iGEM 2016

Molecular Cloning Handbook

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Content

Transformation

Requirements:

—TransGen® Trans5α Chemically Competetent Cell
—LB broth
—iGEM DNA Distribution Kit Plates, Plasmid DNA or DNA ligation mix
—LB agar plates containing 15-100μg/mL antibiotic of choice
—Nuclease-free 1.5mL microcentrifuge tubes
—Nuclease-free 0.2mL PCR tubes
—Water bath of 42°C
—Shaking incubator of 37°C

Before Starting (if you need plasmids from iGEM DNA Distribution Kit Plates):

1. Punch a hole with a pipette tip through the foil cover into the corresponding well of the desired BioBrick part.
2. Add 10μL sterile deionized water, pipette up and down several times.
3. Transfer liquid from Step 2 into a 0.2 mL PCR tube.
4. Repeat Steps 2-3 twice.
5. Store liquid (BioBrick plasmid) at -20°C

Protocol:

1. Add 5-10μL plasmid or ligation system into 50μL fresh competent cells, which is contained in 1.5mL centrifuge tube. Then mix gently.
2. Incubate the tube on ice for 30 minutes
3. Heat shock in the water bath at 42°C for 45 seconds.
4. Incubate on ice for 2 min.
5. Add 450μL fresh LB broth into the tube.
6. Incubate for 1 hour under the condition of 37°C, 200rpm using a shaking incubator.
7. Spread 250μL liquid from Step6 on a LB agar plate, which contains appropriate antibiotics.
8. Incubate overnight at 37°C (about 12 hours, no more than 16 hours).

Growing the Single Colonies from Agar Plates

Requirements:

—LB agar plate containing transformed bacterial colonies incubated overnight

—LB broth

—Antibiotics

—50mL centrifuge tubes

—Shaking incubator of 37°C

Protocol:

1. Add 10mL LB broth into a centrifuge tube, then add the appropriate antibiotic needed.
2. Select the single colony using the pipette tip from the agar plate, which contains the bacterial cells. Then put the pipette tip into the tube from Step 1.
3. Incubate overnight at 37°C (about 12 hours, no more than 16 hours).

Making Glycerol Stocks

Materials and Equipments:

—Bacterial culture

—Glycerol

—1.5mL cryogenic microtubes

Protocol:

1. Add 200μL glycerol into a cryogenic microtube.
2. Pipet 800μL bacterial culture into glycerol in the cryogenic microtube from step 1 and mix by pipetting, save in -20°C freezer.
3. This glycerol stock can be used whenever required, by just adding 10μL glycerol stock into 10mL LB broth.

Plasmid Extraction

Requirements:

—Omega E.Z.N.A.® Plasmid Mini Kit II
—100% ethanol
—Isopropanol
—Microcentrifuge capable of at least 13000 x g
—Nuclease-free 1.5mL microcentrifuge tubes
—Sterile deionized water
—Electric dry oven of 65°C
—Water bath of 65°C

Before Starting:

—Heat sterile deionized water to 65°C using water bath
—Add the vial of RNase A to the bottle of Solution I if there's no mark on the bottle and store at 4°C
—Add some 100% ethanol to the bottle of DNA Wash Buffer if there's no mark on the bottle and store at room temperature
—Add some isopropanol to the bottle of HBC Buffer if there's no mark on the bottle and store at room temperature
(The volume of ethanol and isopropanol is showed on the tag of the bottle)

Protocol:

1. Pellet 1.5mL bacteria in a clean 1.5mL microcentrifuge tube by centrifugation at 10,000 x g for 1 minute at room temperature. Decant or aspirate medium and discard.
2. Add 250µL Solution I/RNase, pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields,
3. Add 250µL Solution II and gently mix by inverting and rotating the tube several times to obtain a clear lysate. A 2 minutes incubation is necessary. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity.
4. Add 350µL Solution III and mix immediately by inverting the tube several times until a flocculent white precipitate forms. Incubate for 2 minutes.
5. Centrifuge at maximum speed (≥13,000 x g) for 10 minutes at room temperature. A compact white pellet will form. Promptly proceed to the next step.
6. Insert a HiBind® DNA Mini Column into a 2mL Collection Tube.
7. Transfer 700µL cleared lysate from Step 5 CAREFULLY aspirating it into the HiBind® DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the HiBind® DNA Mini Column.
8. Centrifuge at maximum speed for 1 minute.
9. Discard the filtrate and reuse the collection tube.
10. Repeat Steps 7-9 until all cleared lysate has been transferred to the HiBind® DNA Mini Column.
11. Add 500µL HBC Buffer.
12. Centrifuge at maximum speed for 1 minute.
13. Discard the filtrate and reuse the collection tube.

14. Add 700μL DNA Wash Buffer.
15. Centrifuge at maximum speed for 1 minute.
16. Discard the filtrate and reuse the collection tube.
17. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.
18. Transfer HiBind® DNA Mini Column to a clean 1.5mL microcentrifuge tube. Open the lid and put it in the electric dry oven for 10 minutes to volatilize alcohol.
19. Add 60mL sterile deionized water directly to the center of the column membrane.
20. Let sit at room temperature for 2 minutes.
21. Centrifuge at maximum speed for 1 minute.
22. Store DNA at -20 °C

Genomic DNA Extraction

Requirements:

—TransGen EasyPure® Bacteria Genomic DNA Kit
—100% ethanol
—70% ethanol (for Gram-positive bacterium)
—Glass bead (for Actinobacillus)
—Lysozyme (produced by BBI Life Sciences®)
—RNase A (produced by Takara®)
—Proteinase K (produced by Takara®)
—Microcentrifuge capable of at least 13,000 x g
—Nuclease-free 1.5mL microcentrifuge tubes
—Sterile deionized water
—Electric dry oven at 65°C
—Water bath at 55°C and 65°C

Before Starting:

—Add 15mL 100% ethanol into BB11 if there's no mark on the bottle and store at room temperature
—Add 48mL 100% ethanol into WB11 if there's no mark on the bottle and store at room temperature
—Weight 4mg lysozyme in a microcentrifuge tube, and add 200μL RB11, store at 4°C
—Heat sterile deionized water to 65°C using water bath

Protocol:

1. Pellet 1.5mL bacteria in a clean 1.5mL microcentrifuge tube by centrifugation at 12,000 x g for 1 minute at room temperature. Decant or aspirate medium and discard.
2. Add 200μL RB11/lysozyme, pipet up and down to mix thoroughly. Incubate at 37°C for 60 minutes.
3. Centrifuge at 10,000 x g for 1 minute. Discard the liquid.
   Note: For Gram-positive bacterium, a re-suspension using 70% ethanol before this step is needed. For Actinobacillus, it's necessary to scatter the hyphostroma.
4. Add 100μL LB11 and 20μL Proteinase K, pipet up and down to mix thoroughly.
5. Incubate at 55°C for 15 minutes, shake the tube every 5 minutes.
   Note: If the liquid is not limpid, another 15 minutes incubation may be necessary.
6. Add 20μL RNase A, mix thoroughly, and incubate at room temperature for 2 minutes.
7. Add 400μL BB11, vortex 30 seconds.
8. Transfer 700μL liquid from Step 7 into a Genomic Spin Column.
9. Centrifuge at 12,000 x g for 30 seconds, discard the filtrate and reuse the collection tube.
10. Repeat Steps 8-9 until all liquid has been transferred to the Genomic Spin Column.
11. Add 500μL CB11 into the Genomic Spin Column, centrifuge at 12,000 x g for 30 seconds, discard the filtrate and reuse the collection tube.
12. Repeat Step 11 once.
13. Add 500μL WB11 into the Genomic Spin Column, centrifuge at 12,000 x g for 30 seconds, discard the filtrate and reuse the collection tube.
14. Repeat Step 13 once.

15. Centrifuge the empty Genomic Spin Column for 2 minutes at 12,000 x g to dry the column matrix.
16. Transfer the column to a clean 1.5mL microcentrifuge tube. Open the lid and put it in the electric dry oven for 10 minutes to volatilize alcohol.
17. Add 75μL sterile deionized water into the column.
18. Let sit at room temperature for 2 minutes.
19. Centrifuge at 12,000 x g for 1 minute.
20. Repeat Steps 17-19 once.
21. Store DNA at -20 °C.

DNA Gel Electrophoresis

Requirements

—50× TAE concentrate Solution (produced by Solarbio®)
—Agarose (produced by Biowest®)
—DNA dye (TransGen® GelStain)
—100mL Erlenmeyer flask
—Distilled water
—Microwave oven
—DNA samples
—10× Loading buffer (produced by Takara®)
—DNA marker (produced by TranGen®)
—Electrophoresis instrument

Before Starting:

—Dilute 50× TAE concentrate Solution to 1× TAE buffer with distilled water
—Add 10× loading buffer into marker and DNA samples. Loading buffer should occupy 10% of total volume.

Protocol:

1. Weigh 0.36g agarose in an Erlenmeyer flask.
2. Add 30mL 1× TAE buffer into the flask from Step 1.
3. Make agarose melt by microwave oven (medium-high heat, about 3 minutes).
4. Add 3μL TransGen® GelStain, mix by shocking.
5. Assemble gel pouring apparatus by inserting gate into slots.
6. Pour agarose gel into the gel tray.
7. Cool for 40 minutes to solidify the DNA agarose gel.
8. Remove the pouring apparatus, put the gel into an electrophoresis instrument.
9. Pipet marker and DNA samples which have been mixed with loading buffer into the slots.
10. Turn on the electrophoresis instrument, set the working electric current at 75-100mA.
11. Electrophoresis for 45-60 minutes.
12. Turn off the instrument, take the gel into the gel formatter to take and save photos.

Gel Extraction

Requirements:

—Omega E.Z.N.A.® Gel Extraction Kit
—DNA agarose gel sliced from the electrophoresed gel
—100% ethanol
—Microcentrifuge capable of at least 13,000 x g
—Nuclease-free 1.5 mL microcentrifuge tubes
—Sterile deionized water
—Electric dry oven of 55°C and 65°C
—Water bath of 65°C

Before starting:

—Heat sterile deionized water to 65°C using water bath
—Add 100mL 100% ethanol to the bottle of SPW Wash Buffer if there's no mark on the bottle, store at room temperature

Protocol:

1. Put the gel slice in a clean 1.5mL microcentrifuge tube.
2. Add Binding Buffer to fill the microcentrifuge tube from Step 1.
3. Incubate at 55°C in a water bath, until the gel has completely melted. Shake the tube every 2-3 minutes.
4. Insert a HiBind® DNA Mini Column in a 2mL Collection Tube.
5. Add 700µL solution from Step 3 to the HiBind® DNA Mini Column.
6. Centrifuge at 10,000 × g for 1 minute at room temperature.
7. Discard the filtrate and reuse collection tube.
8. Repeat Steps 5-7 until all of the sample has been transferred to the column.
9. Add 300µL Binding Buffer.
10. Centrifuge at maximum speed (≥13,000 x g) for 1 minute at room temperature.
11. Discard the filtrate and reuse collection tube.
12. Add 700µL SPW Wash Buffer.
13. Centrifuge at maximum speed for 1 minute at room temperature.
14. Discard the filtrate and reuse collection tube.
15. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.
16. Transfer the HiBind® DNA Mini Column to a clean 1.5mL microcentrifuge tube. Open the lid and put it in the electric dry oven for 10 minutes to volatilize alcohol.
17. Add 35µL sterile deionized water directly to the center of the column membrane.
18. Let sit at room temperature for 2 minutes.
19. Centrifuge at maximum speed for 1 minute.
20. Store DNA at -20°C.

PCR (Polymerase Chain Reaction)

Requirements:

—Takara PrimeSTAR® Max DNA Polymerase

—DNA template

—Primers (synthetized by Sangon Biotech®)

—Nuclease-free 0.2mL PCR tubes

—PCR instrument

Protocol:

1. The choice of reaction system:

Components	Volume
DNA template	2μL
Forward Primer	1μL
Reverse Primer	1μL
PrimeSTAR® Max DNA Polymerase	20μL
Sterile deionized water	16μL

2. The choice of PCR program:

Step	Temperature	Duration	Loops
Preheat	97°C	—	—
1	94°C	5 minutes	
2	94°C	30 seconds	30 loops
3	x	30 seconds	
4	72°C	1 minute/kb	
5	72°C	10 minutes	—
6	15°C	10 minutes	

Note: x is the annealing temperature of the reaction, usually 5°C to 10°C lower than Tm of the primer.

The calculation of Tm: Tm=4(G+C)+2(A+T)

PCR Purification:

Requirements:

—Omega E.Z.N.A.® Cycle Pure Kit
—PCR product
—100% ethanol
—Microcentrifuge capable of at least 13,000 x g
—Nuclease-free 1.5mL microcentrifuge tubes
—Sterile deionized water
—Electric dry oven of 65°C
—Water bath of 65°C

Before Starting:

—Heat sterile deionized water to 65°C using water bath
—Add 100mL 100% ethanol to the bottle of DNA Wash Buffer if there's no mark on the bottle and store at room temperature

Protocol:

1. Determine the volume of PCR product, and transfer the product into a clean 1.5mL microcentrifuge tube.
2. Add 4-5 volumes CP Buffer. For PCR products smaller than 200bp, add 6 volumes CP Buffer.
3. Vortex to mix thoroughly.
4. Insert a HiBind® DNA Mini Column into a 2mL Collection Tube.
5. Add the sample from Step 3 to the HiBind® DNA Mini Column.
6. Centrifuge at maximum speed (≥13,000 x g) for 1 minute at room temperature.
7. Discard the filtrate and reuse collection tube.
8. Add 700μL DNA Wash Buffer.
9. Centrifuge at maximum speed for 1 minute.
10. Discard the filtrate and reuse collection minute.
11. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the column.
12. Transfer the HiBind® DNA Mini Column into a clean 1.5mL microcentrifuge tube. Open the lid and put it in the electric dry oven for 10 minutes to volatilize alcohol.
13. Add 40μL sterile deionized water directly to the center of column matrix.
14. Let sit at room temperature for 2 minutes.
15. Centrifuge at maximum speed for 1 minute.
16. Store DNA at -20°C.

Restriction Digest

Requirements:

—Plasmid DNA or PCR product

—Restriction enzymes and buffers (produced by Takara Bio®)

—Nuclease-free 0.2mL PCR tubes

—Water bath at 37°C

Protocol:

1. Prepare reaction systems in a 0.2mL PCR tube according to the following table:

Components	Volume
Plasmid or PCR product	14μL
Restriction enzyme I	2μL
Restriction enzyme II	2μL
Buffer (10×)	2μL

Choose the buffer according to the following table:

	EcoR I	Xba I	Spe I	Pst I
EcoR I	H	M	H	H
Xba I	M	M+BSA	M	M
Spe I	H	M	M	H
Pst I	H	M	H	H

2. Incubate in the water bath at 37°C for 6-8 hours.

Ligation

Requirements:

—Digested DNA

—T4 ligase and buffer (produced by Takara Bio®)

—Nuclease-free 0.2mL PCR tubes

—Water bath at 16°C or fridge at 4°C

Protocol:

1. Prepare reaction systems in a 0.2mL PCR tube according to the following table:

Components	Volume (uL)
Insert	V_1
Vector	V_2
Buffer	1μL
T4 Ligase	1μL

$$\frac{V_1}{V_2}=\frac{3\times M_1\times c_2}{1\times M_2\times c_1}$$

V_1: insert

V_2:vector

M: molar weight (size)

C: conc. (ng/mL)

2. Incubate in the water bath at 16°C for 6 hours or fridge at 4°C for 12 hours.

Addenda

1. Antibiotics

Antibiotic	Stock Concentration (mg/mL)	Final Concentration (μg/mL)	Solvent
Ampicillin	50	50	Sterile deionized water
Chloramphenicol	50	50	Absolute ethyl alcohol
Kanamycin	50	50	Sterile deionized water

2. Culture Mediums

Component	Amount
Tryptone	1.0g
Yeast Extract	0.5g
NaCl	1.0g
Agar (for solid medium ONLY)	1.5g
Distilled water	100mL