Experiment 6: Plasmid Ligation and Transformation

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Purpose

• To ligase the restricted plasmid and insert DNAs
• To transform the ligated plasmid into competent cells to clone the gene of interest
• To gain experience in bacterial cell culture

Materials

• T4 ligase (ThermoFisher, cat.no. EL0011)
• 10X ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8 at 25°C))
• Restricted insert and vector DNAs (prepared in the prior lab class)
• The cloned plasmid (for BL21(DE3) transformation)
• Competent cell DH5α (enzynomics, cat.no. CP011)
• Competent cell BL21(DE3) (RBCBioscience, cat.no. RH217)
• LB broth and LB agar plate
• NaCl (LPS, cat.no. NACL01)
• Tryptone (LPS, cat.no. CHS-80)
• Yeast Extract (LPS, cat.no. CHS-02)
• Distilled water, ice bucket with ice, Beaker, 500-mL flask, Stirring bar
• Spreader (SPL, cat.no. 90050)
• Parafilm (PARAFILM® M, cat.no. HS234526B)

Machines to use

• Centrifuge (eppendorf, Centrifuge 5418, cat.no. EP5401000137)
• Heat block
• Stirrer
• Autoclave

Principles

1. Plasmid Ligation

The final step in the construction of a recombinant plasmid is connecting the insert DNA (gene of interest) into a compatibly digested vector backbone. This is accomplished by covalently connecting the sugar backbone of the two DNA fragments. This reaction, called ligation, is performed by the T4 DNA ligase enzyme (Figure 1). The DNA ligase catalyzes the formation of covalent phosphodiester linkages, which

permanently join the nucleotides together. After ligation, the insert DNA is physically attached to the backbone and the complete plasmid can be transformed into bacterial cells for propagation.

The majority of ligation reactions involve DNA fragments that have been generated by restriction enzyme digestion. Most restriction enzymes digest DNA asymmetrically across their recognition sequence, which results in a single stranded overhang on the digested end of the DNA fragment. The overhangs, called "sticky ends", are what allow the vector and insert to bind to each other. When the sticky ends are compatible, meaning that the overhanging base pairs on the vector and insert are complementary, the two pieces of DNA connect and ultimately are fused by the ligation reaction.

The example below depicts the ligation of two sticky ends that were generated by EcoRI digestion:

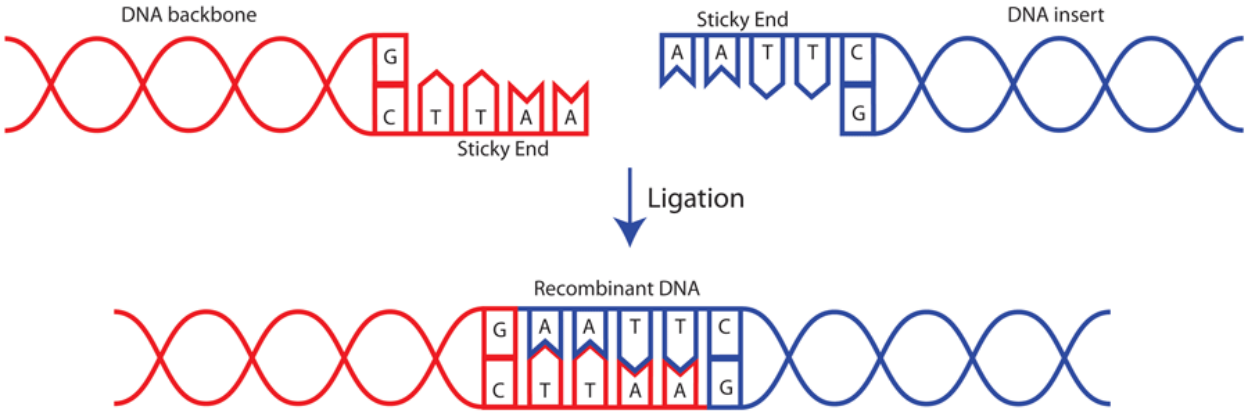

Figure 1 Ligation of DNA backbone and insert having sticky ends

Usually, scientists select two different enzymes for adding an insert into a vector (one enzyme on the 5' end and a different enzyme on the 3' end). This ensures that the insert will be added in the correct orientation and prevents the vector from ligating to itself during the ligation process. If the sticky ends on either side of the vector are compatible with each other, the vector is much more likely to ligate to itself rather than to the desired insert. If you are in this situation, it is important to treat the digested vector backbone with a phosphatase before performing the ligation reaction (phosphatase removes the 5' phosphate and therefore prevents the ligase from being able to fuse the two ends of the vector together).

2. Transformation

One of the cornerstones of modern molecular biology is molecular cloning, the ability to insert DNA from one organism into a different organism. This transfer process is accomplished in a variety of related ways, but the most commonly used is the process of bacterial transformation.

In bacterial transformation, a recombinant plasmid, or circular double-stranded DNA segment, is produced that harbors a novel gene of interest. Plasmids exist naturally as

semi-autonomous replicating entities within bacteria, allowing horizontal gene transfer. The modified plasmid can thus exploit this natural gene transfer system.

To insert a recombinant plasmid into a bacterial cell, we typically use “competent” cells, which are bacterial cells that have been placed into a media with little nutrients and chemicals that cause stress to the cell. This primes them to be more accepting of foreign DNA. The DNA is introduced to the solution surrounding the cells, and a shock is given to disrupt the bacterial cell membrane temporarily either through a rapid change in temperature (“heat shock”) or through an electrical current (“electroporation”) (Figure 2). Once the DNA has entered the cell, it will be recognized and assimilated. If our plasmid contains a gene for antibiotic resistance, this gene product and thus phenotypic trait will become available only to cells that successfully uptake our foreign DNA.

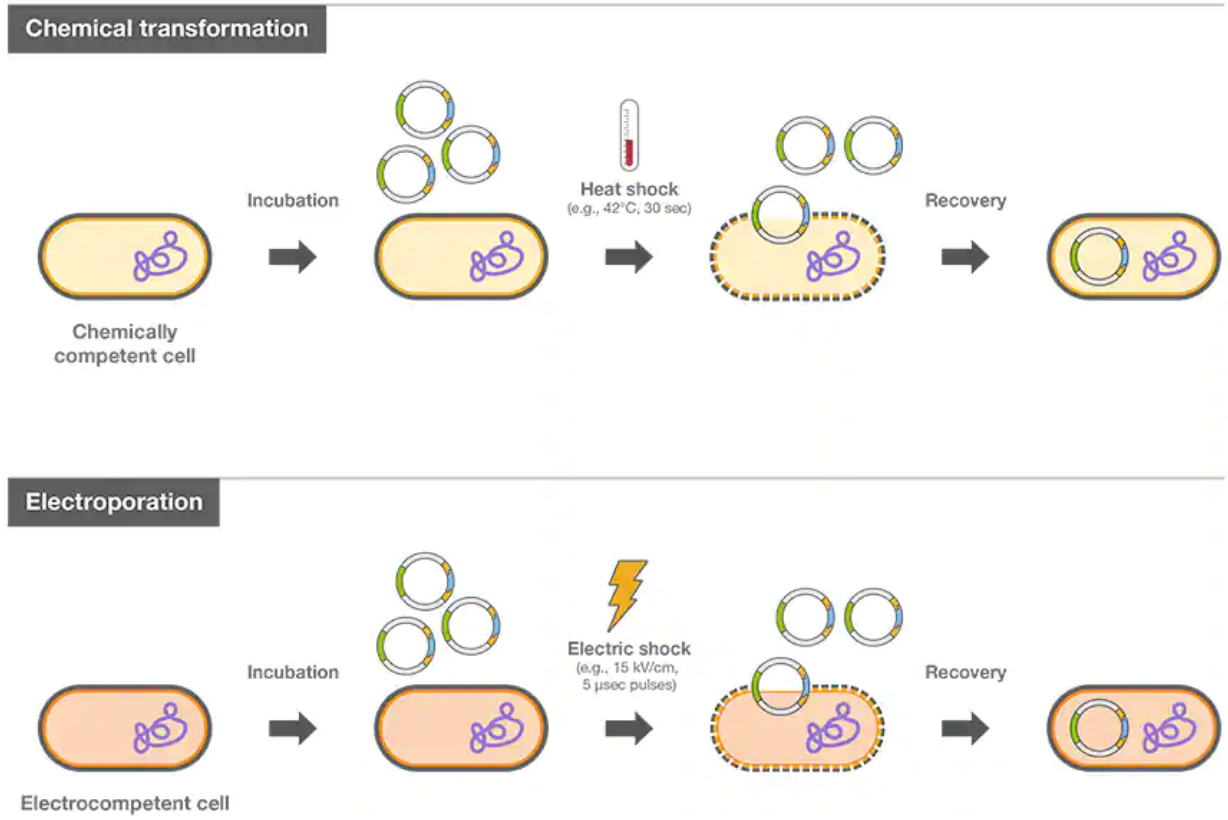

Figure 2 Transformation of plasmids into cells

Procedure

< Plasmid Ligation>

1. Thaw restricted DNAs and 10X ligation buffer at room temperature.

2. To a new 1.5-ml EP tube, make mixtures as in below (molar ratio of insert DNA and plasmid should be 1:1 to 5:1). After adding all the components, mix well with pipetting

Order to add	Component	Volume
1	3rd distilled water	X μℓ (up to final volume)
2	10x ligation buffer	2 μℓ
3	digested plasmid DNA	2 μℓ (subject to change)
4	digested insert DNA	4 μℓ (subject to change)
7 (add later)	T4 ligase	1 μℓ
	Total volume	**20 μℓ**

3. Incubate at room temperature for an hour.

<Transformation>

1. Thaw DH5α & BL21(DE3) competent cells on ice.

2. Add 20μL of ligated sample solution into DH5α competent cell microtube.

3. Add 1μL of the cloned plasmid (provided) into 20 μL of BL21(DE3) competent cell.

4. Incubate both the competent cells on ice for 20~30 min.

5. Warm-up two LB agar plates that contain appropriate antibiotics at 37℃ incubator (each for DH5α and BL21(DE3)).

6. Place both the competent cells in a 42℃ heat block, and incubate for 40~50 sec.

7. Move the microtubes to ice and incubate for 1 min.

8. Add 800μL of LB medium into the DH5α cell and 150μL of LB medium into the BL21(DE3) cell.

9. Incubate the DH5α cells at 37℃ with shaking for 1 hr (BL21(DE3) cells can skip this step).

10. Spread the cells on the LB agar plate with a spreader. Spread the cell until the agar plate surface dries

11. Incubate the plates at a 37℃ incubator for 12~18hr.

12. Check the plates whether bacterial colonies have grown on the plates.

- ▶ Take a picture of the plates to put on the report

12. Seal the plates thoroughly with parafilm and store them at 4℃.

- ■ Pause point: Bacterial colonies can be stored at 4℃ for two weeks.

< Preparation of LB Broth Media>

1. Measure and pour 200mL of DW into a beaker (w/ stirring bar).

2. Measure and add 2g NaCl, 2g tryptone, and 1g yeast extract.

3. Stir until the powder is well dissolved.

4. Transfer the solution to a 500mL flask.

5. Put the flask into autoclave and start autoclave.
   - ■ Pause point: LB broth can be stored at room temperature for one week.

Result

1. Describe your experiment experience by adding details to the procedure above and your observations.

2. Attach the image of the grown agar plates (both DH5α and BL21(DE3))

Analysis

1. How many colonies can you observe on your plate? Are they evenly distributed?

2. How different are the colonies from DH5a and BL21(DE3)?

Discussion

1. We have used DH5α and BL21(DE3). What are the differences between the two stains? When do we use each strain?

2. Calculate the appropriate volume of insert DNA under the following conditions.
   Measured plasmid concentration: 50ng/μL
   Measured insert DNA concentration: 10ng/μL
   Size of the plasmid: 6000bp
   Size of insert DNA: 600bp
   The volume of the plasmid: 2μL
   Target molar ratio: 5:1

3. Heat shock transformation is also called chemical transformation because this competent cell generally contains $CaCl_2$. Search the role of the $CaCl_2$ and the principle of heat shock transformation.

4. In the case of this experiment, which antibiotics would be appropriate? Why?