Transformation

1. Transfer 1 μL of the resuspended DNA into 50 μL competent cells. Thaw the recombinant cells on ice for around 20 minutes before use.

2. While waiting, warm the agar plates containing the desired antibiotics by placing them in 37 °C incubator.

3. Place the tubes containing DNA and competent cells on ice for 20 minutes.

4. Heat shock in water bath (42 °C) for 1.5 minutes.

5. Place the tubes containing DNA and competent cells on ice for 2 minutes.

6. Add 1 ml of LB (without antibiotic) into each tube and grow in 37 °C shaking for 1 hour as recovery for bacteria with plasmids that have CHL or KAN resistance. Bacteria with plasmids that have AMP resistance does not need recovery.

7. Centrifuge all tubes at 7000 rpm for 2.5 minutes.

8. Discard the liquid in 100 ml beaker with some left in the tube.

9. Light the Bunsen Burner. Resuspend the tube and transfer 250 μL of each tube onto agar plate with appropriate antibiotics.

10. Incubate overnight at 37 °C.

Digestion (18 μL reaction)

1. Add water (variable volume, to top to 18ul).

2. Add DNA (variable volume) into the tube. Resuspend.

3. Add 1.8 μL buffer of highest compatibility with restriction enzyme that is used. Resuspend it and centrifuge the tube lightly if there is water drop on the wall of the tube.

4. Add restriction enzyme (volume depends on the amount of DNA added). Resuspend the enzyme before use.

    a. 1 ul of enzyme = 20 unit

    b. 1 unit of enzyme can digest 1 ug of DNA

5. Incubate the tubes for 1 to 2 hours.

6. Add 2ul of 10x Loading buffer

7. Run gel electrophoresis

Preparing agarose gel for gel electrophoresis

1. Mix agarose powder and 1X TAE buffer in a flask. The percentage of the gel depends on the size of DNA sample in the experiment.

2. Melt them in the microwave for 40 seconds to 1 minute until no white-precipitates are shown. Wear gloves when taking the flask out.

3. Add DNA staining material into the gel solution

   a. Midori green : 0.001% of the total gel volume or

   b. SYBRsafe : 0.01% of total gel volume

Colony PCR

1. Preparing the sample:

   - Prepare tubes of 20 µL saline solution.

   - Pick 5 to 10 colonies from the transformed plate.

2. Put all reagents into a master mix (18 µL of master mix for every reaction)

| Component | 20 µl reaction |
|---|---|
| 10X Reaction Buffer | 2 µl |
| 10 mM dNTPs | 0.5 µl |
| 10 µM Forward Primer | 0.5 µl |
| 10 µM Reverse Primer | 0.5 µl |
| Colony-containing saline solution | 2 µl |
| *Taq* DNA Polymerase | 0.15 µl |
| ddH2O | 14.35 µl |

3. Add 2 µL colony-containing saline solution.

4. Run PCR.

Thermocycler settings:

| STEP | TEMP | TIME |
| --- | --- | --- |
| Initial Denaturation | 95°C | 3 minutes |
| 24 Cycles | 95°C<br>45-68°C (depends on primers used)<br>68°C | 15 seconds<br>30 seconds<br>1 minute/kb |
| Final Extension | 68°C | 5 minutes |
| Hold | 4-10°C | |

5. Do gel electrophoresis. Load the 20 µL PCR product into the wells.

6. Keep the colony-containing saline solution left at room temperature, which will be used for plate streaking and inoculation.

Ligation

1. Set up the reaction mixture in the microcentrifuge tubes on ice.
2. Add 0.5µL of T4 ligase into the tube.
3. Mix the reaction mixture and the DNA fragments gently by pipetting up and down.
4. Incubate the microcentrifuge tubes at room temperature for 1 hour.
5. Ligated product is ready to be transformed.

Gibson assembly

1. Prepare the master mix following the recipes below.

Prather Recipe 5x Isothermal Reaction Mix

- 3 ml 1 M Tris-Hcl (pH 7.5)
- 300 µL 1 M MgCl2
- 60 µL 100 mM dGTP
- 60 µL 100 mM dATP
- 60 µL 100 mM dTTP
- 60 µL 100 mM dCTP
- 300 µL1 M DTT
- 1.5 g PEG-8000
- 300 µL100 mM NAD
- balance ddH2O
- 6 ml Total

Assembly Master Mix

- 320 µL 5X Isothermal Master Mix
- 0.64 µL 10 U/µL T5 exonuclease
- 20 µL 2 U/µL Phusion DNA Pol
- 0.16 µL 40 U/µL Taq DNA Ligase
- 860 µL ddH2O
- 1.2 ml Total

RFC57 Recipe 5x isothermal reaction buffer:

- 25% PEG-8000
- 500 mM Tris-HCl pH 7.5
- 50 mM $MgCl_2$,
- 50mM DTT
- 5mM NAD
- 1mM each of the four dNTPs
- 1.33x Gibson Master Mix:
- Taq ligase (40u/ul): 50 ul
- 5x isothermal buffer: 100 ul
- T5 exonuclease (1u/ul): 2 ul
- Phusion polymerase (2u/ul): 6.25 ul
- Nuclease-free water: 216.75 ul

2. Add DNA backbone and inserts with molar ratio 1:5 into a tube of master mix aliquot.
3. Incubate the reaction mixture for 1 hour at 50°C.
4. Gibson assembly product is ready to be transformed.

Miniprep

- Qiagen miniprep kit

Gel Purification

- Favorgen gel purification kit