Aim
Isolation of pUC18 plasmid from TOP10-pUC18 E coli cells.

Principle
Plasmid is an extra chromosomal DNA present in most of the bacteria and in some yeast. Most of them are circular and they vary in size and in numbers. The plasmids are modified and used as vectors (carrier) in rDNA technology. In this experiment one such modified plasmid pUC18 is isolated form an E. coli culture by alkaline lysis method.

This method is based on the principle that exposure of bacterial suspensions to the strongly anionic detergent at high pH opens the cell wall, denatures chromosomal DNA and proteins and release plasmid DNA into the supernatant. Although the alkaline solution completely disturbs chromosomal DNA, the circular plasmids DNA are unable to separate from each other because they are topologically intertwined. During lysis, bacterial proteins, broken cell wall and denatured chromosomal DNA become enmeshed in large complexes that are coated with dodecyl sulphate. These complexes are efficiently precipitated when solution with sodium ions are replaced by potassium ions. After the denatured materials have been removed by centrifugation native DNA plasmid can be recovered from the supernatant by precipitation.

Alkaline lysis is a flexible method that works well with all strains of E. coli and with bacterial culture ranging in size from 1ml to 500ml. The plasmid DNA obtained by this method is devoid of nuclear DNA.

Materials and Reagents

- LB plate with TOP10-pUC18 colonies
- 1.5 ml microcentrifuge tubes
- LB Broth with 100mg/L Amp
- Solution I
  - 50mM Glucose
  - 25mM Tris-Hcl
  - 10mM EDTA
- Solution II
  - 1% SDS
  - 0.2N NaOH (pH 12.0)
• Solution III
  3M Sodium acetate (pH 5.0)
• TE buffer
  10mM Tris-HCl
  10mM EDTA
• Rnase (1mg/ml)
• Phenol: Chloroform: Isoamyl alcohol (25:24:1)
• Absolute Ethanol
• 70% Ethanol
• 1% agarose gel and a electrophoresis apparatus

Procedure
• A loop full of TOP10-pUC18 cells were inoculated in 5 ml of LB broth with 100mg/L amp and kept in 37°C shaker for 16 hrs
• About 1.5 ml of culture were transferred to centrifuge tubes
• Centrifuged for 5min at 6000rpm at 4°C and the supernatant was removed
• To the pellet 100μl of solution I was added and resuspended by vortexing.
• 200μl of freshly prepared solution II was added, mixed by inversion and incubated in ice for 3 min. Complete lysis was ensured by clear and viscous solution
• 150μl of ice cold solution III was added and incubated in ice for 5 min.
• Centrifuged at 15,000 rpm for 5 min at 4°C and the supernatant were transferred carefully to a new tube.
• To the supernatant equal volume of isopropanol was added was added, mixed gently and centrifuged at 15,000 rpm for 10 min at 4°C
• The supernatant was discarded and the pellet was washed with 70% ethanol
• The pellet was dried and resuspended in 50μl TE
• About 10μl of Rnase was added, incubated at 37°C for 1 hr, purified by phenol chloroform extraction as in Annexure II
• 5μl of the plasmid DNA were loaded in 1% agarose gel, electrophoresed as in Annexure I. The remaining samples were stored in -20°C for future use.

Observation
A clear ladder of bans were seen in the marker lane
An orange florescence bands were observed in the well loaded with plasmids.

Inference
The plasmid isolation was successful. The three different bands that we see in the lane loaded with marker are the three different confirmations of the plasmids isolated. The three confirmations are super coiled, covalently closed and circular plasmids.
EXP:2

RESTRICITION DIGESTION OF DNA

Aim
Restriction digestion of pUC 18 and λ DNA

Principle
A restriction enzyme is an enzyme that cuts double-stranded DNA at specific sites known as restriction recognition sequences. Most of the restriction recognition sequences are palindromic and vary in lengths between 4 and 8 nucleotide. Restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. Some restriction enzymes cut the double stranded DNA in two different positions and generate ends that are staggered, with 5’ or 3’ protruding terminal nucleotides; others cut at the same position and produce blunt ends. Discovery of restriction enzymes lead to the development of recombinant DNA technology and are routinely used for DNA modification and manipulation in laboratories.

In this experiment pUC18 and λ DNA are double digested with EcoRI and HindIII and the restriction recognition sites for these enzymes are given below

Eco RI: G/AATTC
ATTAA/C

Hind III: A/AGCTT
TTCGA/A

Materials and Reagents
• DNA source - pUC18 (Plasmid isolated from EXP 1) and λ DNA
• 1.5 ml micro centrifuge tubes
• Double distilled water
• Assay buffers

Hind III-10 mM Tris-HCl, 50mM NaCl, 10 mM MgCl₂, 1mM DTT.
Eco RI - 50 mM Tris-HCl, 100mM NaCl, 10 mM MgCl₂, 5mM Mercaptoethanol
- Hind III and Eco RI restriction enzymes
- 0.5M EDTA
- 1% agarose gel and Electrophoresis apparatus

Procedure

- A clean 1.5ml microcentrifuge is taken and two reactions were set according to the table

<table>
<thead>
<tr>
<th></th>
<th>pUC18 digestion</th>
<th>λ DNA digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18 plasmid (1ug/μl)</td>
<td>1</td>
<td>λ DNA</td>
</tr>
<tr>
<td>Hind III assay buffer (10X)</td>
<td>2</td>
<td>Hind III assay buffer (10X)</td>
</tr>
<tr>
<td>Hind III (10U/μl)</td>
<td>0.5</td>
<td>Hind III (10U/μl)</td>
</tr>
<tr>
<td>Eco R I (10U/μl)</td>
<td>0.5</td>
<td>Eco R I (10U/μl)</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>16</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>Total</td>
<td>20μl</td>
<td>Total</td>
</tr>
</tbody>
</table>

Note: For double digestion, Hind III buffer was used in which both EcoRI and Hind III will work because of the low salt concentration.

- The added reagents were mixed gently and centrifuged briefly
- The tubes were kept at 37°C for 4 hours
- The enzyme reaction was stopped by adding 1μl of 0.5M EDTA
- 5 μl of the samples were loaded in 1% agarose gel, electrophoresed as in Annexure I and the remaining was stored in -20°C for future use

Observation
- A clear marker lane with ladder of bands were seen in the marker lane.
- Multiple bands of different length were seen in the lane loaded with lambda DNA digest
- A single band were seen in the well loaded with pUC 18 digest

Inference
- The clear bands seen in the well loaded with lambda digest shows the lambda DNA is digested completely and no partial digestion and star activity was seen.
- A single band in the well loaded with pUC 18 digest shows the digestion was complete and plasmid has been linearised.
EXP:3 GEL ELUTION OF DNA

Aim
Purifying pUC18/Hind III/ EcoRI digest by gel elution

Principle
DNA can be recovered from agarose gel through different methods such as electroelution, tresqueezes DNA kits and recovering DNA from low melting point agarose gel. In this experiment low melting agarose gel is used for elution.

In this method special grade of agarose that melt at low temperature without significant deterioration in the strength of the hardened gel is used and this grade of agarose are also free of inhibitors and nuclease. The fragments of DNA are first separated by electrophoresis through and agarose gel of the appropriate concentration located by staining with ethidium bromide. The DNA is recovered by melting the agarose, extracting by phenol:Chloroform and precipitating the DNA. The protocol works best for DNA fragments ranging in size from 0.5 to 5.0 Kb.

Materials
- DNA source - pUC18/Hind III/ Eco RI (Samples prepared from EXP 2)
- 1% low melting agarose gel and electrophoresis apparatus
- Scalpel
- 1.5 ml centrifuge tubes
- Phenol:Chloroform (1:1, v/v)
- Absolute Ethanol
- 70% ethanol
- TE buffer
- 1% Agarose gel

Procedure
- The entire volume of pUC18/Hind III/ Eco RI digest was loaded in a 1% low melting agarose gel, electrophoresed and the bands were visualised under UV
- Using a sharp scalpel the DNA band referring the pUC18 digest were cut down and placed in 1.5 ml centrifuge tubes
The gel pieces were chopped nicely and kept in -80C for overnight
The frozen gel pieces were thawed and about 200μl of phenol: chloroform was added and vortexed for 3 min
Centrifuged at 15,000 rpm for 10min and the upper phase was transferred into a new tube.
Equal volume of ice cold ethonol was added and kept at -20ºC for 1 hr
Centrifuged at 15,000 rpm for 10min and the supernatant was discarded
The pellet was washed with 70% ethonal and the pellet was dried
Resuspended in 20μl TE buffer and 2μl of the purified samples were loaded in 1% agarose gel and electrophoresed as in Annexture I
The remaining samples were stored in -20ºC for future use.

**Observation**
A clear marker lane was seen in the well loaded with 1 Kb ladder
A clear single band were seen the well loaded with gel eluted pUC18 digest

**Inference**
A clear band in the well shows the elution was sucessful and plasmid is intact after elution.
EXP:4  LIGATION

Aim
Ligating the linearized plasmid -pUC18 and the insert –λDNA.

Principle
Ligation of a segment of insert DNA to a linearized plasmid vector involves the formation of phosphodiester bonds between DNA molecule. Ligase catalyse the formation of phosphodiester bonds between the directly adjacent 3’ hydroxyl and 5’phosphoryl termini of nuceic acid molecule. The ligation process consumes ATP as energy source. When cohesive ends are present the ligation occurs efficiently, but when blunt-end fragments have to be ligated the efficiency is very low. Salt and phosphate concentration is very important for the efficiency of ligation. Incubation times and temperatures vary a great deal in the literature but the following seem to work well in most cases.

For “sticky ends”: incubate 2-4 hrs at 16°C; for blunt ends incubate overnight at 4°C.

Materials and Reagents
- Linearized vector -pUC18/Hind III/Eco RI (samples prepared from EXP 3)
- Insert - λ DNA/ Hind III/ Eco RI (Samples prepared from EXP3 and purified as in annexure II)
- T4 DNA Ligase
- 5X Ligase buffer (M Tris-Cl(pH 7.6);100mM MgCl2,200mM DTT1,10mM ATP)
- 0.5ml microtube
- Incubator

Procedure
- The following components were added in a 0.5 ml microtube

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18 / EcoRI / Hind III (100ng/µl)</td>
<td>9</td>
</tr>
<tr>
<td>λDNA / EcoRI / Hind III (100ng/µl)</td>
<td>3</td>
</tr>
<tr>
<td>5Xligase buffer</td>
<td>4</td>
</tr>
<tr>
<td>T4 DNA Ligase (0.1U/µl)</td>
<td>1</td>
</tr>
</tbody>
</table>
| Sterile distilled water | 4  
|                        | 20μl |

Mixed gently and centrifuged briefly

- Incubated at 16°C for 2 hrs and after incubation the samples were stored at -20°C for future use
EXP: 5 PREPARATION OF COMPETENT CELLS

Aim
Preparation of TOP10 competent cells by calcium chloride method

Principle
Competence is the ability of a cell to take up extra cellular DNA from its environment. Competency can be artificially induced by treating the cells with CaCl$_2$ prior to adding DNA. The calcium destabilizes the cell membrane and adheres to the cell surface favoring the formation of the pores for the entry of DNA.

Materials
- LB plates with TOP10 cells
- LB broth and plates
- 0.1M CaCl$_2$
- 1.5ml centrifuge tubes
- Spectrophotometer

Procedure
- A single colony of TOP10 cells were inoculated into 2 ml of LB medium and incubated overnight at 37°C and at 150-200 rpm.
- About 0.4 ml of the overnight culture was used to inoculate 40 ml of LB medium and incubated at 37°C at 150-200 rpm until the OD$_{600}$ reaches 0.4 to 0.5.
- About 1.5 ml of cell culture was transferred to centrifuge tubes and the cells were pelleted down at 6000 rpm for 5 min at 4°C.
- The pellet was resuspended in 1ml of ice cold 0.1 M CaCl$_2$ and incubated in ice for 30 min.
- The cells were again pelleted down at 6000 rpm for 5 min at 4°C.
• The pellet was dissolved in 120μl of 0.1 M CaCl2 and 80μl of 50% glycerol.
• The check the viability a loop full of the above suspension were streaked onto a LB plates and incubated at 37°C for overnight
• The remaining cell suspension were immediately transferred to -80°C for future use

Observation
The colonies were seen on the plates

Inference
The colonies growth infer the competent cells were viable and with out contamination
EXP:6 TRANSFORMATION

Aim
Transformation of TOP10 cells with the pUC18-λDNA ligated product

Principle
Changing the genotype of a cell or organism by transferring foreign DNA is called transformation. The transferred DNA may be maintained as extra-chromosomal elements or integrated into the genome. In this experiment, ampicillin susceptible genotype of E.coli strain TOP10 are changed to ampicillin resistant genotype by transferring pUC18 plasmid that carries a gene for ampicillin resistance and the selection is done in a selection medium with ampicillin.

pUC18 vectors have a short segment of E. coli DNA, which contains the regulatory and coding sequences of Lac Z gene that codes for β-galactosidase enzyme. Isopropyl thiogalactoside (IPTG) is an inducer of Lac Z gene expression. β-galactosidase reacts with the chromogenic substrate 5-bromo-4-chloro-β-D-Galactoside (X-gal) and yields a blue colored product. A multiple cloning site (MCS) is engineered inside the coding region of the Lac Z gene. The MCS as such does not disrupt the reading frame and results only in insertion of a few amino acids in the amino terminal fragment of the β-galactosidase. Therefore, the colonies appear blue in color in the presence of IPTG and X-gal. However, when a insert is cloned in the MCS, that becomes a harmful insertion to the functional properties of β-galactosidase and it can no longer react with X-gal, and therefore, the colonies appear white in color. This is a simple visual color test that can be used to screen thousands of colonies to identify the presence of recombinant plasmids.

Materials
- Competent TOP10 cells (Cells prepared from EXP 5)
- Foreign DNA (Ligated product from EXP4)
- LB medium
- LB plates with 100mg/L Amp
- 42°C water bath
- Isopropyl thiogalactoside (IPTG) 100mm, Sterilize by filtration.
- 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) 20mg/ml dissolved in DMSO.

Procedure

- TOP10 competent cells were taken out from -80°C freezer and thawed in the ice.
- About 5ul of the ligated product were mixed with the competent cells and incubated in ice for 30 min
- The mixture was then subjected to heat shock at 42°C for 45 seconds.
- About 500ul of LB broth was added in a sterile condition and incubated under shaking conditions at 37°C for 1 hr
- Meanwhile, 40μl of IPTG and 40μl of X-gal were plated on top of the LB AMP plates, and the plates were dried
- About 100ul of the inoculum were plated on LB AMP plates containing and IPTG / X-gal, incubated overnight at 37°C for 16 hrs.
- About 100ul of untransformed TOP10 cells were plated on a LB AMP plates containing and IPTG / X-gal as a negative control

Observation
Blue white colonies were seen on incubated plates.

Inference
Blue colonies indicate the self ligated product and the white colonies indicate the recombined products.
Aim

To amplify the inserted λDNA digest in pUC18 vector

Principle

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components. Developed in 1984 by Kary Mullis. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 Kb, although some techniques allow for amplification of fragments up to 40 kb in size.

A basic PCR set up requires several components and reagents. These components include:

- DNA template that contains the DNA region to be amplified.
- Two primers, which are complementary to the DNA regions at the 5’ or 3’ ends of the DNA region.
- Taq polymerase to amplify the DNA
- Deoxynucleoside triphosphates (dNTPs): the building blocks from which the DNA polymerases synthesizes a new DNA strand.
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps

- Denaturation step: This step consists of heating the reaction to 94-98°C for 30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.
- Annealing step: The reaction temperature is lowered to 50-65°C for 30 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.
• Extension/elongation step: At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction,
• Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
• Final hold: This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

Materials

• pUC18-λDNA (Plasmid isolated from putative recombinant cells from EXP 6)
• M13 forward
• M13 Reverse
• Tag DNA polymerase
• dntps
• 10X PCR buffer
• Sterile distilled water
• 0.5ml Micro tube

Procedure

The following components were added in a 1.5 ml microcentrifuge tubes

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18-λDNA (100ng/ul)</td>
<td>5</td>
</tr>
<tr>
<td>M13 forward (10mM)</td>
<td>0.5</td>
</tr>
<tr>
<td>M13 Reverse (10mM)</td>
<td>0.5</td>
</tr>
<tr>
<td>dntps (250 uM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Tag DNA polymerase(10U/ul)</td>
<td>0.2</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>11.3</td>
</tr>
<tr>
<td>Total</td>
<td>20ul</td>
</tr>
</tbody>
</table>

• Negative control was maintained replacing the pUC18-λDNA with pUC18.
• The components were mixed gently and centrifuged briefly
• The tubes were placed in a thermocycler and the programme was followed as mentioned below

1. Initial Denaturation   -95°C 3 min
2. Denaturation          -94°C 30 sec
3. Annealing             -55°C 45 sec
4. Elongation            -75°C 1 min
5. From step 2 to 4 were repeated for 30 cycles
6. Final Extension       -72°C 10 min
7. Final hold            -4°C - ∞

• The 5μl of the samples were loaded in 1% gel and electrophorsed as in Annexure 1.
Annexure: I  

**Agarose gel electrophoresis**

**Principle:** Agarose gel electrophoresis is used to analyse and quantitate nucleic acid. The agarose, for agarose gel electrophoresis, is purified from agar. Agarose is a linear polymer made up of repeating units of 1,3-linked β-D galactopyranose and 1,4 linked 3,6 anhydro α-L galactopyranose

\[ \text{P-D-gal (1-4)-3,6 anhydro - a L Gal (1-3) -} \]n

Agarose has an average MW of 12,000 and contains about 35-40 agarobiose units. Agarose in solution exist as a left-handed double helix. About 7 to 11 such helices form bundles which extend as long rods and appear to interwine with one another, further strengthening the framework of the gel. The cross links are held together by hydrogen and hydrophobic bonds. By changing the gel concentration the pre size can be altered. Higher the concentration of agarose smaller the pre size and vice versa. Because of large pore size even at low concentration, agarose gels are widely used for separation of DNA and RNA.

**Effect of agarose concentration on separation ranges**

The following table describes the relationship between agarose concentration and separation range of nucleic acid

<table>
<thead>
<tr>
<th>Agarose Concentration (%)</th>
<th>Separation range (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>5 to 60</td>
</tr>
<tr>
<td>0.6</td>
<td>1-20</td>
</tr>
<tr>
<td>0.8</td>
<td>0.8 to 10</td>
</tr>
<tr>
<td>1.0</td>
<td>0.4 to 8</td>
</tr>
<tr>
<td>1.2</td>
<td>0.3 to 6</td>
</tr>
<tr>
<td>1.5</td>
<td>0.2 to 4</td>
</tr>
</tbody>
</table>

**Factors which affect the rate of migration of nucleic acids in agarose gels**
Rate of migration of nucleic acids in agarose gels depends mainly on four important parameters.

a. Agarose concentrations

Higher concentration of gels are used for the separation of lower molecular weight DNA and RNA fragments and vice-versa

b. Molecular weight

Duplex DNA fragments migrates at rates inversely proportional to the log Molecular weight. A plot of logM.W vs Mobility gives a straight line

c. Conformation

Supercoiled DNA moves fastest followed by linear forms and relaxed open circular forms.

d. Applied Voltage

At low voltage (<5V/cm) the rate of migration is directly proportional to the applied voltage. However, if the voltage is increased, mobility of high molecular weight DNA fragments increased differentially

e. Base composition and temperature

Base composition and running the gel between 4 and 30°C do not change the mobilities

Preparation of stock solutions for DNA gel electrophoresis

To different buffer systems are widely used for separation of nucleic acids by agarose gel electrophoresis. Their composition are given in the table

<p>| TBE buffer | 10X buffer/l |<br />
| Tris borate (1X TBE) |<br />
| 89mm Tris base | 108.0g |
| 89mm Boric acid | 55.0g |
| 25mm Na2-EDTA | 9.3g |</p>
<table>
<thead>
<tr>
<th><strong>Tris acetate (1X TBE)</strong></th>
<th><strong>10X buffer/lit</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>50mm Tris base</td>
<td>302.5g</td>
</tr>
<tr>
<td>25mm acetic acid</td>
<td>71.4g</td>
</tr>
<tr>
<td>1mm Na2-EDTA</td>
<td>18.6g</td>
</tr>
</tbody>
</table>

Sterilize the stock solutions by autoclaving

**Preparations of ethidium bromide (stock solution)**
Weigh 10 mg ethidium bromide into a sterile tube and dissolve in 10 ml sterile distilled water. The stock is stored at 4°C

**Preparation of sample loading dye Glycerol & bromophenol blue (6x)**
3 ml glycerol (30%), 25 mg bromophenol blue (0.25%) dH2O to 10 mL

**Preparation of agarose solution for casting the gel**
Dissolve the agarose by placing the flasks in boiling water both cool to Luke warm. Cover the sides of a tray using cellotape and place the comb about 1 cm from the top of the tray. Pour the agarose with out making any bubes, cool it for 20 mins and take off the combs and uncover the cellotapes
The DNA sample (100 to 200 ng) is mixed with the loading dye (for 5 µl of DNA sample 1 µl of 6x dye is used) and loaded in to the well carefully, using a pipetman or capillary tube. Once the sample is loaded in to the well, the cathode (Black negative terminal) is connected towards the top end of the gel and the anode (Red positive terminal is connected towards the bottom end of the gel. The maximum volume that can be loaded on to a well formed from a 1.5 mm thickness tooth of the comb is 30 µl. The electrophoresis is started by switching on the D. C. Powerpack. The gel is run at 5v/cm. As the bromophenol blue (the tracking dye) has moved 1 cm above the bottom end, the current is switched off, the power supply is disconnected and the gel along with the platform is stained in the plastic tray containing 0.5 µg/ml ethidium bromide in the sterile distilled water (use gloves when handling ethidium bromide). After about 30-45 min, the platform and the gel is rinsed with distilled water and by keeping the platform in a slanting position, the gel is gently pushed onto the UV Transilluminator. (As UV rays are dangerous for the eye, protect your eyes by wearing a UV
face shield, goggles or using glass plate). Now the UV light is switched on and the DNA bands are seen and Photographed at f 5.6 for 10 seconds with an orange filter.
Annexure: II  PhenoL: chloroform extraction

1. Mix the DNA solution with equal volume of phenol: chloroform (1:1 v/v).

2. Centrifuge at 10,000 rpm 5 min.

3. Transfer the aqueous phase to a fresh tube, add equal volume of chloroform, and centrifuge 10,000 rpm for 5 min.

4. Transfer the aqueous phase to a fresh tube and mix with 1/10th volume of 3 M NaOAc and 2.5 volume of ethanol. Leave at -20°C for 1 h for precipitation.

5. Centrifuge the samples at 10,000 rpm for 10 min; decant the supernatant.

6. Add 1 ml of 70% ethanol to the pellet, vortex and centrifuge at 10,000 rpm for 5 min.

7. Air-dry the pellet and dissolved in appropriate volume of 0.1X TE buffer.