Cell Cultures

1. Add 5 mL of LB fresh medium to a 10mL sterile tube or test tube.
2. Add the antibiotic to give the appropriate concentration.
3. Scoop one colony from the plate with a micropipette tip.
4. Put the colony into the sterile tube (or test tube) containing the medium and antibiotic.
5. Incubate for at least 12 h at 37ºC, with shaking at 200 rpm.

Competent cells

1. Take one E.coli DH5α colony and start a 5 mL overnight culture at 37°C, with shaking.
2. Dilute the overnight culture 1:100 into 50 mL LB medium, and continue the incubation for 2~3h until the OD600= 0.4-0.6
3. Let the culture sit on ice for 10 min.
4. Centrifuge at 4000 rpm for 10 min at 4°C.
5. Remove as much as possible of the supernatant without disturbing the pellet.
6. Resuspend the pellet in 10mL of ice-cold 100mM CaCl2 solution.
7. Incubate on ice for 15 min
8. Centrifuge at 4000 rpm for 10 min at 4°C.
9. Again, remove as much as possible of the supernatant without disturbing the pellet, and resuspend the pellet in 10ml of ice-cold 100mM CaCl2/20% glycerol solution.
10. Incubate on ice for 10 min.
11. Aliquot in 40 µL amounts to chilled 1.5 ml tubes.
12. Store immediately at -80ºC

Transformation

1. Take the competent cells from the storage at -80ºC and thaw them on ice for 10 min.
2. Add 5 µL of plasmid solution, or 10ul of ligation reaction mixture, to the 50 µL of competent cells.
3. Incubate for 30 min on ice.
4. Heat shock the cells at 42ºC for 90s
5. Incubate for 5 min on ice.
6. Add 1000 µL of LB media.
7. Incubate for 60 min at 37ºC, with shaking.
8. Spin cells down at 6000 rpm for 1 min.
9. Discard all but ~100 µL of the supernatant and resuspend the pellet in the remaining solution.
10. Plate the remaining suspension on agar plate.

Plasmid DNA Purification (from TIANGEN ® Kit)

1. Add 1-5 mL of E. coli culture in LB media in to a 1.5-2 mL tube. Centrifuge at 12000 rpm for 1 min at room temperature. Remove the supernatant, and add 600 µL of MQ water to the pellet
2. Add 250 µL of Resuspension Solution P1 to the pelleted cells (Ensure RNase A has been added to the Resuspension Solution P1), and resuspend the pellet completely.
3. Add 250 µL of the Lysis Solution P2, and mix thoroughly by inverting the tube 6-8 times until the solution becomes viscous and slightly clear.
4. Add 350 µL of the Neutralization Solution P3 and mix immediately and thoroughly by inverting the tube 6-8 times. The neutralized bacterial lysate should become cloudy.
5. Centrifugate at 12000 rpm for 10 min.
6. Transfer the supernatant to the supplied spin column CP3. Avoid disturbing or transferring the white precipitate.
7. Centrifugate at 12000 rpm for 1 min, and discard the flow-through and place the spin column back into the same collection tube.
8. Optional, for EndA+ strains only, not for DH5α, TOP10: Add 500 µL of Wash Solution PD, and centrifuge for 1 min. Discard the flow-through and place the spin column back into the same collection tube.
9. Add 600 µL of the Wash Solution PW (Ensure ethanol has been added to the Wash Solution PW), and centrifuge for 1 min. Discard the flow-through and place the spin column back into the same collection tube.
10. Repeat the wash procedure (step 9) using 600 µL of the Wash Solution PW
11. Centrifuge for an additional 2 min to remove residual Wash Solution PW. This step is essential to avoid residual ethanol in plasmid preps.
12. Transfer the spin column into a fresh 1.5 mL tube. Add 50-100 µL of the Elution Buffer EB to the center of spin column membrane to elute the plasmid DNA. Incubate for 2 min at room temperature and centrifuge at 12000 rpm for 2 min.
13. Store the purified plasmid DNA at -20°C.

PCR Clean-up (from TIANGEN ® Kit)

1. Estimate the volume of PCR mixture or restriction reaction solution. Add Purification Buffer PB, with 5 times of the volume of PCR mixture solution, and mix thoroughly. i.e. Add 250 µL of Purification Buffer PB to 50 µL of PCR mixture solution.
2. Transfer the solution to a spin column CB2. Incubate for 2 min at room temperature and centrifuge at 12000 rpm for 1 min. Discard the flow-through and place the spin column back into the same collection tube.
3. Add 600 µL of the Wash Solution PW (Ensure ethanol has been added to the Wash Solution PW), and centrifuge for 1 min. Discard the flow-through and place the spin column back into the same collection tube.
4. Repeat the wash procedure (step 4) using 600 µL of the Wash Solution PW
5. Centrifuge for an additional 2 min to remove residual Wash Solution PW. This step is essential to avoid residual ethanol in plasmid preps.
6. Transfer the spin column into a fresh 1.5 mL tube. Add 30-50 µL of the Elution Buffer EB to the center of spin column membrane to elute the plasmid DNA. Incubate for 2 min at room temperature and centrifuge at 12000 rpm for 2 min.
7. Store the purified DNA at -20°C.

Restriction (NEB enzymes)

1. Calculate the volume of the DNA sample containing 1000 ng and the volume of ddH2O to make up to 50 µl.
2. Mix the components below.

|  |  |
| --- | --- |
| ddH2O | μL |
| DNA sample | μL |
| NEB buffer (Cutsmart/3.1) | 5 μL |
| NEB restriction enzyme 1 | 1 μL |
| NEB restriction enzyme 2 | 1 μL |
| Total | 50 μL |

1. Incubate at 37°C for 3 h to overnight (despite the recommendation of only 15 min by NEB).
2. Use the PCR purification kit or gel isolation kit to purify the restriction product.

DNA Electrophoresis

Casting a 30 ml gel

1. For a 1% 30ml agarose gel, weight 0.3 g of agarose in a conical flask.
2. Add 30 ml 1X TAE buffer.
3. Microwave for 1~3 min, and then let the agarose solution cool down.
4. Add 4 µL of Gene\_Green DNA stain to the solution.
5. Pour the gel solution into the gel tray. Remove any air bubbles with a micropipette tip and put in comb.
6. Let the gel solidify.

Running the gel

1. Remove the comb, and place the gel tray into the TAE buffer chamber.
2. Mix the DNA sample (~2 µl) with loading dye (1 µl). This can be done either in PCR tubes, or on a piece of parafilm.
3. Load the samples into the wells. After that, load 2µl of DNA marker into the middle well.
4. Run at 90-130V for 20-30 min. (depends on the length of DNA sample)

Gel Extraction (from MACHEREY-NAGEL® Kit)

1. Visualize the DNA in the agarose gel using a long-wavelength UV lamp. Minimize UV exposure time to avoid damaging the DNA.
2. Take a clean scalpel to excise the DNA fragment from an agarose gel. Remove all excess aga.rose. Weigh the gel slice and transfer it to a clean 1.5 mL tube.
3. For each 100 mg of agarose gel add 200 μL Buffer NTI (Membrane Binding Solution).
4. Incubate mixture solution for 5–10 min at 50 °C. Vortex the mixture solution every 2–3 min until the gel slice is completely dissolved.
5. Place a “NucleoSpin® Gel and PCR Clean-up” column into a 2 mL collection tube, and load up to 700 μL sample.
6. Centrifuge at 12000rpm for 30 s. Discard flow-through and place the column back into the collection tube. Load remaining sample if necessary and repeat the centrifugation step.
7. Add 700 μL Buffer NT3 (Membrane Wash Solution) to the column. Centrifuge at 12000rpm for 30 s. Discard flow-through and place the column back into the collection tube.
8. Centrifuge at 12000rpm for 1 min to remove Buffer NT3 completely.
9. Place the column into a fresh 1.5 mL tube. Add 15–30 μL of the Buffer NE and incubate at room temperature for 1 min. Centrifuge at 12000 rpm for 1 min.
10. Store the purified DNA at -20°C.

Ligation

1. Calculate the volume of the Vector DNA & Fragment DNA, and the volume of ddH2O to make up to 20 µl. The mole ratio of Vector DNA/Fragment DNA should be 1:10-1:100.
2. Mix the components below.

|  |  |
| --- | --- |
| ddH2O | μL |
| Fragment DNA | μL |
| Vector DNA | μL |
| T4 ligase 10X buffer | 2 μL |
| T4 ligase | 1 μL |
| Total | 20 μL |

1. Incubate for 3h at 37ºC(NEB), or overnight at 16ºC. (TAKARA)
2. Transform competent cells. Or use the PCR purification kit or gel isolation kit to purify the ligation product and store at -20 ºC.

PCR (Taq Master MIX® or Q5 High-Fidelity Master Mix®)

1. Design the primers with softwares. i.e. Primer Premier 6.0(要不要给出软件名字？会不会追查盗版-\_-||)
2. Prepare the following reaction mixture:
3. Program the PCR machine
4. Run the PCR reaction.

Colony PCR (Taq PCR Master Mix®)

1. Number the colonies to be tested by marking on the backs of plates.
2. Prepare the pre-mix PCR solution with the volume listed below.
3. Pick a small portion of each colony and add into pre-mix PCR solution.
4. Run the PCR reactions (Protocol PCR\_ Taq Master MIX®).
5. Analyze a 10 µl aliquot of each PCR sample by agarose gel electrophoresis.

Homologous recombination reaction (from ClonExpress® II One Step Cloning kit)

1. Design the ClonExpressTM II primers, with 15~20 bp homologous sequences of the vector introduced into 5’ end of the primer pair and 3’ end of be specific to the inserted fragement.
2. Prepare for linearized cloning vectors with double/single endonucleases digesting (Protocol Restriction).
3. Prepare for insertion fragments with PCR.
4. Set up the reaction mixture below on ice.

|  |  |
| --- | --- |
| ddH2O | μL |
| 5×CE II Buffer | 2 μL |
| Exnase® II | 4 μL |
| Linearized cloning vector | 50~200 ng |
| PCR products of insertions | 50~200 ng |
| Total | 20 μl |

1. Incubate the reaction mixture at 37°C for 30 min and immediately place it on ice for 5 min.
2. Transform the entire recombination products to 200 μL of competent cells. (Protocol Tranformation)

In-Fusion cloning (from In-Fusion HD Cloning Kit)

1. Design the In-Fusion primers, with 15 bp homologous sequences of the vector introduced into 5’ end of the primer pair and 3’ end of be specific to the inserted fragement.
2. Prepare for linearized cloning vectors with double/single endonucleases digesting (Protocol Restriction).
3. Prepare for insertion fragments with PCR.
4. Set up the reaction mixture below on ice.

|  |  |
| --- | --- |
| ddH2O | μl |
| 5X In-Fusion HD Enzyme Premix | 2 μl |
| Linearized cloning vector | 50~200 ng |
| Purified PCR fragment | 10~200 ng |
| Total | 10 μl |

1. Incubate the reaction mixture at 50°C for 15 min and immediately place it on ice for 5 min.
2. Transform the entire recombination products to 200 μL of competent cells. (Protocol Tranformation)