Equal volume of TE buffer (pH 7.5) was added and carried out extraction and precipitation.

Reagents needed

- 10 X Polynucleotide kinase buffer
- 10 mM ATP
- 100 mM DTT
- T4 Polynucleotide kinase (1units/µl)

### 2.3.7 Dephosphorylation of DNA

(Kaufman *et al.*, 1995)

1. Plasmid DNA (10-20 µg) was digested with 2-3 fold excess of the desired RE.
2. After complete digestion, DNA was purified with phenol:chloroform, precipitated with ethanol and finally dissolved in 100 µl of sterile milliQ water.
3. Dephosphorylation reaction was set up the in a microfuge tube on ice:
   - Digested plasmid DNA (~10 µg in 50 µl)
   - 10X CIP dephosphorylation buffer (10 µl)
   - CIP (7units)
   - dd. $H_2O$ to a final volume of 100 µl.
4. Incubated at 37°C for one hour.

5. To inactivate CIP, 1/10 volume of 200 mM EGTA was added to the reaction mix and heated to 65°C for 10 minutes.
6. The dephosphorylated DNA was purified with phenol:chloroform, precipitated with ethanol, and dissolved in sterile milliQ water for further use (ligation).

Reagents needed

- 10X CIP dephosphorylation buffer
- CIP (1unit/µl)
- 200 mM EGTA

**2.3.8 Elution of DNA from gel**

1. Digested vector and DNA to be inserted was electrophoresed on 0.8 to 2% agarose gel depending upon the DNA sizes.
2. The gel was transferred onto a long-wavelength UV transilluminator to visualize the DNA bands. The band(s) of interest was excised with a clean razor blade.
3. A hole was made at the bottom of a 0.5 ml microfuge tube with the help of a needle and the tube was packed with sterile glass wool.
4. The gel slice was placed into the packed tube. This tube was placed into a 1.5 ml microfuge tube and spun at 8000 rpm for 10 minutes at 4°C.
5. Equal volume of Tris buffer-equlibrated phenol was added to the liquid phase containing the eluted DNA. Spun at 8000 rpm for 5 minutes, the supernatant was transferred to a fresh tube.
6. Equal volume of phenol: chloroform: isoamyl alcohol was added and mixed by vortexing. Centrifuged at 8000 rpm for 5 minutes, supernatant was transferred to a fresh tube.
7. DNA was precipitated with 2 volumes of ethanol, incubated at RT for 15 minutes, and centrifuged at 10000 rpm for 20 minutes.
8. Supernatant was removed and the DNA pellet was washed with 500 µl of 70% ethanol.
9. Ethanol was removed by gentle aspiration after centrifuging at 10000 rpm for 5 minutes.

10. DNA pellet was dried in air/ speed vac for 10 minutes and finally dissolved in appropriate volume dd. $H_2O$

Reagents needed

- Glass wool (autoclaved)
- Agarose
- 1XTAE buffer
- Ethidium Bromide (10mg/mi)
- 5X Loading buffer
- TE-Saturated Phenol
- Chloroform:Isoamyl Alcohol (24:1)
- Ethanol (100% and 70%)

### 2.3.9 Preparation of blunt-end DNA fragments

#### 2.3.9.1 End filling activity

(Kaufman *et al.*, 1995)

1. 5' overhang reaction was set up in a microfuge tube on ice:
   - DNA fragment or vector DNA (2 to 4 μg in 4 μl)
   - 10X 5' overhang buffer (2μl)
   - 2 mM dNTP mixture (2μl)
   - DNA polymerase I (*Klenow* fragment) (2 to 3 units)
   - dd. $H_2O$ to a final volume of 20μl.
2. Incubated at room temparature for 20 minutes and heated at 70°C for 5 minutes to stop the reaction.
3. Equal volume of TE buffer (pH 7.5) was added and carried out phenol:chloroform extraction and precipitation.

Reagents needed

- 10X overhang buffer
- 2mM dNTP mixture
- DNA polymerase I (*Klenow* fragment)

### 2.3.9.2 Exonuclease activity

(Kaufman *et al.*, 1995)

1. 3' overhang reaction was set up in a microfuge tube on ice:
   - DNA fragment or vector DNA (2 to 4 μg in 4 μl)
   - 10X 5' overhang buffer (2μl)
   - 2 mM dNTP mixture (2μl) (optional)
   - DNA polymerase I (*Klenow* fragment) (2 to 3 units)
   - dd. $H_2O$ to a final volume of 20μl.
2. Incubated at 12°C for 20 minutes and heated at 70°C for 5 minutes to stop the reaction.
3. Equal volume of TE buffer (pH 7.5) was added and carried out phenol:chloroform extraction and precipitation.

Reagents needed

- 10X overhang buffer
- 2mM dNTP mixture
- DNA polymerase I (*Klenow* fragment)

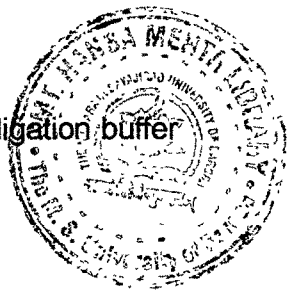
### 2.3.10 Ligation

(Kaufman *et al.*, 1995)

1. Ligation reaction was set up in a microfuge tube on ice:
   - Vector DNA (500 ng in 1 μl)
   - Insert DNA (3 μl)
   - 10X Ligation buffer (1 μl)
   - 10 mM ATP (1 μl)
   - Bacteriophage T4 DNA ligase (1 unit/μl)
   - dd. $H_2O$ to a final volume of 10μl.
2. Incubated overnight at 4°C.
3. Equal volume of TE buffer (pH 7.5) was added and carried out phenol:chloroform extraction and precipitation.

For our experiments the vector: insert ratio was kept 1:3. For blunt end ligation, more ligase (2 units) was used. Although ATP is present in the ligation buffer, it was

added in the reaction mix because repeated freezing and thawing of ligation buffer might lead to degradation of ATP in the buffer.



Reagents needed

- 10X Ligation buffer
- 10 mM ATP
- Bacteriophage T4 DNA ligase (1 unit/µl)

**2.3.11 Polymerase chain reaction (PCR)**

(Kaufman *et al.*, 1995)

1. A standard PCR reaction was set in a 0.5 ml microfuge tube on ice, by adding the following:
   - dd. $H_2O$ (27µl)
   - 10X Amplification buffer (5µl)
   - $MgCl_2$ (25 mM) (3µl)
   - dNTPs (2.5 mM) (8µl)
   - Primer 1 (10 pmoles) in dd. H2O (2µl)
   - Primer 2 (10 pmoles) in dd. H2O (2µl)
   - Template (10 ng) (3µl)
   - *Taq* DNA polymerase (1 unit) (1µl)
2. 30 cycles of PCR amplification was carried out for plasmid DNA in a PCR machine, which was programmed as

| Cycle | Denaturation | Annealing | Polymerization |
|---|---|---|---|
| First 94°C | 2 min at 94°C | - | - |
| Subsequent | 20 sec at 94°C | 30 sec at 63°C | 2 min at 72°C |
| Last | - | - | 10 min at 72°C |

Reagents needed

- 10X Amplification buffer
- $MgCl_2$ (25 mM)
- dNTPs (2.5 mM each)
- Primer 1 in dd. H2O

- Primer 2 in dd. $H_2O$
- *Taq* DNA polymerase

### 2.3.12 Ligation of PCR amplified DNA into pGEM-T vector

The pGEM-T vector system is a convenient system for cloning of PCR products. The 3' terminal thymidine overhangs at the insertion site greatly improves the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3'-ends of the amplified fragments. pGEM-T vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones (white) to be directly identified by colour screening on indicator plates.

1. Ligation reaction was set up as described below in 0.5 ml tubes known to have low DNA binding capacity:
   - 2X Rapid ligation buffer (5μl)
   - pGEM-T vector (50ng) (1μl)
   - PCR product (500ng) (2μl)
   - $T_4$ DNA Ligase (3 Weiss units/μl) (1μl)
   - dd. $H_2O$ to a final volume of 10μl

   (Vortex the 2X Rapid ligation buffer vigorously before each use)
2. Reaction mixture was mixed by pipetting. Incubated for 1 hour at room temperature. Generally incubation overnight at 4°C produces the maximum number of transformants.
3. Centrifuged the tubes containing the ligation.
4. Tubes of frozen competent cells were removed from -70°C storage and placed in an ice bath until just thawed. Cells were mixed by gently flicking the tube.
5. DNA was added to the cells, mixed well and transferred to precooled cuvett on ice.
6. Electroporated the cells at 2.5 V ( Biorad multiporator).

7. 1ml of SOC medium was added to the cells and then cells were transferred to a microfuge tube. Incubated at 37°C for 30 minutes.
8. For blue/white selection of recombinants 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal was added to the cells and mixed well.
9. Appropriate volume (100-200µl) of transformed cells were plated on LA + amp plates and incubated overnight at 37°C.

Reagents needed

- 2X Rapid ligation buffer
- pGEM-T vector
- $T_4$ DNA Ligase (3 Weiss units/µl)
- SOC medium
- 100 mM IPTG
- 50 mg/ml X-Gal
- LA+AMP plates

### 2.3.13 Screening by colony blot

(Sambrook *et al.*, 1989, vol. 2)

Nick translation

1. Dilution of DNase I:

   10 mg/ ml stock solution of DNase I

   ↓ 1: 50,000 dilution in 1 X Nick translation buffer

   200 ng/ ml solution of DNase I
2. Nick translation reaction was set up as follows in a microfuge tube on ice:
   - DNA fragment (1- 0.25 µg in 4 µl)
   - 10 X Nick translation buffer (2 µl)
   - dNTPs – dATP (33 mM each) (1 µl)
   - [$\alpha$-$^{32}$P] dATP (1 µl)
   - DNA Polymerase I (*E .coli*) (1 unit)
   - DNaseI (diluted) (200 pg in 1 µl)
   - dd. $H_2O$ to a final volume of 20 µl.

3. Mixed well and incubated at 16°C for 1 hour.
4. To stop the reaction, 1µl 10 mM EGTA (pH 8.0) was added and incubated at 16°C for 15 minutes.
5. Preparation of column- bottom of a 1 ml disposable syringe was plugged with a small amount of sterile glass wool. The syringe was filled with Sephadex G-50, equilibrated in 1X TEN buffer (pH 8.0), inserted the syringe into a 15 ml tube and spun at 1600g for 4 minutes at RT collecting the effluent from the bottom of the syringe (100 µl) into a de-capped microcentrifuge tube
6. DNA sample (0.1 ml) was applied to the column. The spun column was placed in a tube containing a de-capped microcentrifuge tube.
7. Spun at 1600g, Added 1 µl of NAD (1 mM), 1 µl of DNA ligase (*E. coli*, 10U/µl) and incubated at 16°C for 15 minutes.
8. Added 88 µl of low TE.
9. Probe was transferred to 30-35 ml of hybridization buffer and stored at 4°C.

Replica plating

1. Colonies were replica plated as dots (arranged in grid pattern) on LA plates with antibiotic.
2. Incubated overnight at 37°C.

Colony hybridization

1. A nylon membrane was placed slowly on the plate with colonies and left for 5 minutes.
2. A filter paper was cut and soaked in denaturing buffer on a tray.
3. Membrane with colonies was placed on the soaked filter paper preventing any air bubbles, incubated for 5 minutes and blotted the underside of the membrane on a tissue paper.
4. Another filter paper was soaked in neutralizing buffer, placed the membrane on it, incubated for 5 minutes and again blotted the underside of the membrane on a tissue paper.
6. Membrane was rinsed in 70% ethanol, blotted the underside of the membrane on a tissue paper and baked at 80°C for two hours.

7. Baked membrane was placed in a petri plate with cells facing upper side, added prehybridization buffer (15-20 ml), incubated at 42°C for 30 minutes.
8. The labelled probe was boiled in hybridization buffer for 5 minutes and snap cooled on ice.
9. The probe was added to the membrane after removing the prehybridizing buffer, incubated overnight at 42°C.
10. Membrane was washed in prewashing buffer for 20 minutes with slow shaking.
11. Membrane was transferred to preheated washing buffer (65°C) and incubated at 65°C for 20 minutes.
12. A filter paper was wrapped in saran wrap, placed the membrane on it, again wrapped with saran wrap and placed it in a cassette.
13. In the dark room X-ray film was placed on the membrane and incubated at -80°C overnight.
14. Film was developed next day.

Reagents needed

- 10 X Nick-translation buffer
- dNTPs – dATP (33 mM each)
- [$\alpha$-$^{32}$P] dATP
- DNA Polymerase I (*E .coli*)
- Dnase I
- 10 mM EGTA
- Sephadex G-50
- 1 mM NAD
- DNA Ligase (*E .coli* 10U/$\mu$l)
- TE buffer
- Denaturing solution
- Neutralizing solution
- Prehybridizing buffer
- Hybridizing buffer
- Prewashing buffer (2X SSC)
- Washing buffer

### 2.2.14 Sequencing of DNA

Sequence of the vectors constructed were confirmed by DNA sequencing. 100 ng of template was PCR amplified using the PCR mix containing fluorescent dNTPs provided by ABI PRISM. Purified PCR product was sequenced by ABI PRISM sequencer (courtesy Dr. Thomas Winkler, Germany).

## 2.4 Transformation and clonal seletion in *Dictyostelium discoideum*

(Nellen *et al.*, 1984)

($CaPO_4$ method)

1. Cells ($1X10^6$) were inoculated in a plate (90 mm) in 10 ml HL-5 medium and grown till the plate was full ($2X10^7$ cells).
2. Full grown plates were marked at one end for removing the medium (marking avoids loss of cells from many places). After completely removing the medium from the plate with pasture pipette, 10 ml of MES-HL5 was added to each plate and incubated at 21°C for 45 minutes. In the mean time DNA precipitate was prepared:
   - DNA (7-9 μg)
   - 2X HBS (300 μl)
   - dd. $H_2O$ to 600 μl

   To this mixture added 38 μl of 2 M $CaCl_2$ dropwise with vortexing and incubated at RT for 25 minutes.
3. After 45 minutes of incubation, MES-HL5 was removed completely and DNA precipitate was added dropwise in the centre of the plate, incubated for 30 minutes at 21°C. After 30 minutes, 10 ml of MES-HL5 was added and incubated for 3 hours at 21°C.
4. In the mean time glycerol shock (2 ml/plate) was prepared:
   - milliQ (2ml)
   - 2X HBS (5 ml)
   - glycerol (60%) (3 ml)
4. Medium was removed and glycerol shock was given to the cells by adding dropwise in the centre of the plate and incubated for 8 minutes.
5. Glycerol was removed thoroughly from the plate, added 10ml HL5 and incubated overnight at 21°C.

6. Next day medium was replaced with fresh medium with 10 μg of G418/ml.
7. After 48 hours, medium was replaced with fresh medium with 5μg of G418/ml.
8. Medium was replaced every 48 hours till the clones were visible (transformation efficiency upto $10^{-6}$). Once the clones were visible (within 8-10 days), medium was not changed any more.
9. Fully grown clones were pipetted out along with the medium (50-100 μl) and dropped in a well of coaster well plate, incubated for 1 hour and 300 μl of medium with 5 μg of G418/ml was added. Next day again 200 μl of medium with 5μg μg/ml was added. Cells were allowed to grow till the wells were full. From the full grown wells cells were inoculated in flasks/plates for further experiments.

Reagents needed

- MES-HL5
- 2X HBS

  NaCl (4 gm)

  KCl (0.18 gm)

  Na2HPO4 (0.05 gm)

  HEPES (2.5 gm)

  Glucose (0.5 gm)

  dd. $H_2O$ to 250 ml

  pH 7.1
- 2 M $CaCl_2$
- Glycerol (18% in 1X HBS; made up fresh for each transformation)

**2.5 Confirmation of *D. discoideum* transformants**

**2.5.1 TCA precipitation of growth medium (*D. discoideum*)**

1. Cells were grown to log phase ($5x10^6$/ml) in HL5 medium.
2. 10 ml of culture was centrifuged at 1000 rpm for 5 min.

3. Supernatant was transferred to a fresh tube.
4. TCA solution to a final concentration of 10% was added to the collected medium and incubated overnight at 4°C.
5. TCA precipitated medium was centrifuged at 8000 rpm for 20 min. at 4°C.
6. Supernatant was discarded and pellet was dissolved in minimum volume of 0.1N NaOH.

Reagents needed

- 50% TCA solution
- 0.1 N NaOH

### 2.5.2 Extraction of genomic DNA (*D. discoideum*)

Mini DNA prep

1. $10^7$ to $10^8$ cells were harvested by centrifuging at 1,000 rpm for 5 minutes.
2. Resuspended in 1 ml of Nuclei Buffer.
3. 200 ml 20% triton X-100 was added, incubated on ice for 5 minutes.
4. Centrifuged at 5000 rpm, 5 minutes.
5. Nuclear pellet was resuspended in 300 ml Proteinase K buffer. Incubated at 65° C 20 min.
6. Extracted with an equal volume of phenol:CHCl3 (1:1), followed by CHCl3-isoamyl alcohol.
7. Ethanol precipitated by adding 2.5 volumes of ethanol at room temperature. Incubated for 5 minutes.
8. DNA was pelleted by centrifuging at 10000 rpm for 15 minutes, dried and suspended in appropriate volume of TE .
9. 50 µl of 10 mg/ml RNAse A was added to remove RNA.
10. Ethanol precipitated and dried the DNA pellet.
11. Resuspended in appropriate volume of TE.

Reagents needed

- Nuclei Buffer

  20 mM Tris-HCL (pH 7.4)

  5 mM MgOAc

  0.5 mM EDTA (pH 8.0)

  5% Sucrose

  pH to 7.6

- Protease K Buffer

  100 mM Tris-HCL (pH 7.4)

  5 mM EDTA (pH 8.0)

  0.1 mg/ml Proteinase K

  1% SDS

  pH to 7.5

- 20% Triton X-100

### 2.5.3 Genomic DNA PCR for detecting GFP insert

(Kaufman *et al.*,1995)

Genomic DNA was PCR-amplified as per section 2.3.11 except for the amount of template DNA and number of amplification cycle - 1μg of genomic DNA was used as a template and amplified for 40 cycles.

### 2.5.4 Extraction of total cell RNA (*D. discoideum*)

(Padh *et al.*, unpublished)

1. $10^9$ cells were harvested by centrifuging at 1,000 rpm for 5 minutes.
2. Pellet was resuspended in 9 ml of 5 mM glycine-NaOH buffer, at a cell density of $2X10^8$ cells/ml.
3. Cells were lysed by passing through a layer of 2 polycarbonate Nucleopore filters
4. 1ml of 10 X HMK containing SDS to a final concentration of 0.2% was added.

5. 10 ml of HMK-saturated phenol and 0.1 ml of 4 M Sodium acetate was added and vortexed.

6. 10 ml of chloroform was added, vortexed and spun at 8000 rpm for 10 min. at 4°C to separate the phases

7. The aqueous phase was re-extract with equal volume of phenol:chloroform and the phenol phase was extracted with equal volume of HMK.

8. Centrifuged at 8000 rpm for 10 min. at 4°C, pooled up the aqueous phase and extracted with equal volume of phenol:chloroform twice.

9. Aqueous phase was extracted with equal volume of chloroform twice.

10. 2.5 volumes of 95% ethanol was added and incubated overnight at -20°C to precipitate the RNA.

11. Centrifuged at 10,000 rpm for 25 min. at 4°C. Pellet was washed with chilled 95% ethanol, dried and suspended in appropriate volume of sterile dd. water.

12. RNA prep was confirmed with RNase digestion.

13. Yield and quality of RNA prepration was checked by spectrophotometric analysis at 260 nm and 280 nm.

Reagents needed

- 5 mM glycine-NaOH buffer, pH 8.5
- 10X HMK
  50 mM Hepes-N-(2-Hydroxyethyl) piperazine-N-(2ethane sulfonic acid)
  40 mM Magnesium acetate
  20 mM Potassium chloride
  pH 7.5
- 2% SDS
- HMK-saturated phenol
- Chloroform

- 4 M Sodium acetate, pH 7.5
- 95% Ethanol

### 2.5.5 RT-PCR for detecting GFP mRNA

(Kaufman *et al.*, 1995)

1. 1 µg total RNA template was annealed with primer (20pmol) by heating at 70°C for 5 min.
2. The first-strand cDNAs were synthesized from the isolated total RNA using Reverse transcriptase (RT):
   - 5X RT buffer (2 µl)
   - dNTPs (2.5 mM each) (2 µl)
   - $MgCl_2$ (25 mM) (5 µl)
   - AMV reverse transcriptase (15 units/ µg RNA)
   - nuclease free dd. H2O to a volume of 20 µl
2. Incubated at 42°C for 60 min.
3. RT was inactivated by incubating at 95°C for 5 min.
4. cDNA (20 µl) was PCR-amplified in a total volume of 100 µl for 40 cycles **(section 2.3.11)**.

Reagents needed

- 5X RT buffer
- AMV reverse transcriptase
- 10X Amplification buffer
- $MgCl_2$ (25 mM)
- dNTPs (2.5 mM each)
- Primer 1 in dd. $H_2O$
- Primer 2 in dd. $H_2O$

### 2.5.6 Drop test for confirming the effect of the expressed recombinant protein on development of *D. discoideum*

1. *Klebsiella* cells were grown on SM plates for 48 hours at 21°C (full plate).
2. Growing Ax-2 cells were washed once with KK2 buffer and finally suspended at a concentration of $2x10^5$ /ml of KK2 buffer.

3. 50 µl of cell mix was dropped on the *Klebsiella* lawn, dried in laminar flow and incubated at 21°C for 3-4 days (till the fruiting bodies appeared).

Reagents needed

- SM agar
- KK2 buffer

**2.5.7 SDS-PAGE for detecting the expressed recombinant proteins**

(Sambrook *et al.*, 1989, vol.3)

1. Acrylamide resolving gel of required concentration (depending upon the size of the protein) was prepared and poured.
2. The gel was overlayed with water-saturated butanol and allowed to polymerise (2-3 hours).
3. Once polymerisation was over, upper surface was washed with distilled water and blot-dried.
4. 5% stacking gel was prepared and poured, inserted the comb and allowed to polymerise (1hour).
5. The comb was removed and washed the wells with distilled water.
6. Samples were boiled in 1X Denaturing buffer, boiled for 1 min. and centrifuged to remove the cell debries.
7. Samples were loaded in the wells along with the standards and the gel was electrophoresed at 8 V/cm for stacking and 15 V/cm for resolving gel.
8. Gel was removed and stained in staining solution for 30-45 min.
9. Gel was destained overnight in destaining solution.

Reagents needed

- Acrylamide:bis-acrylamide (29:1) solution
- Running gel buffer
- Stacking gel buffer
- 10% SDS
- 10% Ammonium Persulfate
- TEMED
- Water-saturated Butanol

- 2X Denaturing buffer
- Staining solution
- Destaining solution

**2.5.8 Western blotting for detecting the expressed recombinant proteins**

(Sambrook *et al.*, 1989, vol.3)

Blotting (use gloves)

1. Six pieces of Whatman 3MM filter paper and one piece of nitrocellulose filter (Millipore HAWP) of the exact size of the SDS-polyacrylamide gel were cut. One end of the filter was marked.
2. Nitrocellulose filter was floated in deionized water and allowed to wet.
3. Six pieces of 3MM paper was soaked in a small amount of transfer buffer.
4. Three sheets of 3MM paper were placed on the bottom electrode (anode), one on top of the other. Using a glass pipette as a roller, squeezed out air bubbles.
5. The nitrocellulose filter was placed on the stack of 3MM paper. Squeezed out any air bubbles.
6. SDS-polyacrylamide gel was transferred exactly on top of the nitrocellulose filter. Squeezed out any air bubbles.
7. The final three sheets of 3 MM paper were placed on the gel making sure that no air bubbles were trapped.
8. The upper electrode was fixed and applied a current of 0.65 mA/ sq. cm of gel for a period of 1.5-2 hours.
9. After blotting the nitrocellulose filter was incubated in 3% BSA overnight.

Binding of the primary and secondary antibodies to the target protein

1. Primary antibody was diluted in freshly prepared 3% BSA (1:1000) and incubated the nitrocellulose filter in it for 1 hour.
2. The nitrocellulose filter was washed thrice in 1X TTBS with gentle agitation (10 minutes each washing).
3. The nitrocellulose filter was washed four times in 5% (w / v) non-fat milk solution with gentle agitation (10 minutes each washing).

4. The Horseraddish Peroxidase conjugated secondary antibody (anti-rabbit IgG) was diluted in 5% milk (1:10000) and incubated the nitrocellulose filter in it for 1 hour.
5. The nitrocellulose filter was washed thrice in 5% milk with gentle agitation (10 minutes each washing).
6. The nitrocellulose filter was washed twice in 1X TTBS with gentle agitation (10 minutes each washing).
7. The light detection kit was an ECL kit from Amersham. Nitrocellulose filter was developed as per the Kit's instruction. (Exposure time depends on amount of protein on the blot (i.e. signal strength). The enzyme oxidizes Luminol).
8. Light was detected with Kodak X-Omat films.

Reagents needed

- 3% BSA
- Transfer buffer
- Primary antibody
- Secondary antiboby
- 1X TTBS
- 5% Milk
- ECL Kit

**2.5.9 cAMP Induced expression of DNase I**

(Kastein and Thomas, unpublished)

1. Cells were grown in shaken cultures to 7-9 x $10^6$/ ml.
2. Grown cells were washed twice with Soerensen phosphate buffer and adjusted the cell count to 2 x $10^7$/ml.
3. Cells were shaken at 21°C at 150 rpm for 6 hours and then 200 µM cAMP final concentration was added and cells were shaken for additional 4 hours.
4. 5 ml of the cultures (1x$10^8$ cells) was transferred to a 9 cm perti dish containing phosphate-buffered agar and allowed the cells to settle for about 20 minutes.

5. Supernatant was removed carefully and incubated the plate at 21°C until multicellular stages were observed.

Reagents needed

- Soerensen phosphate buffer
- cAMP
- KK2 agar