Objectives:

- To learn plasmid preparation by alkaline lysis method.
- To analyze plasmid DNA by agarose gel electrophoresis.

Principle:

Plasmids are double stranded, circular, self-replicating extra chromosomal DNA molecules. They are commonly used as cloning vectors in molecular biology. Many methods are used to isolate plasmid DNA, essentially involving the following steps:

- Growth
- Harvest and lysis of bacteria
- Isolation of plasmid DNA

Alkaline lysis method for rapid purification of plasmids exploits the topological difference between plasmid circles and linear chromosomal fragments. Cells are lysed by treating with alkali (NaOH) and a detergent, sodium dodecyl sulphate (SDS). SDS denatures bacterial proteins and NaOH denatures the plasmid and chromosomal DNA. However, in the case of plasmids the strands remain closely circularized since they are linked by the interwined backbones of double helix. In contrast, strands of linear/nicked DNA are free to separate completely. When this mixture of denatured plasmid and chromosomal DNA is neutralized by the addition of sodium acetate, renaturation occurs. Renaturation of plasmid is rapid and accurate since the strands are already in close physical proximity. Linear molecules generated by random shearing of chromosomal DNA renature less accurately forming networks of DNA that can be removed from lysate by centrifugation, together with denatured protein and RNA. Plasmid DNA remains in solution and can be precipitated using ethanol/isopropanol.
Agarose gel electrophoresis separates plasmid DNA by its size and shape. A preparation of plasmid DNA has predominantly supercoiled form that runs faster by virtue of its compact structure. Introduction of single/double strand breaks lead to presence of relaxed and linear forms respectively. These forms run more slowly since their open structures experience more resistance passing through the gel matrix. These are seen as bands above the supercoiled form (Refer figure 1). However, the presence of linear form is very rarely seen in a plasmid preparation.

Kit Description:
Using this kit, students will purify pUC18 plasmid from E. coli strain. It is a commonly used cloning vector of size 2686 bp with an ampicillin resistance gene. Following isolation and RNase treatment, samples will be analyzed by agarose gel electrophoresis.

The kit contains following solutions required for isolation of plasmid by alkaline lysis method:

Solution I
A buffer which maintains pH, preventing immediate lysis of cells.

Solution II
An alkali & a detergent that disrupts cell membrane & denatures chromosomal & plasmid DNA.

Solution III
A buffer which renatures the plasmid DNA.

Solution IV
An alcohol which precipitates the plasmid DNA.

RNase A: Degrades RNA without affecting DNA.
Plasmid Preparation

**KT06** : Kit is designed to carry out 5 sets of experiments using a single lyophilized vial (host) supplied. Each set of experiment consists of 5 plasmid DNA extractions. The kit also includes electrophoresis equipment (ETS-1) required for agarose gel electrophoresis.

**KT06A** : Kit is designed to carry out 5 sets of experiments using a single lyophilized vial (host) supplied. Each set of experiment consists of 5 plasmid DNA extractions.

**KT06B** : Kit is designed to carry out 20 sets of experiments. Two lyophilized vials (host) are supplied, each vial to be used for 10 sets of experiments. Each set of experiment consists of 5 plasmid DNA extractions.

**Note** : Electrophoresis equipment is required for KT06A and KT06B

**Duration of experiment**: Experiment is carried out over a span of 3 days, approximate time taken on each day is indicated below:

Day 1: 2 hours (Preparation of media and Revival of strain)
Day 2: 15 minutes (Inoculation of media)
Day 3: 3 hours (Plasmid preparation and Agarose gel electrophoresis)

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**Materials Provided:**

The list below provides information about the materials supplied in the kit. The products should be stored as suggested. Use the kit within 6 months of arrival.

<table>
<thead>
<tr>
<th>Materials</th>
<th>KT06/06A (5 sets of expts.)</th>
<th>KT06B (20 sets of expts.)</th>
<th>Store</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg</td>
<td>2 x 100 mg</td>
<td>4°C</td>
</tr>
<tr>
<td>Control DNA (ready to use)</td>
<td>0.15 ml</td>
<td>0.6 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Solution I, II and III</td>
<td>5 ml each</td>
<td>20 ml each</td>
<td>4°C</td>
</tr>
<tr>
<td>2.5X Gel loading buffer</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Host (Lyophilized vial)</td>
<td>1 vial</td>
<td>2 vials</td>
<td>4°C</td>
</tr>
<tr>
<td>RNase A</td>
<td>50 µl</td>
<td>0.2 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>1X TE</td>
<td>0.5 ml</td>
<td>2.0 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Agarose</td>
<td>2.5 g</td>
<td>10 g</td>
<td>RT</td>
</tr>
<tr>
<td>6X Staining dye</td>
<td>40 ml</td>
<td>160 ml</td>
<td>RT</td>
</tr>
<tr>
<td>Solution IV</td>
<td>15 ml</td>
<td>50 ml</td>
<td>RT</td>
</tr>
<tr>
<td>50X TAE</td>
<td>20 ml</td>
<td>80 ml</td>
<td>RT</td>
</tr>
<tr>
<td>LB Broth</td>
<td>5 g</td>
<td>15 g</td>
<td>RT</td>
</tr>
<tr>
<td>Agar</td>
<td>1 g</td>
<td>1 g</td>
<td>RT</td>
</tr>
<tr>
<td>1.5 ml vials</td>
<td>50 Nos.</td>
<td>4 x 50 Nos.</td>
<td>RT</td>
</tr>
</tbody>
</table>
Plasmid Preparation

Materials Required:
Equipment: Gel Rocker (optional), Incubator, Shaker,
Microcentrifuge, Vortex mixer.
Glassware: Beaker, Conical flask, Test tubes,
Measuring cylinder, Petriplates, Staining tray.
Reagent: Distilled water.
Other Requirements: Crushed ice, Micropipettes, Tips.

Note:
- Read the entire procedure before starting the experiment.
- All microbiological operations should be strictly done under aseptic conditions.
- Revive the strain as soon as the lyophilized vial is opened.
- Carry out the experiments within 2 weeks of reviving the strain.
- Thaw solution II before use.
- Control DNA supplied is ready to use and can be loaded directly onto agarose gel.
- Ensure that all components are ready prior to starting the experiment.
- For preparation of media and antibiotic, refer appendix.
- For preparation of gel, staining and destaining, refer agarose gel electrophoresis.

Plasmid Preparation

Procedure:
Day 1: Revival of Host
1. Break open the lyophilized vial and resuspend the sample by adding 0.1 ml of LB broth.
2. Streak a loopful (each) of this suspension on to two LB plates containing ampicillin at a concentration of 100 µg/ml.
3. Incubate the plates at 37°C, overnight.

Day 2
4. Pick a single colony from the LB plate and inoculate into 10 ml LB broth containing ampicillin (100 µg/ml).
5. Incubate in a shaker set at 37°C, overnight.

Note: Store the revived plates at 4°C, use within 2 weeks to carry out 5 sets of experiments in case of KT06/KT06A and 10 sets of experiments in case of KT06B.

Day 3: Plasmid Preparation
Begin alkaline lysis as outlined below (carry out 5 extractions at a time).
6. Pipette 1.5 ml culture each into 5 individual 1.5 ml vials.
7. Spin at 6000 rpm for 8-10 minutes. Discard the supernatant and invert the vial on blotting paper to drain out left over supernatant. Place on ice.
8. Resuspend cell pellet in 100 µl of ice-cold solution I. Vortex gently. Place on ice for 5 minutes and shift to room temperature (RT).
9. Add 200 µl of solution II at RT. Mix gently by inverting the vial five times. (Do not vortex).
10. Add 150 µl of solution III. Mix gently by inverting the vial. Place on ice for 5 minutes.
11. Spin at 6000 - 8000 rpm for 10 minutes.
12. Transfer the supernatant immediately to a fresh vial and add 450 µl of solution IV to precipitate the DNA. Mix by inverting the vial. Incubate at RT for 10-15 minutes.
13. Spin at 10,000 rpm for 20 minutes or at 6,000 rpm for 30 minutes. Decant the supernatant. Invert the vial on blotting paper to drain out left over supernatant. DNA will be seen as white precipitate, sticking to the side of the vial.
14. Dry the sample at 37°C for 10-15 minutes till there is no trace of solution IV.
15. Resuspend the pellet in 20 µl of 1X TE (add along the sides); mix by tapping the vial so that DNA goes into solution.
16. Add 5 µl of RNase A to three vials and incubate at 42°C for 5 minutes or 37°C for 20 minutes. Label these vials as RNase treated. To the remaining two vials, do not add RNase A.
17. Meanwhile, prepare 1% agarose gel.
18. Add 2 µl of gel loading buffer to each of the 5 samples.
19. Load 20 µl of extracted DNA (5 samples) along with 30 µl of control DNA sample on 1% agarose gel and electrophorese at 100 volts for 1-2 hours.
20. Stain with 1X staining dye.
21. Destain to visualize the bands.

**Observation:**

Compare the extracted DNA samples with that of control DNA and observe for bands corresponding to supercoiled and nicked forms. Also observe for the presence or absence of RNA in samples untreated or treated with RNase A (Refer fig 1).

**Interpretation:**

- On analyzing plasmid DNA after electrophoresis, one observes two bands corresponding to nicked and supercoiled DNA. As is seen, the supercoiled form runs faster than the nicked form due to its compact structure.
- RNA, which is a small molecule, is seen migrating faster than the supercoiled DNA in samples not treated with RNase A.

![Fig 1: pUC18 plasmid DNA run on 1% agarose gel. (Stained with EtBr)](image)
Appendix:

**Preparation of LB broth/Agar (1 litre):** Dissolve 25 g of media in 800 ml of distilled water. Adjust the pH to 7.0 with 5N NaOH (if necessary) and make up the volume to 1000 ml, sterilize by autoclaving.

For LB agar, add 1.5% agar and autoclave.

**Ampicillin Preparation:** Dissolve 100 mg of the antibiotic (Ampicillin) in 1 ml of sterile water to get a stock concentration of 100 mg/ml. Store at 4°C. Use the antibiotic solution within two weeks.

**For Ampicillin LB media:** Add ampicillin to LB broth or agar at a final concentration of 100 µg/ml, when the temperature of the media is around 40-45°C.
Introduction:
Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their molecular weight and is an intrinsic part of almost all routine experiments carried out in molecular biology.

The technique consists of 3 basic steps:
- Preparation of agarose gel
- Electrophoresis of the DNA fragments
- Visualization of DNA fragments

Principle:
Preparation of Agarose Gel:
Agarose is a linear polymer extracted from seaweeds. Its basic structure is shown in the figure.

![Basic unit structure of agarose.](image)

Figure: Basic unit structure of agarose.

Purified agarose is a powder insoluble in water or buffer at room temperature but dissolves on boiling. Molten solution is then poured into a mould and allowed to solidify. As it cools, agarose undergoes polymerization i.e., sugar polymers cross-link with each other and cause the solution to gel, the density or pore size of which is determined by concentration of agarose.
Visualization of DNA fragments:

Since DNA is not naturally coloured, it will not be visible on the gel. Hence the gel, after electrophoresis, is stained with a dye specific to the DNA. Discrete bands are observed when there is enough DNA material present to bind the dye to make it visible, otherwise the band is not detected. The gel is observed against a light background wherein DNA appears as dark coloured bands.

Alternatively, an intercalating dye like Ethidium bromide is added to agarose gel and location of bands determined by examining the gel under UV light, wherein DNA fluoresces.

Note: Ethidium bromide must be handled carefully as it is a mutagen and a carcinogen. Wear gloves while handling EtBr solution & gels stained with EtBr.

Electrophoresis of DNA fragments:

Electrophoresis is a technique used to separate charged molecules. DNA is negatively charged at neutral pH and when an electric field is applied across the gel, DNA migrates towards the anode. Migration of DNA through the gel is dependent upon:

1. Molecular size of DNA
2. Agarose concentration
3. Conformation of DNA
4. Applied current

Matrix of agarose gel acts as a molecular sieve through which DNA fragments move on application of electric current. Higher concentration of agarose gives firmer gels, i.e., spaces between cross-linked molecules is less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases, it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps in movements of larger DNA fragments as the spaces between the cross-linked molecules is more. The progress of gel electrophoresis is monitored by observing the migration of a visible dye (tracking dye) through the gel. Two commonly used dyes are Xylene cyanol and Bromophenol blue that migrate at the same speed as double stranded DNA of size 5000 bp and 300 bp respectively. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of run, when the tracking dye reaches towards the anode, run is terminated.
Procedure:

**Preparation of 1% Agarose Gel**
1. Prepare 1X TAE by diluting appropriate amount of 50X TAE buffer. (For one experiment, approximately 200 ml of 1X TAE is required. Make up 4 ml of 50X TAE to 200 ml with distilled water).
2. Weigh 0.5 g of agarose and add to 50 ml of 1X TAE. This gives 1% agarose gel.
3. Boil till agarose dissolves completely and a clear solution results.
4. Meanwhile place the combs of electrophoresis set such that it is approximately 2 cm away from the cathode.
5. Pour the agarose solution in the central part of tank when the temperature reaches approximately 60°C. Do not generate air bubbles. The thickness of the gel should be around 0.5 to 0.9 cm. Keep the gel undisturbed at room temperature for the agarose to solidify.
6. Pour 1X TAE buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
7. Gently lift the combs, ensuring that wells remain intact.

**Electrophoresis**
8. Connect the power cord to the electrophoretic power supply according to the convention **red: anode, black: cathode**.
9. Load the samples in the wells in the desired order.
10. Set the voltage to 50 V and switch on the power supply.
11. Switch off the power when the tracking dye (bromophenol blue) from the well reaches ¾th of the gel. This takes approximately one hour.

**Staining Procedure to Visualize DNA**
12. Prepare 1X staining dye by diluting 6X dye (1:6) with distilled water. (Approximately 50 ml of 1X staining dye is required for one experiment. Therefore, make up 8 ml of 6X dye to 48 ml with distilled water).
13. Carefully transfer the gel (from gel tank) into a tray containing 1X staining solution. Make sure that the gel is completely immersed.
14. For uniform staining, place the tray on a rocker for approximately one hour or shake intermittently every 10 to 15 minutes.
15. Pour out the staining dye into a container. (The dye can be reused twice). Destain the gel by washing with tap water several times till the DNA is visible as a dark band against a light blue background.

**Note:** Alternatively, **Ethidium bromide can be used for visualizing DNA fragments. Add Ethidium bromide to molten agarose to a final concentration of 0.5 µg/ml (from a stock of 10 mg/ml in water), when temperature is around 50°C. Mix and cast the gel. After electrophoresis, DNA samples can be visualized under UV light, they appear fluorescent. No destaining is required in this case.**
### Ordering Information

<table>
<thead>
<tr>
<th>Product</th>
<th>Size</th>
<th>Cat #</th>
</tr>
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<tbody>
<tr>
<td>GeNei™ Plasmid Preparation Teaching Kit (Consumables for 5 experiments &amp; Elpho Kit (ETS 1))</td>
<td>1 Pack</td>
<td>KT06</td>
</tr>
<tr>
<td>GeNei™ Plasmid Preparation Teaching Kit (Consumables 5 experiments)</td>
<td>1 Pack</td>
<td>KT06A</td>
</tr>
<tr>
<td>GeNei™ Plasmid Preparation Teaching Kit Includes consumables (Consumables 20 experiments)</td>
<td>1 Pack</td>
<td>KT06B</td>
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</tbody>
</table>

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