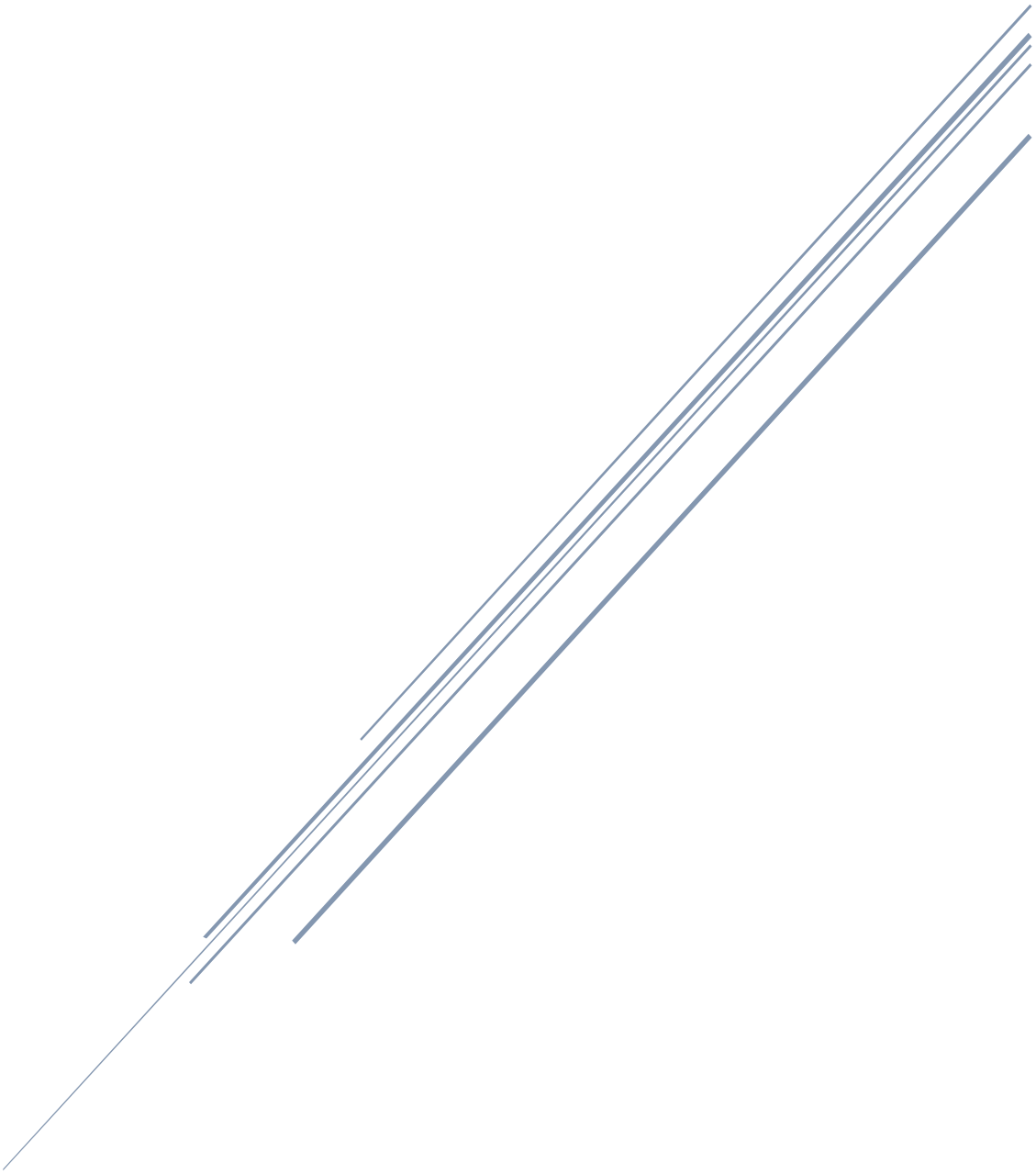
PROTOCOL

Greatbay_Shenzhen



Glycerol stock preparation

1. Do this before amplification
2. Label the sterilized Eppendorf tube with exactly correct name, pipette 300μL glycerol into it
3. Add 300μL of bacteria solution into the tube
4. Blow with a pipette multiple times to mix thoroughly
5. Update strain list

Plasmid extraction

1. Prepare 1950μl of bacteria solution in 2ml Eppendorf tube, labeling with [00n], and centrifuge at 12000rpm for 1 min, discard the supernatant (Repeat the step two times until bacteria from 4ml bacteria solution is obtained)
2. Add 150μL of Buffer P1 and vortex to blend until no bulk precipitation visible
3. Add 150μL of Buffer P2 and gently invert 6~8 times to mix. At this point the solution should be observed as pink in color
4. Immediately add 350μL of Buffer P5 within 2 min and gently invert 6~8 times. At this time the color of solution should change from pink to yellow with precipitations (no bubbles are allowed)
5. Centrifuge at 12000rpm for 5min to sperate plasmid and impurity.
6. Apply the supernatants from step 5 to a CP3 spin column and centrifuge 12000rpm for 1 min, discard the liquid in the collection tube.
7. Wash the CP3 spin column by adding 300μL of Buffer PWT. Let stand for 2 min and centrifuge at 12000 rpm for 1 min, discard the liquid in the collection tube.
8. Put back CP3 spin column and repeat step 7 without adding Buffer PWT. Discard the ethanol impurities.
9. Obtain CP3 spin column and placing it in a clean Eppendorf tube with the lid open (already labeling the name and code of the sample on the lid). Dry in 50˚C for 3-5min
10. Pipette 50μL of Buffer TB on the film of CPR. Close the lid and place under 50˚C for 5min. Centrifuge at 12000rpm for 3min and discard the CP3 spin column. At this time plasmid is in the Eppendorf tube.
11. Obtain its OD value using Microplate reader and note the value on the body of the tube. Place it in the -20˚C refrigerator.

Gel Extraction

1. Electrophoresis 20min at 120V
2. Obtain Gel image by using Gel imaging system
3. Prepare a 1.5mL Centrifuge tube already named
4. Cut the Gel and place the fragment in Centrifuge tube
5. Compress the gel on the dip of the tube using a microcentrifuge. Estimate Gel volume
6. Add equal volume of Buffer PN into the tube, mix with vertex, and place the tube inside a 700rmp, 55℃ metal bath until the gel fragment is fully dissolved
7.Prepare a Spin column and a Collection tube
8.Balance the Spin column with 500μL Buffer BL
9.Centrifuge the Spin column for 1min at 12000rpm, discard the flow-through
10.Lay the dissolved Gel solution into the spin column, letting stand for 2min and centrifuging at 12000rpm for 1min

11.Place all the flow-through back into the spin column again and centrifuging at 12000rpm for 1min
12.Discard the flow-through and add 600μL Buffer PW. Let stand for 2min then centrifuge at 12000rpm for 1min, discard the flow- through
13.Add 600μL Buffer PW again, centrifuge at 12000rpm for 1min, discard the flow- through
14.Centrifuge at 12000rpm for 3min without adding anything
15.Change a new Centrifuge tube and name it
16.Put inside a metal bath with lid open at 50℃ for 5min (Until the smell of alcohol is disappear)
17.Add 35μL Buffer EB to the thin film of the spin column, let stand in a 50℃ metal bath with lid closed for 3min
18.Centrifuge at soft mode 12000rmp for 3min
19.Obtain DNA concentration with a Microplate reader, note it on the body of the tube just under the name of plasmid

Enzyme Digestion

1. Set up the system as shown. Carry out the process on ice
2. Blend with vortex then place in a metal bath at 37℃ for 1h

RD Mix	30μL
Buffer	3μL
Enzyme 1	1μL
Enzyme 2	1μL
ddH2O	Up to 30μL

Extraction

1.Add triple volume of Buffer PN into digested product
2.Prepare a 1.5mL Centrifuge tube already named
3.Cut the Gel and place the fragment in Centrifuge tube
4.Compress the gel on the dip of the tube using a microcentrifuge. Estimate Gel volume
5.Add equal volume of Buffer PN into the tube, mix with vertex, and place the tube inside a 700rmp, 55℃ metal bath until the gel fragment is fully dissolved
6.Prepare a Spin column and a Collection tube
7.Balance the Spin column with 500μL Buffer BL
8.Centrifuge the Spin column for 1min at 12000rpm, discard the flow-through
9.Lay the dissolved Gel solution into the spin column, letting stand for 2min and centrifuging at 12000rpm for 1min
10.Place all the flow-through back into the spin column again and centrifuging at 12000rpm for 1min
11.Discard the flow-through and add 600μL Buffer PW. Let stand for 2min then centrifuge at 12000rpm for 1min, discard the flow- through
12.Add 600μL Buffer PW again, centrifuge at 12000rpm for 1min, discard the flow- through
13.Centrifuge at 12000rpm for 3min without adding anything
14.Change a new Centrifuge tube and name it
15.Put inside a metal bath with lid open at 50℃ for 5min (Until the smell of alcohol is disappear)
16.Add 35μL Buffer H_2O to the thin film of the spin column, let stand in a 50℃ metal bath with lid

closed for 3min

17.Centrifuge at soft mode 12000rmp for 3min

18.Obtain DNA concentration with a Microplate reader, note it on the body of the tube just under the name of plasmid

Pigment extraction

1. Add the bacteria solution into two 50mL centrifuge tubes, centrifuge for 10 min, 2000G, the bias between two tubes should be within 0.01g
2. Pour the supernatant into an empty Erlenmeyer flask
3. Add 4000μL absolute ethanol and resuspend it
4. Extract the ethanol which dissolves the pigment into new 50mL tube, do not extract the sediment
5. Put the supernatant of bacteria solution into 2mL centrifuge tubes and centrifuge for 12000rmp, 2min and repeat the step 1-4

Protein gels

1. Make underlayer gel for 10%, total 15ml for two gels

	Volume
ddH2O	6100ul
30%Acr-Bis	5000ul
Underlayer gel buffer	3750ul
10%gel polymerization catalyst	150ul
TEMED	6ul

2. Put in to the model, about 2/3 volume, flattened gels with water
3. Make top layer gel for 5%, total 5ml for two gels

	Volume
ddH2O	3500ul
30%Acr-Bis	1000ul
Top layer gel buffer	1500ul
10% gel polymerization catalyst	60ul
TEMED	6ul

Sample for SDS-PAGE

1. Lift 1ml bacteria solution into a clean Eppendorf tube
2. Centrifuge 12000rmp for 2min
3. Remove the supernatant
4. Add Tris Buffer (1x), and resuspend and mix
5. Lift 20μL and add 5μL loading (5x) into small tube
6. 98℃ cooking in PCR instrument (bacteria solution for 15min, only protein for 10min)

SDS-PAGE

1. Pour in buffer, the inner liquid is full and cannot be reused, the outer liquid has to pass the half of the "door" and can be reused.
2. 4μL ladder (put back to -20℃ refrigerator), 6μL sample
3. Electrophoresis 60min 130V
4. The remaining glue which has not been used can be put back to 4℃ refrigerator. It cannot be used after 48 hours.
5. After the electrophoresis, take out the glass with gel and cut the upper gel
6. Wash the rest of the gel for three times
7. Add Coomassie Blue Fast Staining Solution, shake for 40 min
8. Add pure water to decolor the gel in 4℃ refrigerator overnight

Measure OD value

1. Put flasks into a 30-degree shaker and take out to measure OD after 2 hours.
2. Take out a new 96-well plate and open it in the clean bench.
3. 200ml LB as control group, 2 holes per sample, each hole should have 200μL bacteria solution. (When OD>0.5, the Microplate reader's measurement may be not accurate, then dilute the sample into 100 μL LB+100 μL sample.)

IPTG induced protein

1. When OD=0.6-0.8, add IPTG solution. (concentration of IPTG solution* sample volume=0.3Mm*flask volume)
2. Put into the 25℃ shaker

Collection of bacteria

1. Pour the bacteria solution into to 50 mL centrifuge tubes and balance them within 0.01g
2. Centrifuge for 5min 10000rmp
3. Remove the supernatant
4. Add pH=8 Tris Buffer and resuspend
5. Put the tubes into -20℃ refrigerator

Histag protein purification

1. Two 2ml centrifuge tubes with bacteria solution, centrifuge 12000rmp, 2min, throw the supernatant and keep the sediment, until all the solution is collected.
2. Save 1mL supernatant, label as CT-1
3. Weight the sediment, every 1g sediment add nondenaturing lysate 4mL
4. Add 1000μL nondenaturing lysate and resuspend it
5. Add 10μL (100x) inhibitor, mix it
6. Add 10μL lysozyme (100mg/mL) to 1mg/mL and mix gently
7. Put on the ice for 1hour
8. Repeat freezing and thawing for three times (instead of ultrasonic treatment), -80℃ 15min for freezing, room temperature for melting.
9. Centrifuge 4℃. 12000rmp for 5min, place on ice for 30s, repeat 6 times

10. Collect the supernatant (label as CT-2), take 20μL as sample
11. 0.5mL 50%BeyoGold His-tag Purification Resin centrifuge 4℃ 12000rmp for 1min, throw the supernatant. Add 0.25mL nondenaturing lysate solution and mix it. Centrifuge 12000rmp for 1min, throw the supernatant and repeat for 1 or 2 times.
12. Add all CT-2 in to resin and mix
13. 4℃ shaker to shake for 1 hour
14. Put the mixture into affinity layer folding column
15. Open the bottom of the column and collect the flow through, take 20μL label as FT-1
16. Add washing buffer 1mL 5 times, collect the flow through in a new tube every time, label as W 1-5
17. Add nondenaturing elution buffer 0.5mL 6-10 times, collect the flow through in a new tube every time, label as P 1-8
18. Take 20μL from W 1-5 and P1-8 put in PCR tubes for test

BCA concentration determination

1. Protein standard sample dilution: dilute to 0.5mg/mL with buffer
2. BCA solution: BCA-A: BCA-B=50:1
3. Protein standard sample: from A-H: 0, 1, 2, 4, 8, 12, 16, 20μL, each hole is made up to 20μL with dilution, so that the concentration of standard sample should be 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0,5
4. Sample: every hole has 20μL purified protein (if less than 20μL, use dilution to supplemented)
5. Add 200μL BCA solution to each hole (both standard sample and protein sample), place at 37℃ for 20-30min
6. Use Microplate reader to obtain the concentration, sensitivity= 562nm
7. Calculate the standard concentration of the sample according to the standard concentration equation

Concentration of protein and change buffer to Tris buffer

1. Mixed sample protein 1-8 combine with the Purification Resin, each resin has 2mL protein
2. Centrifuge 4℃ for 30min 2000G after balancing, in order to concentrate the volume to 200μL (10%)
3. Collect the flow through (label as Waste-1) to 1.5mL centrifuge tube
4. Add Tris buffer (pH=8) 2mL
5. Centrifuge 4℃ for 30min 2000G after balancing, in order to concentrate the volume
6. Collect the flow through (label as Tris-1 FT) to 1.5mL centrifuge tube
7. Repeat steps 4-6 for two times, label as Tris-2/3 FT
8. Centrifuge 4℃ for 30min 2000G after balancing, in order to concentrate the volume
9. According to C1V1=C2V2, calculate the volume of Tris buffer needed. (C1=0.63, V1=2000μL)
10. Mix the mixture
11. Put all the protein solution in one new tube.
12. Due to residue, add 200μL of diluent Tris (pH=8) buffer to each tube and collect the diluted protein liquid (Tris-3) for detection concentration.

Dialysis of concentrated protein

1. Pick a piece of dialysis bag and put in a 500mL beaker, add EDTA & $NaHCO_3$ solution
2. Put into microwave oven, boiling for 10min
3. Pour out the solution and use pure water to wash 3 times
4. Add EDTA solution and put into microwave oven, boiling for 10min
5. Pour out the solution and use pure water to wash 2 times
6. Add Tris buffer in the beaker
7. Fold a small section of a side of the dialysis bag, clamp it with clip, and make sure it will not leak
8. Add protein solution and clamp the other side, put it into big beaker with Tris buffer
9. Place the beaker on the shaker in 4℃ refrigerator, cover the beaker with a film
10. After 1 hour, change the Tris buffer and keep shaking over night
11. After dialysis, pour the Tris buffer, carefully remove the clip and take out the protein
12. Use to pure water to clean the clips and throw away the bag

Violacein production standard curve

1. Streak plate, put in 37℃ incubator overnight.
2. Pre-culture: put 5mL LB and 5μL antibiotic in a 15mL tube and pick single colony to mix in the tube, put in 37℃shaker overnight.
3. Seed-culture: put 100mL LB, 100μL and 100μL antibiotic in a clean Erlenmeyer flask, 30℃, the sample will be collected at 6 hours, 18 hours, 24 hours, 30 hours, 42 hours, 48 hours, each collect 1mL sample.
4. Centrifuge 12000rmp, until the supernatant is pure.
5. Pour the supernatant, add 1000μL absolute ethanol, resuspend it and place for 5min, centrifuge again, for 12000rmp (to make sure all the sediment is in the bottom of the tube, the bacteria will affect the final result)
6. Pigment standard sample: from A-H: 80, 60, 40, 10, 4, 0μL, each hole is made up to 200μL with absolute ethanol, so that the concentration of standard sample should be 0.125, 0.1, 0.075, 0.05, 0.025, 0.0125, 0.005
7. 200μL sample in each hole
8. Calculate the standard concentration of the sample according to the standard concentration equation

Indigo production standard curve

1. Streak plate, put in 37℃ incubator overnight.
2. Pre-culture: put 5mL LB and 5μL antibiotic in a 15mL tube and pick single colony to mix in the tube, put in 37℃shaker overnight.
3. Seed-culture: put 100mL LB, 100μL and 100μL antibiotic in a clean Erlenmeyer flask, 30℃, the sample will be collected at 6 hours, 18 hours, 24 hours, 30 hours, 42 hours, 48 hours, each collect 1mL sample.
4. Centrifuge 12000rmp, until the supernatant is pure.
5. Pour the supernatant, add 300μL DMSO solution, resuspend it and place for 5min, centrifuge again, for 12000rmp (to make sure all the sediment is in the bottom of the tube, the bacteria will affect the final result)
6. Pigment standard sample: from A-H:, 0μL, each hole is made up to 200μL with absolute ethanol,

so that the concentration of standard sample should be 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.04, 0.03, 0.02, 0.01, 0

7. 200μL sample in each hole
8. Calculate the standard concentration of the sample according to the standard concentration equation