Categary

Molecular Cloning

1.Growing overnight cultures:

1) Prepare 5ml of broth in 15ml Falcon tubes (in the case of yeast, 3ml liquid culture);
2) Add 5μL of antibiotics (in the case of yeast, 3μL antibiotics)[1];
3) Incubate at 37℃ for 24h(E.Coli) or 30℃ for 48-72h(yeast) on shakers:
   a. In the case of a single colony: Use a sterile tip to pick single colonies and then dip it underneath the surface of the broth. Blend multiple times;
   b. In the case of preserved bacteria culture: Pipette 10μL of culture into the broth, blend multiple times.

2.Glycerol stock preparation:

1) Prepare sterile 1.5ml Eppendorf tubes;
2) Pipette 300μL 50% glycerol into 1.5ml Eppendorf tubes after labeling them;
3) Add 300μL of bacteria culture into 1.5ml Eppendorf tubes;
4) Gently invert the tube multiple times to mix thoroughly;
5) Store the tube in -80℃ refrigerators;

3.Plasmid extraction:

1) For E.Coli plasmid extraction, please refer to E.Z.N.A. ® Plasmid DNA Kit I Spin Protocol of Omega Bio-tek, Inc.
2) For yeast plasmid extraction, please refer to E.Z.N.A. ® Yeast Plasmid Mini Spin Protocol of Omega Bio-tek, Inc.

4.PCR:

- Gradient PCR:

1) Set up PCR programs;

[1] Concentration of antibiotics: 0.1% (1μL of antibiotics per mL of liquid culture medium);

2) Prepare reaction systems:

Reagent	Volume/μL	Negative Control
Forward Primer	2	2
Reverse Primer	2	2
Template	5	/
2×High-Fidelity Master Mix[2]	25	25
ddH_2O	16	21
Total	50	50

3) Mix thoroughly and centrifuge to clear away bubbles;
4) Run gradient PCR programs, adjust annealing and extension time and temperature accordingly;
5) Perform electrophoresis to verify the results;

· Amplification PCR:

1) Please refer to chapter "Gradient PCR";
2) Please note that volume of template may be adjusted according to their concentrations;
3) Annealing temperature may be adjusted according to the results of gradient PCR;

· Colony PCR:

1) Set up PCR programs:

Process	Temperature/℃	Duration	Note
Initial Denaturation	98	2 min	In yeast colony PCR, adjust initial denaturation time to 10 minutes and run at 98℃
Denaturation	98	10 s	
Annealing	Discrepant		
Extension	72	Discrepant	
Final Extension	72	3 min	
Hold	4	∞	

2) Prepare reaction systems:

Reagent	Volume/μL	Negative Control
Forward Primer	0.4	0.4

[2] From Tsingke Bio-tek, Inc.

Reverse Primer	0.4	0.4
Template	2	/
2×T5 Super PCR Mix[3]	5	5
ddH_2O	2.2	4.2
Total	10	10

3) Mix thoroughly and centrifuge to clear away bubbles;
4) Run colony PCR programs, adjust annealing and extension time and temperature accordingly;
5) Perform electrophoresis to verify the results;

5.Agarose gel preparation:

1) Determine concentration of agarose gels according to lengths of DNA fragments and calculate the weight of agarose needed according to gel volume and concentration;
2) Add corresponding weight of agarose into 1×TAE buffer;
3) Boil with microwave oven until the liquid becomes transparent and no suspended solids can be observed;
4) Add 1% of ethidium bromide dye into the gel and mix up thoroughly after cooling the gel down;
5) Gently add the gel into the mould. Make sure the liquid is evenly spreaded and no bubble is formed;

6.Nucleic acid electrophoresis:

1) Emerge the gel completely in 1×TAE buffer and make sure the holes are placed towards the cathode;
2) Mix loading buffers into the samples, blend thoroughly;
3) Add markers and mixtures of samples into holes, the volume can be adjusted accordingly;
4) Run electrophoresis at 120V for 20-60min;
5) Obtain and process images of electrophoresis via Gel Doc™ XR+ System of Bio-Rad, Inc.

[3] From Tsingke Bio-tek, Inc.

7.Gel extraction:

1) Please refer to E.Z.N.A. ® Gel Extraction Kit - Spin Protocol of Omega Bio-tek, Inc.

8.Purification of nucletic products:

1) Please refer to E.Z.N.A. ® Cycle Pure Kit Centrifugation Protocol of Omega Bio-tek, Inc.

9.Enzyme digestion:

1) Set up reaction systems as follows:

Reagent	**Volume/μL**
Enzyme 1	1
Enzyme 2	1
10×Buffer[4]	2
Template	6
ddH_2O	10
Total	20

2) Incubate at 37℃ for 60-120 minutes, hold at 4℃;

10.Enzyme ligation:

1) Set up reaction systems as follows:

Reagent	**Volume/μL**
10×Buffer[5]	2
Fragments	Discrepant
T4 Ligation Enzyme[6]	1

[4] From Takara Bio-tek, Inc.
[5] From Takara Bio-tek, Inc.
[6] From Takara Bio-tek, Inc. Volume of fragments to be ligated can be determined after concentration measuring.

ddH_2O	Discrepant
Total	20

2) Incubate overnight at 15℃;

11.Plasmid transformation into E.Coli:

1) Prepare sterile 1.5ml Eppendorf tubes;
2) Add 50μL competent E.Coli and 10 μL plasmid into the tubes. Negative controls contain 50μL competent E.Coli and 10 μL ddH2O;
3) Incubate on ice for 30 minutes;
4) Heat shock at 40-42℃ for 65-85 seconds;
5) Instantly transfer the tubes on ice and let sit for 10 minutes;
6) Add 400μL liquid LB medium into each tube and vibrate on a shaker for 60 minutes at 37℃;
7) Add 200μL cultured medium onto solid LB plate with 0.1% Ampicilin and incubate at 37℃ overnight;

12.Extraction of yeast genome:

1) Please refer to E.Z.N.A. ® Yeast DNA Vacuum/Spin Protocol of Omega Bio-tek, Inc.

13.Extraction of total RNA[7]:

1) Add 1mL overnight yeast culture into 1.5mL Rnase-free Eppendorf tubes, centrifuge at 10000rpm at room temperature for 1 minute. Discard the culture medium and collect yeast cells;
2) Add 500μL DEPC-treated ddH2O into the tubes to remove any residue culture medium. Centrifuge at room temperature for 1 minute and discard the supernatant;
3) Add 600μL Snailase Reaction Buffer per 20mg yeast cell into the tubes.
4) Add 50μL Snailase and mix thoroughly. Incubate in water at 37℃ for 5 minutes.

[7] Using yeast total RNA rapid extraction kit from Sangon Biotech, Inc.

Centrifuge at 10000rpm at 4℃ for 2 minutes, discard the supernatant;

5) Immediately add 400μL Buffer Rlysis-Y into the system, vortex to mix thoroughly. Incubate in water at 65℃ for 5 minutes;
6) Put 1.5mL Eppendorf tubes in ice for 5 minutes, add 200μL Buffer YCA into the system and mix thoroughly. Centrifuge at 12000rpm at 4℃ for 5 minutes and collect the supernatant;
7) Add phenol: chloroform(24:25, pH=4.5) of equilevant volume into the supernatant. Vortex to mix thoroughly, centrifuge at 12000rpm at 4℃ for 4 minutes. Collect the supernatant;
8) Add 1/3 the volume of the supernatant water-free ethanol into the tube and mix up. Centrifuge at 12000rpm at 4℃ for 3 minutes, carefully discard the superenatant;
9) Add 700μL 75% ethanol(formulated with DEPC-treated ddH2O) to wash the sediments, centrifuge at 12000rpm at 4℃ for 3 minutes. Carefully discard the supernatant;
10) Repeat step 9) for once;
11) Invert the tube at room temperature for 10 minutes to let residue ethanol evaporate. Add 50μL DEPC-treated ddH2O to dissolve the sediments.
12) Use immediately or store at -70℃;

14.Reverse transcription PCR[8]:

1) Set up reaction system as follows on ice:

Reagent	Volume	Note
5×PrimeScript™ RT Master Mix (Perfect Real Time)	2μL	*Reaction system can be enlarged. 500ng of total RNA can be added into 10μL system in maximum.
Total RNA	*	
RNase free ddH_2O	Up to 10μL	

2) Gently invert the tubes several times to mix thoroughly, centrifuge to clear away bubbles;
3) Set up the reaction program as follows:

Temperature/℃	Duration	Note
37	15 min	Reverse transcription
85	5 s	Inactivation of enzyme

[8] Using PrimeScript™ RT Master Mix (Perfect Real Time) from Takara Bio-tek, Inc.

4	∞	Hold

4) Please note that products acquired in this step can be directly added into real time PCR systems. However, volume added cannot exceed 1/10(V/V) the total volume of real time PCR system;

15.Quantitive real time PCR[9]:

1) Set up reaction system as follows on ice:

Reagent	Volume/μL	Note
TB Green® Premix Ex Taq™ II (Tli RNaseH Plus)(2×)	12.5	a) A primer concentration of 0.4μM could produce optimal yields; b) Add no more than 100ng of DNA template.
PCR Forward Primer(10μM)	1	
PCR Reverse Primer(10μM)	1	
DNA template	2	
ddH2O	8.5	
Total	25	

2) Gently invert the tubes several times to mix thoroughly, centrifuge to clear away bubbles;

3) Set up the reaction program as follows:

Procedure	Temperature/℃	Duration
Initial denaturation	95	30 s
PCR reaction (40 cycles)	95	5 s
	60	30 s
Melt curve	/	/

4) Obtain amplication curve and melt curve. Data can be processed to create standard curve.

[9] Using TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) from Takara Bio-tek, Inc. The instrument applied is CFX96 Real-Time PCR Detection System（Bio-Rad）.

Protein Expression

1.Ultrasonic lysis of Yeast (According to splitting parameters of E.coli)

1) Using 5-10 ml culture of Yeast for centrifugation (4000-6000G, 4 ℃, 10 min). After centrifugation, discarding the supernatant to remove medium.
2) Using 2-3 ml lysis buffer to resuspend Yeast sediments.
3) Filling a 100 ml beaker with ice. Then, inserting a 50ml ep tube which contains resuspended Yeast solution into the beaker, after that, inserting a supersonic probe into the Yeast solution. (Attention: The probe should be under the liquid level and avoid touching the beaker wall)
4) Setting parameters of ultrasonic cracking instrument as 30min total cracking time; 5s cracking time; 5s pause; 50% power. Then, pressing ON button and starting lysis.
5) After lysis, covering the lid of the 50ml ep tube. Then, centrifuging it with 13000G, 4 ℃, 10min.
6) After centrifugation, collecting supernatant with 1.5ml ep tube. Then, adding lysis buffer (Quantity equal to step 2) to resuspend sediments and gain sediments sample.
7) Detecting the protein concentration of that sample.

Attention:

- Controlling the cultivating time. Too short cultivating time may lead to no or a low expression of aimed protein, too long cultivating time, emission of protease from death Yeast probably degenerate protein.
- In order to avoid protein lysis by self-protease and maintain activity of protein, all of experimental steps shall be quickly operated on the ice.
- When collecting lytic supernatant, please ensure supernatant clean and lengthening centrifugal time is needed If supernatant is not clean enough.
- Protein sample is easy to be degraded, please proceed following experiments as soon as possible or storage at -20 ℃ for several days.

2.SDS-PAGE

2.1. Pretreatment of protein sample

1) Fetching 40- 50 ug protein sample (volume depends on concentration), then, according to mix proportion to add 4x SDS-PAGE Loading and mix uniformly. After that, setting in 100℃ water bath for 10 min.
2) Taking out the protein sample for loading.

2.2. Preparation of acrylamide gel

1) Assembling loading plate and detecting weeping.
2) According to formula and your demand to prepare proper acrylamide gel. Loading it into the plate until about 2cm away from top plate edge. Finally, filling the residual space with isopropanol.
3) After acrylamide gel solidification, pouring off any isopropanol. Then, according to formula to prepare spacer gel and filling in plate until overflow. Finally, inserting a comb and waiting for spacer gel solidification.

2.3. Electrophoresis of protein

1) Setting the concretionary SDS-PAGE gel into electrophoresis apparatus. Using new 1x running buffer to fill inside groove until overflow. Filling outside groove with 1x running buffer to standard level.
2) Applying samples and markers into gel hole.
3) Starting electrophoresis with 80V until protein samples are pressed as bands and reach dividing line of spacer gel and separation gel. Then, changing the voltage into 120V, stopping electrophoresis until bromophenol blue band reaches the bottom of ??(spacer gel or separation gel).

2.4. Dye and decolorization of CBB (Neglecting this step for Western Blot)

2.4.1. Rapid staining

1) After electrophoresis, putting SDS-PAGE in appropriate CBB dye liquor and heating for 1min through microwave. Then, observing staining condition, heating for 15-30s each time until the gel is stained in dark blue.
2) Putting staining gel in destaining solution and heating by microwave until background of gel changes into light blue. It’s available to gain a cleaner gel after putting it into destaining solution for a overnight shaking.

2.4.2. Slow staining

1) After electrophoresis, putting SDS-PAGE in appropriate CBB dye liquor and shaking it overnight on shaker. After that, whole gel is stained in dark blue.
2) Putting staining gel in destaining solution and shaking it overnight on shaker. After that, observing a colorless, clear background and dark blue protein band. Please lengthening destaining time if the background is not clear.

Attention:

- Pretreatment of protein is necessary, which aims at unfolding spatial structure of protein.
- APS is easy to degrade. To use SDS-PAGE right after it was ready.
- When preparing SDS-PAGE in low temperature (for example: winter), adding 50% more TEMED to promote gel solidification. Meanwhile, in order to dissolve SDS, any precipitation of it needs to be heated.
- In most of cases, problem of APS or/and TEMED will lead to dissolution of gel.
- Avoid large voltage in SDS-PAGE electrophoresis, especially in spacer gel.
- Please be careful in gel melting when use CBB for rapid staining.
- Avoid excessive destaining of target bands when use CBB for slow staining.

3.Western Blot

1) Steps of SDS-PAGE electrophoresis are the same as those mentioned above, but without CBB dye.
2) In order to tell direction of gel, cutting one of corners of the gel after electrophoresis.
3) Soaking PVDF membrane in methyl alcohol for several seconds.
4) Soaking sponge, filter paper, PVDF membrane and gel in appropriate 1x transfer buffer.
5) Using clips, in turn, to fix sponge, filter paper, gel, PVDF membrane, filter paper and sponge from bottom to top.
6) Putting fixed clips in transfer membrane tank, then adding 1x transfer buffer in the tank until reaches scale line. Both the inside and outside of transfer membrane tank shall be set in ice.
7) Turn on the system for 2h at 100V. Replenishing ice to outer transfer membrane tank in time.

8) After the transfer is complete, remove the cassette holder from the tank. Using forceps, carefully disassemble the transfer stack.
   (Optional step: After the transfer is complete, put the membrane in Ponceau's satin liquid and shaking for 30 min on shaker. Then, use ultrapure water/TBST to rinse it. If protein transfer is successful, you will see red protein bands. Don't stop rinsing until color of the membrane turns into white)
9) After PVDF membrane transfer is complete, soaking it in 4% skimmed milk powder (TBST as solution) to seal those unspecific sites and shaking it for 1h on shaker.
10) Place the PVDF membrane in TBST and shake for 10 minutes. Repeat twice with fresh TBST.
11) Place the PVDF membrane in the primary antibody solution and incubate with agitation for overnight in 4 ℃.
12) Place the PVDF membrane in TBST and shake for 10 minutes. Repeat twice with fresh TBST.
13) Place the PVDF membrane in the secondary antibody solution and incubate with agitation for 1 hour at room temperature.
14) Place the PVDF membrane in TBST and shake for 10 minutes. Repeat twice with fresh TBST.
15) Photograph the PVDF membrane with ECL developer (1:1 mixture).

Attention:

- Confirm direction consistency of gel and PVDF membrane.
- When transfer time is too short, large protein can't transfer on the membrane. When transfer time is too long, small protein may across membrane.
- When process protein transfer, set up the cooling unit on the tank transfer unit or the gel may melt.
- Place whole PVDF membrane in the antibody solution and incubate with agitation if unable to confirm protein sites.
- Lightly rinse is needed for Ponceau's dye.
- Dilution of primary antibody needs 4% BSA/4% skimmed milk powder. (Dilution rate: 1:5000 or more)
- Place the PVDF membrane in the primary antibody solution and incubate with agitation for 3-4h in room temperature.
- Rapid photography and dark environment can reduce fluorescence quenching.

Verify the Product COR/PTN

1.Thin-layer Chromatography

1) Accurately weigh the sample powder 0.1g, put it into 100 ml sample bottle, add 5.00 ml normal saline and 5.00 ml absolute ethyl alcohol, shake it up. Microwave over medium heat for 2 minutes, then centrifuge at 3000r/min for 5 minutes. The precipitate is removed by centrifugation and the clear supernatant extract is collected as the standard solution.
2) Take 5μl sample solution and 5μl our product COR, and sample on the same thin layer plate (the thin layer plate should be put under 110 ℃ for 30 minutes), then put the plate into the camag until the developing solvent have run 9 cm (the component of developing solvent is: chloroform: ethyl acetate: isopropanol: water: stronger ammonia water=8.0 : 2.0 : 6.0 : 0.3 : 0.2, remember to saturate for 15minutes before we use it.) Take out the plate and use 254nm UV to find the spots . Calculate the Rf and compare them if they are the same.

2.High Performance Liquid Chromatography

1) Accurately weigh the sample powder 5 mg, add 10 ml purified water and make into 0.5mg/ml standard solution (store in 4 C°).
2) Ultrasonic processing our product for 5 minutes, then centrifuge at 12000r/min for 10 minutes. Repeat for 3 times. The precipitate is removed by centrifugation and the clear supernatant extract is collected as the sample solution. Merge the clear supernatant then through the 0.45 um filtration membrane.
3) Begin testing.

COR:

chromatographic column	ZORBAX SB—C(4．6 mm× 250mm，5um)
column temperature	30℃
Sample size	20μl
moving phase	purified water : methyl alcohol = 85:15

detestor wave length	260nm
flow rate	1.0ml/min

PTN:

chromatographic column	Hyper-silODS2(4．6 mm× 250mm，5um)
column temperature	30℃
Sample size	10μl
moving phase	methyl alcohol: acetonitrile(10mmol/L)：ammonium acetate (pH7.6) = 2.5:2.5:95
detestor wave length	280nm
flow rate	1.0ml/min

4) dilute standard reserve solution with purified water gradient. Each concentration gradient is sampled three times, every sample is 20 μl. Recording peak area.
5) The standard curve was drawn and the regression equation was calculated with the injection concentration as the abscissa (x) and the average integral value of the peak area (Y) as the ordinate.
6) Use SPSS to collating of data.