iGEM 2020

Molecular Cloning Handbook 6.0 BETA

XMU-China

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Content

Transformation

Requirements:

—TransGen® Trans5α Chemically Competent Cell

Note: Trans5α is usually used in amplifying plasmids. If you want to obtain an adequate gene expression product, you may choose BL21 as your competent cells.

—LB broth

—iGEM DNA Distribution Kit Plates, Plasmid DNA or DNA ligation mix

—LB agar plates containing 15~100 μg/mL antibiotics of choice

—Nuclease-free 1.5-mL microcentrifuge 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 10 μL sterile deionized water, pipette up and down several times.
3. Transfer liquid from Step 2 into a 1.5-mL microcentrifuge tube.
4. Repeat Steps 2~3 twice.

   Note: This step is not always necessary because the concentration of plasmids you extract from the kit plates may be too weak to get a good transformation.
5. Store liquid (BioBrick plasmid) at -20 °C.

Protocol:

1. Add 5~10 μL plasmid or ligation system into 50 μL fresh competent cells, which is contained in 1.5-mL centrifuge tube. Then mix gently.

   Note: You may need to make a distribution of the competent cells in advance because a tube of commercial competent cell is usually 100 μL in volume.
2. Incubate the tubes on ice for 30 minutes.
3. Heat shock in the water bath at 42 °C for 45 seconds.

4. Incubate on ice for 2 minutes.
5. Add 450 μL fresh LB broth into the tube.
6. Incubate for 1 hour under the condition of 37 °C, 200 rpm using a shaking incubator.

   Note: Please use the waiting time efficiently to make LB agar plates which will be used in the next step. Add 100 μL antibiotics into 100 mL LB agar broth. The prescriptions of antibiotics are attached in the addenda.

7. Spread 100~125 μL liquid from Step 6 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 10 mL LB broth into a 50-mL centrifuge tube, then add the appropriate antibiotics needed.

   Note: In general, 100 mL LB broth is entirely used in a time, so adding 100 μL antibiotics into 100 mL broth is recommended before you make distributions of the LB broth. The prescriptions of antibiotics are attached in the addenda.

2. Select the single colony using the **10-μL 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

Requirements:

—Bacterial culture

—80% Glycerol

—1.5-mL cryogenic microtubes

Protocol:

1. Add 200 µL glycerol into a cryogenic microtube.
2. Pipet 800 µL 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 10 µL glycerol stock into 10 mL LB broth.

Plasmid Extraction

Requirements:

—Omega E.Z.N.A.® Plasmid Mini Kit II

—100% ethanol

—Isopropanol

—Microcentrifuge capable of at least 13000 × g

—Nuclease-free 1.5-mL microcentrifuge tubes

—Sterile deionized water

—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.

Note: The volume that should be added of ethanol or isopropanol is showed on the label of the bottle.

Protocol:

1. Pellet 1.5 mL bacteria in a clean 1.5-mL microcentrifuge tube by centrifugation at 10,000 × g for 1 minute at room temperature. Decant or aspirate medium and discard.

   Note: This step may need to be repeated in accordance with the introduction of the kit you're using.
2. Add 250 µL Solution I/RNase, pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.
3. Add 250 µL Solution II and gently mix by inverting and rotating the tube several times to obtain a clear lysate. A 2-minute incubation is necessary.

Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity.

4. Add 350 µL 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 × 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 2 mL Collection Tube.
7. Transfer 700 µL 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 500 µL HBC Buffer.
12. Centrifuge at maximum speed for 1 minute.
13. Discard the filtrate and reuse the collection tube.
14. Add 700 µL DNA Wash Buffer.
15. Centrifuge at maximum speed for 1 minute.
16. Discard the filtrate and reuse the collection tube.
17. Repeat Steps 14~16.
18. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.
19. Transfer HiBind® DNA Mini Column to a clean 1.5-mL microcentrifuge tube. Open the lid and put it in room temperature for 3 minutes to volatilize alcohol.

    *Note: Before transferring, you need to **MARK** the new 1.5-mL microcentrifuge tubes. It's necessary.*

20. Add 60 µL 65 °C sterile deionized water directly to the center of the column membrane.
21. Let sit at room temperature for 2 minutes.
22. Centrifuge at maximum speed for 1 minute.

23. Store DNA at -20 °C.

RNA extraction

Requirements:

—Omega Bio-Tek E.Z.N.A.® Bacterial RNA Kit

—High speed refrigerated centrifuge

—1.5-mL centrifuge tube

—70% ethanol

—β-mercaptoethanol (β-ME)

—Bacteria in log phase (grown in LB media)

Before Starting:

—Prepare a stock solution of lysozyme (provided) at 15 mg/mL with TE buffer and —aliquot into adequate portions. Store aliquots at -20 ℃.

—β-mercaptoethanol (β-ME) must be added to Buffer BRK before use.

—Add 48 mL absolute ethanol to RNA Wash Buffer II.

Protocol:

1. Grow Bacteria in LB media to log phase. (Do not use overnight culture.)

2. Harvest 2-mL culture by centrifugation at 5000×g for 10 min at 4℃.

3. Discard medium and resuspend cells in 100μL Lysozyme/TE Buffer. Mix by vortexing at maxi speed for 30 seconds.

4. Incubate at 30℃ for 10 minutes. Incubate on a shaker-incubator or vortex 20 seconds for every 2 minutes during incubation.

5. Add 350 μL Buffer BRK/2-Me and 30 mg Glass Powder to the sample and vortex vigorously for 5 minutes. Centrifuge for 5 minutes at 13,000×g in a micro-centrifuge.

6. Transfer 400 µL of the supernatant into a new 1.5 mL tube. Add 400 µL 70% ethanol to the lysate and mix well by pipetting.

7. Apply sample, including any precipitate that may have formed, to a HiBind® RNA mini column inserted in a 2-mL collection tube. Centrifuge for 60 seconds at 10,000 x g. Discard the liquid and reuse the collection tube for next step.

8. Place column in the collection tube, and add 300 µL RNA Wash Buffer I. Centrifuge at 10,000×g for 60 seconds at room temperature and discard flowthrough.

9. Place column in a clean 2 mL collection tube, and add 500 µL RNA Wash Buffer I. Wait for 10 minutes. Centrifuge at 10,000×g for 60 seconds at room temperature and discard flow-through.

10. Place column in the same 2-mL collection tube, and add 500 µL RNA Wash Buffer II diluted with ethanol. Centrifuge at 10,000×g for 60 seconds at room temperature and discard the flow-through.

11. Wash column with a second 500 µL of RNA Wash Buffer II as in step 11. Centrifuge at 10,000×g for 60 seconds at room temperature and discard the flow-through. Then with the collection tube empty, centrifuge the spin cartridge at 10,000×g for 2 min at room temperature to completely dry the HiBind matrix.

12. Transfer the column to a clean 1.5-mL microfuge tube and elute the RNA with 30-50 µL of DEPC-treated water. Make sure to add water directly onto column matrix. Centrifuge at 10,000 x g for 1 min at room temperature.

Restriction Digest

Requirements:

—Plasmid DNA or PCR product

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

—Nuclease-free 0.2-mL PCR tubes

—Water bath at 37 °C

Protocol:

1. Prepare reaction systems (20 μL, Large System) in a 0.2-mL PCR tube according to the following table:

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

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

Note: If your reaction system is a Small System (10 μL), the volume of reagents used should be halved.

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

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 Bio®)

—DNA marker (produced by TransGen®)

—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 0.36 g agarose in an Erlenmeyer flask.
2. Add 30 mL 1 × TAE buffer into the flask from Step 1.
3. Make agarose melt by microwave oven (medium-high heat, about 3 minutes).

   Note: In order to make the gel more even, reheating the agarose is recommended.
4. Add 3 μL TransGen® GelStain, mix by shocking. Or you can put the Erlenmeyer flask on a decolorizing shaker to shake for 1.5 minutes (do not shake too fiercely) to achieve mixing. To make the gel even is **VITAL** to obtain a good result of electrophoresis.

   Note: The Large System needs 0.72 g agarose, 60 mL 1 × TAE buffer and 6 μL TransGen® GelStain while the Small System needs 0.36 g agarose, 30 mL 1 × TAE buffer and 3 μL

TransGen® GelStain.

5. Assemble gel pouring apparatus by inserting gate into slots.
6. Pour agarose gel into the gel tray.
7. Cool for 40 minutes to **FULLY** 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 120~150 mA and the working voltage at 120~150 V.
11. Electrophoresis for 35~60 minutes.

    Note: When the blue strips move to two-thirds of the gel, the electrophoresis could be stopped.
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 × g

—Nuclease-free 1.5-mL microcentrifuge tubes

—Sterile deionized water

—Water bath of 55 °C and 65 °C

Before starting:

—Heat sterile deionized water to 65 °C using water bath.

—Add 100 mL 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 1.5-mL 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 2-mL Collection Tube.
5. Add 700 µL solution from Step 3 to the HiBind® DNA Mini Column.
6. Centrifuge at 10,000 × g for 1 minute at room temperature.
7. Pour the filtrate into the HiBind® DNA Mini Column used then centrifuge at 10,000 × g for 1 minute at room temperature again.
8. Discard the filtrate and reuse collection tube.
9. Repeat Steps 5~8 until all of the sample has been transferred to the column.
10. Add 300 µL Binding Buffer.

11. Centrifuge at maximum speed (≥13,000 × g) for 1 minute at room temperature.
12. Discard the filtrate and reuse collection tube.
13. Add 700 μL SPW Wash Buffer.
14. Centrifuge at maximum speed for 1 minute at room temperature.
15. Discard the filtrate and reuse collection tube.
16. Repeat Steps 13~15.
17. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.
18. Transfer the HiBind® DNA Mini Column to a clean 1.5-mL microcentrifuge tube. Open the lid and put it in room temperature for 3 minutes to volatilize alcohol.

    *Note: Before transferring, you need to **MARK** the new 1.5-mL microcentrifuge tubes. It's necessary.*

19. Add 35 μL 65 °C 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.

Reverse transcription

Requirements:

—Total RNA

—TransGen Biotech TransScript® Reverse Transcriptase

—TransGen Biotech High Pure dNTPs

—TransGen Biotech Ribonuclease Inhibitor

—Nuclease-free 200-μL PCR tubes

—Biometra TAdvanced Thermal Cycler (Analyticjena®)

Before Experiment:

None.

Protocol:

1. Add different components into PCR tubes according to following table

Component	Volume
Total RNA	50 ng-5 μg
Anchored Oligo$(dT)_{18}$ Primer (0.5 μg/μL)	1 μL
10 mM dNTPs	1 μL
5×TS RT Buffer	4 μL
Ribonuclease Inhibitor (50 units/ μL)	0.5 μL
TransScript® RT	1 μL
RNase-free Water	Variable
Total volume	20 μL

2. Mix well, incubate at 42℃ for 30 minutes.

3. Heat at 85℃ for 5 seconds.

PCR (Polymerase Chain Reaction)

Requirements:

—Takara PrimeSTAR® Max DNA Polymerase

—DNA template

—Primers (synthetized by Biosune®)

—Nuclease-free 0.2-mL PCR tubes

—PCR instrument

Protocol:

1. The choice of reaction system:

Components	Volume
DNA template	1 μL(<200 ng)
10 μM Forward Primer	1 μL
10 μM Reverse Primer	1 μL
PrimeSTAR® Max DNA Polymerase	25 μL
Sterile deionized water	22 μL

2. The choice of PCR program:

Step	Temperature	Duration	Loops
Preheat	98 °C	—	—
1	95 °C	4 minutes	
2	98 °C	10 seconds	35 loops
3	X	5 seconds	
4	72 °C	5 seconds/kb	
5	72 °C	10 minutes	—
6	15 °C	Hold	

Note: x is the annealing temperature of the reaction, usually 2 °C to 3 °C lower than T_m of the primer.

The calculation of T_m: *$T_m = 4(G+C) + 2(A+T)$*

Colony PCR

Requirements:

—Takara PrimeSTAR® Max DNA Polymerase

—Colony template

—Primers (synthetized by Biosune®)

—Nuclease-free 0.2-mL PCR tubes

—PCR instrument

Protocol:

1. The choice of reaction system:

Components	Volume
DNA template	Few colony
10 μM Forward Primer	1 μL
10 μM Reverse Primer	1 μL
PrimeSTAR® Max DNA Polymerase	5 μL
Sterile deionized water	3 μL

2. The choice of PCR program:

Step	Temperature	Duration	Loops
Preheat	98 °C	—	—
1	98 °C	5 minutes	30 loops
2	X	1 minute	
3	72 °C	2 minutes	
4	72 °C	10 minutes	—
5	15 °C	Hold	

Note: x is the annealing temperature of the reaction, usually 2 °C to 3 °C lower than T_m of the primer.

The calculation of T_m: *$T_m = 4(G+C) + 2(A+T)$*

qPCR

Requirements:

—cDNA template, Forward Primer, Reverse Primer

—TransGen Biotech TranStart® Top Green qPCR Supermix

—Axygen® PCR 8 strip tubes

—Analyticjena® qTOWER3/G

Before Experiment:

None.

Protocol:

1. Add different components into 8 strip PCR tubes according to following table

Component	Volume
Template	10 pg-1 μg
Forward Primer (10 μM)	0.4 μL
Reverse Primer (10 μM)	0.4 μL
2× *TranStart*® Top Green qPCR Supermix	10 μL
Nuclease-free water	Variable
Total volume	20 μL

2. The qPCR program is shown in following table

steps	scan	temp (°C)	time (m:s)	goto	loops	delta Temp (°C)	delta Time (s)	ramp (°C/s)
1		95	00:30	0	0	0	0	8
2		95	00:10	0	0	0	0	8
3	*	60	00:30	2	39	0	0	6
4	*	60	00:15	4	35	1	0	5

PCR Purification:

Requirements:

—Omega E.Z.N.A.® Cycle Pure Kit

—PCR product

—100% ethanol

—Microcentrifuge capable of at least 13,000 × g

—Nuclease-free 1.5-mL microcentrifuge tubes

—Sterile deionized water

—Water bath of 65 °C

Before Starting:

—Heat sterile deionized water to 65 °C using water bath.

—Add 100 mL 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 1.5-mL microcentrifuge tube.
2. Add 4~5 volumes CP Buffer. For PCR products smaller than 200 bp, add 6 volumes CP Buffer.
3. Vortex to mix thoroughly.
4. Insert a HiBind® DNA Mini Column into a 2-mL Collection Tube.
5. Add the sample from Step 3 to the HiBind® DNA Mini Column.
6. Centrifuge at maximum speed (≥13,000 × g) for 1 minute at room temperature.
7. Pour the filtrate into the HiBind® DNA Mini Column used then centrifuge at maximum speed (≥13,000 × g) for 1 minute at room temperature again.
8. Discard the filtrate and reuse collection tube.
9. Add 700 µL DNA Wash Buffer.

10. Centrifuge at maximum speed for 1 minute.
11. Discard the filtrate and reuse collection minute.
12. Repeat Steps 9~11.
13. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the column.
14. Transfer the HiBind® DNA Mini Column into a clean 1.5-mL microcentrifuge tube. Open the lid and put it in room temperature for 3 minutes to volatilize alcohol.

    *Note: Before transferring, you need to **MARK** the new 1.5-mL microcentrifuge tubes. It's necessary.*
15. Add 40 μL sterile deionized water directly to the center of column matrix.
16. Let sit at room temperature for 2 minutes.
17. Centrifuge at maximum speed for 1 minute.
18. Store DNA at -20 °C.

Ligation

Requirements:

—Digested DNA

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

—Nuclease-free 0.2-mL PCR tubes

—Water bath at 16 °C or fridge at 4 °C

Protocol:

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

Components	Volume (μL)
Insert	V_1
Vector	V_2
Buffer	1 μL
T4 Ligase	1 μL
Total	10 μL

Note: The formula below is used to calculate the volumes of insert and vector after you measure the concentration of them. Actually, the molar weight could be replaced by the number of base pairs to simplify the calculation.

$$\frac{V_1}{V_2} = \frac{3 \cdot M_1 \cdot C_2}{1 \cdot M_2 \cdot C_1}$$

V_1: the volume of insert (μL)

V_2: the volume of vector (μL)

M: molar weight (size)

C: concentration (ng/μL)

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

Note: Add 0.500 g antibiotics into 10 mL solvent in a 15mL centrifuge tube. Then filter the solution through a 0.22-μm filter membrane to distribute the antibiotics into a number of clean sterile 1.5-mL microcentrifuge tubes.

2. Culture Mediums

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