HiPer® Plasmid DNA Cloning Teaching Kit

Product Code: HTBM022

Number of experiments that can be performed: 5

Duration of Experiment: 4 days
Day 1- Preparation of media and revival of *E. coli* Host
Day 2- Inoculation of host strain from single colony and ligation of insert to vector
Day 3- Preparation of competent cells and transformation of the ligation mix
Day 4- Observation and interpretation

Storage Instructions:
- The kit is stable for 12 months from the date of manufacture
- Store *E. coli* Host at 2-8°C
- Store control plasmid DNA, vector, insert, 10X DNA ligase buffer, T4 DNA ligase and ampicillin at -20°C
- Other kit contents can be stored at room temperature (15-25°C)
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**Aim:**

To learn the process of cloning a foreign gene into a vector.

**Introduction:**

The cloning experiments of Herbert Boyer, Stanley Cohen, Paul Berg, and their colleagues in the early 1970s ushered in the era of recombinant DNA technology. Recombinant DNA is a DNA that has been created artificially. DNA from two or more sources is incorporated into a single recombinant molecule. The basic procedure of molecular cloning involves a series of steps. First, the DNA fragments to be cloned are generated by using restriction endonucleases. Second, the fragments produced by digestion with restriction enzymes are ligated to other DNA molecules that serve as vectors. Vectors can replicate autonomously (independent of host genome replication) in host cells and facilitate the manipulation of the newly created recombinant DNA molecule. Third, the recombinant DNA molecule is transferred to a host cell. Within this cell, the recombinant DNA molecule replicates, producing dozens of identical copies known as clones. As the host cells replicate, the recombinant DNA is passed on to all progeny cells, creating a population of identical cells, all carrying the cloned sequence. Finally, the cloned DNA segments can be recovered from the host cell, purified, and analyzed in various ways.

**Principle:**

Cloning refers to the process of creating an exact copy of an organ, whole organism, single cell or a piece of DNA. Gene cloning is a process through which an exact copy of a particular gene is made. The process through which a foreign piece of DNA is transferred into another DNA led to the development of recombinant DNA technologies. The entire cloning procedure involves the following steps:

1. Isolation of pure vector and insert DNA
2. Restriction digestion of the DNAs
3. Ligation of the two linear DNA fragments
4. Transformation of the ligated product
5. Screening for the right clone

**Isolation of pure vector and insert DNA:**

During cloning the foreign DNA is isolated after following the specific DNA isolation procedure. The insert DNA which contains the gene of interest is obtained after restriction enzyme digestion. Several naturally occurring plasmids have been engineered to make different types of cloning vectors. Cloning vectors are circular DNA molecules in which DNA fragments/insert are maintained and amplified. Vectors should have the following features that make them compatible for a variety of uses in recombinant DNA procedures:

**Size:** Vectors are relatively small molecules; most are only 2.5 - 3 kb in size.

**Ori:** A vector should contain an origin of replication (ori) in order to maintain its autonomous state.

**Marker:** To facilitate selection of cells that contain the vector, a vector should carry at least one genetic marker for which an easy assay exists (e.g., ampicillin resistance, Ampr).

**Cloning sites:** A vector should contain several different restriction endonuclease sites that allow the insertion of a gene of interest during cloning. Many cloning vectors like pUC19 contain a synthetic DNA sequence
called a polylinker, which consists of 21 closely-packed RE recognition sites in a small region of DNA.

2. Restriction digestion of the DNAs: During cloning procedure an insert is added to a vector and for this reason both of them have to be cleaved by restriction endonucleases (REs). Restriction endonucleases recognize a specific, rather short, nucleotide sequence on a double-stranded DNA molecule, called a restriction site, and cleave the DNA at this recognition site or elsewhere, depending on the type of enzyme.

The target site or sequence which the restriction enzyme recognizes is generally from 4 to 6 base pairs and arranged in a palindromic sequence. Generally both the vector and the foreign DNA are digested with the same RE. The restriction digested vector DNA has to be dephosphorylated to prevent self-ligation.

3. Ligation of two linear DNA fragments: Two linear DNA molecule ends (vector and insert) can be joined together through a process called ligation. This process involves the formation of a covalent bond between two DNA fragments (having blunt or overhanging, complementary, 'sticky' ends) with the help of an enzyme named as ligase. DNA ligase forms a phosphodiester bond between the 3’ hydroxyl of one nucleotide and the 5’ phosphate of another. Ligation can be directional or non-directional based upon the restriction enzyme used. When both the vector and the insert are digested with a single RE then the ligation can occur in either direction and when they are digested with two REs then ligation takes place only in one direction.

4. Transformation of the ligated product: The ligation reaction mixture is introduced into bacterial cells in a process called transformation. In this method cells are incubated in a concentrated calcium salt solution to make their membranes leaky. The permeable “competent” cells are then mixed with DNA (ligation mix) to allow entry of the DNA into the bacterial cell. Alternatively, a process called as electroporation can be used that drives DNA into cells by a strong electric current. Successfully transformed bacteria will carry either recombinant or nonrecombinant plasmid DNA. Multiplication of the plasmid DNA occurs within each
transformed cell. A single bacterial cell placed on a solid surface (agar plate) containing nutrients can multiply to form a visible colony made of millions of identical cells. As the host cell divides, the plasmid vectors are passed on to progeny, where they continue to replicate.

5. Screening of the right clone: The last step during cloning is screening for the right clone. Screening means to detect the right clone among a population of colonies and the screening procedure depends upon the vector used for cloning. The *E. coli* plasmid pUC19 carries sites for different restriction enzymes (Multiple Cloning Site, MCS) within the N-terminal coding sequence for β-galactosidase of the lac operon. When pUC19 is transformed into a competent host cell (which has a deletion at the amino terminal end of the LacZ gene, which codes for β-galactosidase), the truncated products from both complement each other and as a result enzymatically active β-galactosidase is produced. This is called α-complementation. The transformants turn blue on X-gal and IPTG containing plates due to the production of β-galactosidase. X-gal is a chromogenic substrate of β-galactosidase and IPTG acts as an inducer for the expression of this enzyme. If an insert is cloned within the MCS of pUC19 then β-galactosidase is not produced and as a result the transformants turn white on X-gal and IPTG containing plates. As a result one can directly screen for the insert containing vector (recombinant).

**Kit Contents:**

This kit can be used to clone GFP (Green Fluorescent Protein) gene in pUC19 vector and to screen for the right clone under UV light.

**Table 1: Enlists the materials provided in this kit with their quantity and recommended storage**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Product Code</th>
<th>Materials provided</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MB104</td>
<td>Ampicillin</td>
<td>0.03 g</td>
<td>-20°C</td>
</tr>
<tr>
<td>2</td>
<td>M1245</td>
<td>Luria Bertani (LB) Broth</td>
<td>8.5 g</td>
<td>R T</td>
</tr>
<tr>
<td>3</td>
<td>M1151</td>
<td>Luria Bertani Agar</td>
<td>25 g</td>
<td>R T</td>
</tr>
<tr>
<td>4</td>
<td>TKC027</td>
<td><em>E. coli</em> Host</td>
<td>1 No.</td>
<td>2-8°C</td>
</tr>
<tr>
<td>5</td>
<td>TKC195</td>
<td>Vector</td>
<td>0.012 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td>6</td>
<td>TKC196</td>
<td>Insert</td>
<td>0.024 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td>7</td>
<td>TKC197</td>
<td>Positive Control Plasmid</td>
<td>0.012 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td>8</td>
<td>*TKC198</td>
<td>10X Ligase Assay Buffer</td>
<td>0.012 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td>9</td>
<td>*TKC199</td>
<td>T4 DNA Ligase</td>
<td>0.006 ml</td>
<td>-20°C</td>
</tr>
<tr>
<td>10</td>
<td>TKC065</td>
<td>1M Calcium chloride (Sterile)</td>
<td>20 ml</td>
<td>R T</td>
</tr>
<tr>
<td>11</td>
<td>ML024</td>
<td>Molecular Biology Grade Water</td>
<td>0.075 ml</td>
<td>R T</td>
</tr>
<tr>
<td>12</td>
<td>PW1139</td>
<td>Collection Tubes, Polypropylene</td>
<td>15 Nos.</td>
<td>R T</td>
</tr>
<tr>
<td>13</td>
<td>CG282</td>
<td>Polypropylene Tube (0.2 ml)</td>
<td>5 Nos.</td>
<td>R T</td>
</tr>
</tbody>
</table>

* Always give a quick spin before opening the vial as the liquid material may stick to the wall or to the cap of the vial

**Materials Required But Not Provided:**

**Glasswares:** Conical flask, Measuring cylinder, Beaker
**Other requirements:** Micropipettes, Tips, 50 ml Centrifuge Tubes, Water bath (42°C), 37°C Incubator, 37°C Shaker, Centrifuge, UV Transilluminator, Crushed ice, Sterile double distilled water, Sterile loop and spreader

**Storage:**

HiPer® Plasmid DNA Cloning Teaching Kit is stable for 12 months from the date of manufacture without
showing any reduction in performance. On receipt, store *E. coli* Host at 2-8°C. Vector, insert, control Plasmid, 10X ligase buffer, T4 DNA Ligase and Ampicillin should be stored at –20°C. All other kit contents can be stored at room temperature (15-25°C).

**Important Instructions:**

1. Read the entire procedure carefully before starting the experiment. The entire procedure should be carried out under sterile conditions.
2. **Preparation of 0.1M Calcium chloride (Sterile) (200 ml):** To prepare 200 ml of 0.1M Calcium chloride, take 20 ml of 1M Calcium chloride and add 180 ml of sterile distilled water. Store at 2-8°C.
3. Prior to the preparation of competent cells, pre-chill the tubes, 0.1M Calcium chloride solution and centrifuge tubes. Set the centrifuge at 4°C and water bath at 42°C.
4. Transformation should be carried out as soon as possible after the competent cells are prepared. Storage of competent cells leads to poor or no transformants. 
5. **Preparation of LB (Luria Bertani) broth (55 ml):** Dissolve 1.38 g of LB media in 55 ml of distilled water. Sterilize by autoclaving.
6. **Preparation of LB (Luria Bertani) agar plates (20 ml):** Dissolve 0.8 g of LB agar in 20 ml of sterile distilled water. Sterilize by autoclaving and pour on sterile petri plate.
7. **Preparation of Ampicillin:** Dissolve 30 mg of ampicillin powder in 600 μl of sterile double distilled water to give a concentration of 50 mg/ml.
8. **Preparation of LB (Luria Bertani) Agar plates containing Ampicillin (100 ml):** Dissolve 2.5 g of LB media and 1.5 g of agar in 100 ml of distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45°C. Add 100 μl of ampicillin, to 100 ml of autoclaved LB agar media, mix well and pour on sterile petri plates.

**Procedure:**

**Day 1:**
1. Open the vial containing culture and resuspend the cells with 0.25 ml of LB broth.
2. Pick up a loopful of culture and streak onto LB agar plate.
3. Incubate overnight at 37°C.

**Day 2:**
1. Inoculate a single colony from the revived plate in 1 ml LB broth.
2. Incubate at 37°C overnight.
3. Thaw 10X Ligase Buffer, T4 DNA Ligase, Vector and insert on ice and then set up the ligation reaction as follows:

   - Molecular biology grade Water : 11 μl
   - Vector DNA : 2 μl
   - Insert DNA : 4 μl
   - 10X Ligase buffer : 2 μl
   - T4 DNA ligase : 1 μl

Mix the contents by tapping gently and incubate at 16°C water bath, overnight.
Day 3:

4. Take 50 ml of LB broth in a sterile flask. Transfer 1 ml of overnight grown culture into this flask.

5. Incubate at 37°C shaker at 300 rpm for 3-4 hours till the O.D\textsubscript{600} reaches ~ 0.6.

A) Preparation of Competent Cells:

   Note: Prepare competent cells within 3 days of reviving the strain.

1. Transfer the above culture into a prechilled 50 ml polypropylene tube (not provided).

2. Allow the culture to cool down to 4°C by storing on ice for 10 minutes.

3. Centrifuge at 4100 rpm for 10 minutes at 4°C.

4. Decant the medium completely. No traces of medium should be left.

5. Resuspend the cell pellet in 30 ml prechilled sterile 0.1 M Calcium chloride solution.

6. Incubate on ice for 30 minutes.

7. Centrifuge at 4100 rpm for 10 minutes at 4°C.

8. Decant the calcium chloride solution completely. No traces of solution should be left.

9. Resuspend the pellet in 2 ml prechilled sterile 0.1M Calcium chloride solution.

10. This cell suspension contains competent cells and can be used for transformation.

B) Transformation of cells:

1. Take 200 \(\mu\)l of the above cell suspension in three 2.0 ml collection tubes and label them as control, plasmid and ligation mix. Add 2 \(\mu\)l of positive control plasmid DNA to the tube labeled as plasmid and mix well. Add 10 \(\mu\)l of the ligation mix to the tube labeled as ligation mix and mix well.

2. Incubate all the 3 tubes on ice for 30 minutes.

3. Transfer them to a preheated water bath set at a temperature of 42°C for 2 minutes (heat shock).

4. Rapidly transfer the tubes on ice. Allow the cells to chill for 5 minutes.

5. Add 800 \(\mu\)l of LB Broth to all the tubes. Incubate the tubes for 1 hour at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

6. Plate 200\(\mu\)l of cell culture from each tube on LB agar plate containing ampicillin using a sterile spreader.

7. Store at room temperature till the plates are dry.

8. Incubate the plates overnight at 37°C.
**Flowchart:**

1. **Gene for antibiotic resistance**
2. **Restriction digestion**
3. **Sticky ends**
4. **Ligation**
5. **Recombinant plasmid**
6. **Transformation**
7. **Bacterial chromosome**
8. **Recombinant plasmid**
9. **Only recombinant plasmid containing cells grow on the antibiotic containing media**

**Observation and Result:**

After incubation observe the plates under UV light (on a UV transilluminator) and look for the glowing green colonies on the plasmid plate. For the ligation mix plate look for both glowing and non-glowing colonies.
Interpretation:

On transformation of cells with positive control plasmid, antibiotic resistance is conferred on the host as this plasmid carries gene for ampicillin resistance. As a result, those cells that grow in presence of ampicillin are transformed cells. In contrast, no growth is observed on plating competent cells untransformed with vector DNA as the host *E. coli* cell is sensitive to ampicillin. The transformed colonies of the positive control plate glow when exposed to UV light as it contains GFP gene. The unique 3 dimensional structure of the GFP releases energy in the form of visible green light on exposure to UV light. For the ligation mix plate, the colonies which carry the recombinant plasmid (vector+insert) glow under UV light and those which carry only the vector (self-ligation) do not fluoresce under UV light.

Troubleshooting Guide:

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<th>Sr. No.</th>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Transformants from the ligation mix do not fluoresce under UV light</td>
<td>Ligation is not done properly</td>
<td>Set up the ligation assay as mentioned in the protocol. Always thaw the ligase buffer and the enzyme on ice. Try to maintain the temperature during ligation</td>
</tr>
<tr>
<td>2</td>
<td>Few or no transformants</td>
<td>Cold chain not maintained properly during the procedure</td>
<td>Always keep the cells on ice &amp; make sure the centrifuge machine is cold throughout the experiment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat shock treatment not done accurately</td>
<td>Follow the procedure, with exact time duration for heat shock treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ampicillin concentration used is not correct</td>
<td>While making the plates add accurate amount of ampicillin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plates were not incubated at proper temperature for sufficient time</td>
<td>Incubate the plates at 37°C for minimum 18 hours</td>
</tr>
<tr>
<td>3</td>
<td>Observed bacterial growth on control plate</td>
<td>Ampicillin is inactivated</td>
<td>Add ampicillin to the media after it cools down to 40-45°C</td>
</tr>
<tr>
<td>4</td>
<td>Contamination observed on plates</td>
<td>Sterility not maintained during the experiment</td>
<td>Make sure that the entire procedure is performed aseptically</td>
</tr>
</tbody>
</table>
**Technical Assistance:**

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com

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**Storage temperature**

15°C

25°C

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Do not use if package is damaged

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