Protocol

Competent cell preparation

A. Preparing glassware and media eliminate detergent
   1. Autoclaving glassware filled 3/4 with DD-H2O to remove most detergent residue
   2. Media and buffers in detergent free glassware and cultures grown up in detergent free glassware

B. Preparation of the competent cells

Reagents:
- glycerol stock
- LB plate
- MgCl2-CaCl2 solution

<table>
<thead>
<tr>
<th>MgCl2-CaCl2 solution</th>
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Procedure:
Day 1:
1. Flame the metal inoculating loop until it is red hot and then cool it down
2. Scrape off a portion from the top of the frozen glycerol stock [DO NOT THAW]
3. Streak it onto the LB plate
4. Put the stock back to -80C immediately
5. Leave the plates for 5 minutes and place them upside down in the 37C incubator for 16-20 hours

**Day2**

6. Pick a single colony into 5ml of LB medium
7. inoculate the culture overnight at 37C with shaking at 250rpm

**Day3**

8. Inoculate 100ml LB medium with 1ml of saturated overnight culture
9. Shake at 37C until OD600=0.4 (usually 2-3 hours)
10. Place in an ice bath for 10 minutes [After this point the cells should never touch anything that is warm]
11. Pre-cool solution, centrifuge, pipette tips, falcon, eppendorf
12. Transfer the culture into two pre-chilled 50ml falcon
13. Centrifuge at 2700x g for 10 minutes at 4C
14. Remove the medium, resuspend the cell pellet with 1.6ml ice0cold 100mM CaCl2 by swirling on ice gently
15. Incubate on ice gor 30 minutes
16. Centrifuge at 2700x g for 10 minutes at 4C
17. Remove the medium, resuspend the cell pellet with 1.6ml ice-cold 100mM CaCl3 by swirling on ice gently
18. Incubate on ice for 20 minutes
19. Combine cells to one tube and add 0.5 ml ice-cold 80% glycerol and swirl to mix
20. Freeze 100ul aliquots in liquid nitrogen
21. Store in -80C

**Transformation protocol**

1. Thaw DH5-alpha cells on ice.
2. Add 2 ul of DNA (usually 20 ng- 100 ng) to the cells
3. Stand for 30 minutes on ice.
   *(Hint: Turn on the 37°C shaker to warm up)*
4. Cells are incubated for 60 seconds at 42°C.
5. Put back cells on ice for 5 min.
6. Add 900 ul LB broth.
7. Incubate for 1.5 - 2 hour at 37°C on 125 rpm shaker.
   *(Hint: Warm up the LB agar plate by transferring it from 4°C fridge to room temperature)*
8. (To increase colony number): centrifuge cells at 5000 rpm for 45 s.
9. Remove 600 ul LB, and resuspend the solution.
10. Spread 100 μl onto the petri dish with LB agar (with /without antibiotic, depends on the experiment).
11. Grow overnight at 37 °C.

Protocol of Restriction cutting and ligation

For QC/ backbone preparation:

For each sample,
1. Add 500-1000 ng samples
2. Add ddH₂O up to 42.5 ul
3. Add 5 ul 10X buffer 2
4. Add 0.5 ul 100X BSA
5. Add 1 ul EcoRI
6. Add 1 ul PstI
7. Perform restriction cutting temperature protocol

For 3A assembly:

- Prefix:
1. Add 1000 ng samples
2. Add ddH₂O up to 42.5 ul
3. Add 5 ul 10X buffer 2
4. Add 0.5 ul 100X BSA
5. Add 1 ul EcoRI
6. Add 1 ul SpeI
7. Perform restriction cutting temperature protocol

- Suffix:
1. Add 1000 ng samples
2. Add ddH₂O up to 42.5 ul
3. Add 5 ul 10X buffer 2
4. Add 0.5 ul 100X BSA
5. Add 1 ul XbaI
6. Add 1 ul PstI
7. Perform restriction cutting temperature protocol

For standard assembly:

- Prefix, Suffix with backbone:
1. Add 1000 ng samples
2. Add ddH₂O up to 42.5 ul
3. Add 5 ul 10X buffer 2
4. Add 0.5 ul 100X BSA
5. Add 1 ul EcoRI
6. Add 1 ul XbaI
7. Perform restriction cutting temperature protocol

- Prefix with backbone, Suffix:

1. Add 1000 ng samples
2. Add ddH₂O up to 42.5 ul
3. Add 5 ul 10X buffer 2
4. Add 0.5 ul 100X BSA
5. Add 1 ul SpeI
6. Add 1 ul PstI
7. Perform restriction cutting temperature protocol

Restriction cutting temperature protocol:

1. 37°C for 2.5 hours
2. 80 °C for 20 mins (to denature all the restrictive enzyme)
3. 4 °C forever (to store the sample temporarily)

If time is urgent, the following temperature protocol is also alright:

1. 37°C for 2 hours
2. 80 °C for 15 mins
3. 4 °C forever

Ligation protocol:

For 3A assembly:

1. Add 50ng restriction cut backbone
2. Add 100ng prefix
3. Add 100ng suffix
4. Add ddH₂O up to 17 ul
5. Add 2 ul 10X ligation buffer (HINT: check it should smell rotten)
6. Add 1 ul DNA ligase
7. Perform ligation temperature protocol

For standard assembly:

1. Add 50ng restriction cut suffix-backbone
2. Add 100ng prefix
3. Add ddH₂O up to 17 ul
4. Add 2 ul 10X ligation buffer (HINT: check it should smell rotten)
5. Add 1 ul DNA ligase
6. Perform ligation temperature protocol

**ligation temperature protocol:**

1. 16°C for 12 hours
2. 80°C for 20 mins (to denature all ligase which inhibits transformation)
3. 4°C forever

**QIAGEN gel extraction method (modified from QIAquick Gel Extraction Kit Protocol)**

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a 2 ml centrifuge tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 μl).
3. Incubate at 50°C until the gel slice has completely dissolved (around 10 min). Mix by vortexing the tube every 2 mins during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 5-10 μl of 3 M sodium acetate, pH 5.0, and mix until it turn to yellow.
5. Add 1 gel volume of isopropanol to the sample and mix (for DNA fragments <500 bp and >4 kb).
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 30 s.
   (The maximum volume of the column reservoir is 800 μl. For sample volumes of more than 800 μl, simply load and spin again).
8. Discard flow-through and place QIAquick column back in the same collection tube.
    Collection tubes are re-used to reduce plastic waste.
9. Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 30 s to remove all traces of agarose.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column, stand for 2 mins and centrifuge for 30 s.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 30 s and air-dry for 2 mins (This step can ensure all ethanol is removed and the column is NOT over-dry).
12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, add 35 μl of 50 °C ddH2O to the center of the QIAquick membrane, wait 2 mins and centrifuge the column for 2 mins at maximum speed.
    (35 ul 50 °C ddH2O can increase the eluted DNA concentration as the efficiency of the gel extraction is not high)
**LB broth preparation**
1. Weigh 20 g LB broth
2. Add it into 1 L deionized H₂O in 1 L bottle
3. Label and autoclave the solution (120 °C/15 psi/20 mins)

**LB agar preparation:**
1. Weigh 5 g LB broth
2. Weigh 5 g Agar (Bacteriological grade)
3. Add them into 250 ml deionized H₂O in a 500 ml bottle
4. Label and autoclave the solution (120 °C/15 psi/20 mins)
5. Cool down until it reach around 50 °C and add the respective antibiotic
6. Pour around each 15 ml into 12 cm petric dish (can make around 18 dishes)

**Antibiotic shock preparation:**

**Ampicillin: Stock Concentration** - 50mg/ml in H₂O
**Working Concentration** - 50μg/ml
- Weigh 0.4g of ampicillin into a small weigh boat.
- Add 8 ml of double autoclave H₂O(ddH₂O) to a 15ml centrifuge tube.
- Add the ampicillin to the ddH₂O.
- Vortex to dissolve all the ampicillin into solution.
- Filter sterilize the solution with 10ml syringe, linked with 0.2 um filter.
- Aliquot each 0.8ml into 1.7ml centrifuge tubes.
- Cover the tubes with aluminium foil.
- Store at -20°C.

**Tetracycline: Stock Concentration** - 5mg/ml in 70% Ethanol
**Working Concentration** - 20μg/ml
- Weigh 0.04 g of tetracyline HCl.
- Add 8 ml of 70% Ethanol to a 15ml centrifuge tube.
- Add the tetracycline HCl to the 70% ethanol.
- Vortex vigorously to dissolve all the tetracycline HCl.
- Filter sterilize the solution with 10ml syringe, linked with 0.2 um filter.
- Aliquot each 0.8ml into 1.7ml centrifuge tubes.
- Cover the tubes with aluminium foil.
Store at -20C.

**Chloramphenicol**

**Stock concentration** - 34mg/ml in 100% Ethanol

**Working concentration** = 25μ/ml

- Weight 0.272g of chloramphenicol sulphate.
- Add 8 ml of 100% Ethanol to a 15ml centrifuge tube.
- Add the chloramphenicol sulphate to the 100% ethanol.
- Vortex vigorously to dissolve all the chloramphenicol into solution.
- Aliquot each 0.8ml into 1.7ml centrifuge tubes.
- Cover the tubes with aluminium foil
- Store at -20C.