Beijing_4ELEVEN Experiment Protocols（iGEM2020）Experiments

- ## Plasmid extraction

1. Collect 1-4 mL cell culture into 1.5 mL tube, 1,2000 rpm, 1 min, discard the supernatant.
2. Add 150 μL Buffer P1 and resuspend the cells.
3. Add 150 μL Buffer P2 and invert 6~8 times to mix.
4. Immediately add 350 μL Buffer P5 and gently invert 6~8 times. 1,2000rpm, 5min, save the supernatant.
5. Add the supernatant from step 4 to a CP3 spin column, 1,2000 rpm, 1 min, discard the liquid in the collection tube.
6. Wash the CP3 spin column by adding 300 μL Buffer PWT. 12000 rpm, 1 min, discard the liquid in the collection tube.
7. Put back CP3 spin column and without adding Buffer PWT, 1,2000 rpm, 2 min.
8. Obtain CP3 spin column and place it in a clean 1.5 mL tube with the lid open to let the residual ethanol impurities evaporate.
9. Add 50 μL H2O on the film of CP3, 1,2000 rpm, 1min and discard the CP3 spin column. Plasmid DNA is collected in the tube.
10. Measure the concentration of the DNA solution using Microplate reader and place it in the -20°C refrigerator.

- ## Polymerase Chain Reaction (PCR)

High fidelity PCR

1. PCR Reaction System

Components	50 μL System	Final Concentration
Forward primer (10 μM)	2.5 μL	0.5 μM
Reverse primer (10 μM)	2.5 μL	0.5 μM
Template	Variable	<1,000 ng
5X TransStart FastPfu Buffer	10 μL	1×

2.5 mM dNTPs	4 µL	0.2mM
TransStart FastPfu	1 µL	2.5 units
ddH2O	up to 50 µL	

2. PCR Reaction Condition

	A	B	C	D
1	Step	TEMP.	Time	Cycle
2	Initial Denaturation	98 °C	2 min	1 cycle
3	Denaturation	98 °C	30 s	
4	Renaturation	50 - 72 °C	30 s	30 cycles
5	Extension	72 °C	1 kb/30 s	
6	Final Extension	72 °C	2 min	1 cycle
7	Hold	12 °C	∞	

Colony PCR (Using Taq PCR Star Mix with Loading Dye)

1. PCR Reaction System

Components	**50 µL System**	**Final Concentration**
Forward primer (10 µM)	2.5 µL	0.5 µM
Reverse primer (10 µM)	2.5 µL	0.5 µM
Template	Variable	<1,000 ng
2×Taq PCR Star Mix with Loading Dye	10 µL	1×
ddH2O	up to 50 µL	

- The single colonies on plate were picked as template directly.

2. PCR Reaction Condition

	A	B	C	D
1	Step	TEMP.	Time	Cycle
2	Initial Denaturation	96 °C	10 min	1 cycle
3	Denaturation	96 °C	30 s	30 cycles
4	Renaturation	50 - 72 °C	30 s	
5	Extension	72 °C	1 kb/60 s	
6	Final Extension	72 °C	2 min	1 cycle
7	Hold	12 °C	∞	

Agarose Gel Electrophoresis

1. Put the gel tray into the tape on a level surface and insert the comb into the gel tray at the end of ~1 cm from the end. Make sure that the bottom edge of the comb maintained 0.5~1mm gap to the surface of gel tray.
2. For a 1% 50 mL agarose gel, weigh 0.5 g agarose into a conical flask, add 50 mL TAE buffer. Microwave the mixture for a few minutes until all the agarose melted completely.
3. Cool down the solution to a touchable temperature (60-70 °C). Add appropriate amount of SYBR® safe DNA Gel Stain (10000×) to a final concentration 1×. Mix the solution gently and pour it into the gel tray. Remove any air bubbles with a pipette tip.
4. After 30~60 min, the gel will solidify at room temperature. Remove the comb carefully and separated sample wells were formed.
5. Release the gel tray from the tape, put them into the electrophoresis chamber, add TAE buffer until 2-3 mm immersion of the gel surface.
6. Take part of the DNA samples and mix with loading dye. Load the samples into the wells.
7. Put the lid onto the electrophoresis chamber and connect it to the power, make sure to put it in the right direction so that the DNA runs from the negative electrode (black) towards the positive electrode (red).
8. Run the gel at 120V for about 15~30 min, stop the electrophoresis when the dyes move to about 2/3 of the gel.
9. Bring the gel from the gel tray to a Blu-ray table to visualize the gel bands.

DNA Gel Extraction

1. Carefully excise the target DNA fragment using a clean scalpel and put it into a clean 1.5 mL microfuge tube.
2. Weigh the gel slice to determine the volume of gel dissolution buffer. Assuming a quality of 0.1 g gel, add 100 µL gel dissolution buffer. Incubate the mixture at 50 °C for a few minutes until the gel has completely melted. Mix by shaking or inverting the tube every 2~3 min.

3. Add 500 µL equilibrium buffer to the adsorption column (assembly the adsorption column into a collection tube), centrifuge at 1,2000 rmp for 1 min, discard the waste liquid.
4. Cool down the gel solution to room temperature and transfer it to adsorption column, incubate at room temperature for 2 min, centrifuge at 1,2000 rmp for 30~60 s, discard the waste liquid.
5. Add 600 µL of wash buffer diluted with absolute ethanol for 2 times, centrifuge at 1,2000 rmp for 30~60 s, discard the waste liquid.
6. Reusing the collection tube, centrifuge the empty column at 1,2000 rmp for 2 min to dry the column matrix.
7. Place the column into a clean 1.5 mL microfuge tube, add 50~100 µL ddH2O (Sterilized， pre-heated to 50 °C) directly into the center of the column matrix, then incubate 2 min at 50 °C, centrifuge at 1,2000 rmp for 1 min to elute DNA.
8. The concentration of DNA fragments can be measured by Microplate reader.

• Restrict Enzyme Digestion & Ligation

Digestion：

Components	**Volume**
DNA	Variable (1µg)
Cutsmart Buffer (10×)	5 µL
Restriction Enzyme I	1 µL
Restriction Enzyme II	1 µL
ddH2O	Add to 50 µL
Temperature	**Time**
37 °C	15 min
65 °C	20 min

Ligation：

Components	**Volume**
T4 DNA ligase buffer (10×)	2 µL

Vector DNA	2 μL
Insert DNA	8 μL
T4 DNA ligase	1 μL
ddH2O	Add to 20 μL
Temperature	**Time**
16 °C	∞

Gibson Assembly

<table>
<tr><th>Components</th><th>Volume</th></tr>
<tr><td>Backbone</td><td rowspan="4">Mole Ratio of each fragments 1:1
Total moles (2-3 fragments): 0.01—0.25 pmol
Total moles (4-6 fragments): 0.1—0.5 pmol</td></tr>
<tr><td>Fragment 1</td></tr>
<tr><td>Fragment 2</td></tr>
<tr><td>。。。</td></tr>
<tr><td>Gibson Assembly Mix</td><td>58 μL</td></tr>
<tr><td>ddH2O</td><td>Add to 10 μL</td></tr>
<tr><td>Temperature</td><td>Time</td></tr>
<tr><td>50 °C</td><td>30 min</td></tr>
</table>

- ## GoldenGate

1. GoldenGate Reaction System

Components	Volume
T4 DNA ligase buffer (10×)	2 μL
Vector DNA	30-60 ng
Fragment	1 μL

Bsa I Enzyme	1 µL
T4 DNA ligase	1 µL
ddH2O	Add to 20 µL

2. GoldenGate Reaction Condition

	A	B	C	D
1	Step	TEMP.	Time	Cycle
2	Digestion	37 °C	5 min	10 cycle
3	Ligation	16 °C	10 min	
4	Final Digestion	37 °C	10 min	1 cycle
5	Renaturation	50 °C	5 min	1 cycle
6	Iactivation	85 °C	5 min	1 cycle
7	Hold	12 °C	∞	

Competent cell transformation

1. Mix target DNA (ligation reaction mixture, plasmids or controls) with 50 µL of competent cells, incubate for 30 min on ice..
2. Heat shock for 60 s at 42 °C , then incubate for 5 min on ice immediately. Do not shake the tube at this procedure.
3. Add 150 µL LB medium without antibiotic.
4. Incubate for 40-60 min at 37 °C .
5. Resuspend the cells and spread the suspension on an agar plate containing the appropriate antibiotic.
6. Incubate for 16-18 h at 37 °C.

Protein expression in E.coli

1. Pick several single colony to 3mL medium and incubate overnight.
2. Inoculate (1%) the culture up to 100 mL medium, inducer should add at 0 h or appreciate OD600 value.
3. Incubate the culture for 20h at 25°C.
4. After the incubation, collect the culture for 20 minutes at 9000rm, 4°C.

SDS-PAGE Protein Gel electrophoresis

1. Prepare SDS-PAGE running buffer(1X): 10mL.

2. Prepare gels and assemble the electrophoresis device.
3. Prepare samples: 20 μL for each.
4. Heat samples for 5 minutes at 94 °C.
5. Cover the chamber and firmly connect both the anode and the cathode. Set the voltage on the electrophoresis power supply to a constant voltage of 120 V.
6. Run the gel electrophoresis cell for 30-60 min.
7. After electrophoresis, remove the gel by floating it off the plate into water.
8. Stain the SDS-PAGE with Coomassie blue dyes, then destain it.
9. Observe the electrophoresis results that show up in the E-gel imager.

- ## Transformation of Pichia pastoris

DNA Preparation: Plasmid（10-20μg), restriction enzyme SacI, 80% Ethanol, 3M Sodium acetate，Anhydrous ethanol
Demonstration with pPIC9K plasmid:

1. Plasmid extraction: Prepare 50ml of bacteria solution，extract plasmid with standard methods of operation to acquire 20 μg plasmids (100 μL).
2. Enzyme digestion: Single restriction digest all samples with Sacl, 37°C, 15min-1h, heat treatment. Take 3μl sample to verify via gel electrophoresis.
3. Recycle: Reclaim remaining samples, add 1/10 of the sample's volume of 3M Sodium acetate, add 2.5 times the sample's volume of anhydrous ethanol, mix.
4. 13,000 rmp, centrifuge for 10min. Finally, elute 2 times with 80% ethanol, dry, add 20μL ddH2O, dissolve DNA (about 5-10μg) place in -20°C for future use.

LiCl Transformation
Preparation：100mM LiCl, ddH2O, Salmon sperm DNA, 50% PEG3350 (filtration sterilization) 1M LiCl, YPD culture
LiCl competent cell preparation

1. Inoculate 150μL Pichia pastoris seed liquid into shake flask containing 50mL YPD liquid culture, cultivate in 30°C, 220rpm until OD becomes approximately 0.3 (actual measured value of ELIASA), about 12h required.
2. Harvest cells, resuspend with 25ml ddH2O, room temperature, 5000r, 10min.
3. Resuspend cells in 1mL 100mM LiCl solution, transfer resuspension liquid into a 1.5mL centrifuge tube.
4. 12000r, 15s, subside cells, resuspend in 400ul 100mM LiCl solution.
5. Collect 50ul, transform immediately.

Transformation

1. Boil 1ml Salmon sperm DNA for 5min, quickly place in ice bath to prepare single chain DNA, used to prevent gene degradation and therefore facilitate target gene transformation.
2. Centrifuge competent cells at 8000r for 1min to remove remaining LiCl solution.
3. Add the following sequentially to every every transformation

50% PEG3350 240μL
1M LiCl 36μL
2mg/ml single chain Salmon sperm DNA 25μL
5-10μg linearized plasmid DNA

4. Vortex strongly until cells are evenly dispersed.
5. 30°C warm bath for 30min.
6. 42°C water bath heat shock for 20-25min.
7. Centrifuge at 8000r for 10min, collect cells.
8. Resuspend cells in 500μL YPD culture, put in shaker at 30°C for 1-4h.

Plating

Preparation: G418 resistance YPD plate of various concentrations (lowest being 0.25g/L) coat gradiently according to different resistance concentration, for instance：G418 0.25g/L, 0.5g/L, 1g/L. Add 100μL bacteria solution to plates of each G418 concentration, spread evenly with spreading rod, cultivate at 30°C invertedly for 2-5d, then verify. (A pinhead sized monoclone should appear in 2 days of cultivation, followed by emergence of other monoclones)

Strain PCR verification

Preparation: Lyticase

1)Select monoclones and add into 10μL ddH2O, add 10U Lyticase, 30°C, 30min, set at -80°C for 1min, extract 1μL right after taking it out of the refrigerator, verify with PCR.

2)For pPIC9K plasmids, the verification primers are usually:

5’ *AOX1* GACTGGTTCCAATTGACAAGC

3’ *AOX1* GCAAATGGCATTCTGACATCC

Notice: to distinguish from wild type, verify again with internal primers.

Bacteria solution extracted genome DNA PCR verification

Preparation: Lysis buffer (10mM Tris, pH 8.0, 1mM EDTA, 100mM NaCl, 1% SDS, 2% Triton X-100), Phenol:Chloroform:Isoamyl alcohol=25:24:1, acid cleaned glass beads（0.5mm), 95% Ethanol, RnaseA, 5M Nacl

1. Cultivate 5ml bacteria solution overnight.
2. Centrifuge at 13000r for 1min, dispose of supernatant, add 230μL Lysis buffer.

3. Add 0.4g acid cleaned glass beads, then add 200μL Phenol: chloroform: isoamyl alcohol, vortex for 2-3min.
4. Centrifuge at 13000r for 5min, transfer supernatant to a new centrifuge tube.
5. Add 600μL 95% ethanol, set at -20°C for 30 min.
6. Centrifuge at 13000r for 15min, air dry until ethanol completely evaporates.
7. Resuspend DNA with 200μL TE, add 5μL RnaseA, incubate at 37°C for 10min.
8. Add 8μL 5M Nacl, centrifuge at 13000r for 15min, dispose of supernatant, air dry, then resuspend with TE.

Expression of protein of Pichia pastoris

Preparation: BMGY, BMMY

1. Inoculate monoclones into BGMY culture, 30°C, grow for 36h, bacteria solution should become turbid.
2. Transfer inoculate, wait until OD600=0.6-0.8.
3. Centrifuge at 2000g for 10min， dispose of supernatant, resuspend subsided cells in 90mL BMMY culture for 5 days, add 0.5% methanol each day and collect 1mL sample for SDS-PAGE protein sample test.
4. In our project, the proteins are extracellular expression: Centrifuge 1mL bacteria solution at 13000r for 10min, mix 50μL sample with SDS-PAGE loading buffer, boil for 10min, load sample.

Medium

1. LB medium: Tryptone 10g/L, Yeast extract 5g/L, Nacl 10g/L. Add 15-20 g/L agar for solid medium. 121°C steam sterilization for 20min.
2. **YPD medium**: 1% Yeast extraction (10g), 2% Peptone (20g), 2% Glucose
   Preparation：
   a. Dissolve 10g yeast extraction and 20g peptone in 900mL deionized water, if making solid culture, add 20g agar, sterilize at 121°C for 20min.
   b. After cooling down, add 100mL 20% glucose solution。
3. **BGMY medium**: 1% Yeast extraction (10g), 2% Peptone (20g), 100 mM Potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10-5% Biotin, 1% Glycerin.
4. **BMMYmedium** : Replace Glycerin in above formula with methanol, the rest ingredients remain the same.
   Preparation:
   a. Dissolve 10g yeast extraction and 20g peptone in 700mL deionized water, sterilize at 121°C for 20min.
   b. Cool down to room temperature and add the following:

100ml 1M potassium phosphate buffer, pH 6.0
100ml 10X YNB（filtration sterilizated）
2ml 500X Biotin（filtration sterilizatied）
100ml 10% glycerol

c. For BMMY, replace glycerol with 100ml 5% methanol.

· Cryopreservation

Bacteria liquid and 50% glycerin are added in 1:1 volume ratio into the bacteria preservation tube and stored in the refrigerator at -80°C.

· Large scale His tag protein purification in non-denaturing condition

1. Collect bacteria cells, add 4mL (or any volume between 2-5mL) non-denaturing lysis buffer to every gram of bacteria, resuspend bacteria. If necessary, add protease inhibitor to lysis buffer.
2. Add lysozyme until concentration is 1mg/mL, mix and set in ice bath or on ice for 30min.
   Notice: Lysosome can be combined with lysis buffer into mother liquor of 200mg/mL and added. The mother liquor can be dispensed into moderate containers and preserved at -20°C.
3. Ultrasonic lysis bacteria on ice at 200-300W, 10s per duration, 10s between each time of lysis, process 6 times in total.
   Notice: Process of ultrasonic lysis should be further moderated according to type of ultrasonic
   lysis equipment.
4. (Optional) If solution is viscous after ultrasonic lysis, add 10μg/mL RNase A and 5μg/mL DNase and set on ice for 10-15min. Or use syringe to repeatedly aspirate and inject solution and dissect viscous genome DNA.
5. Centrifuge at 4°C, 10000g for 20-30min, collect supernatant and set in ice bath or on ice. Extract and preserve 20μL supernatant for future verification.
6. Centrifuge 1mL evenly mixed 50% BeyoGoldTM His-tag Purification Resin (Reduction-resistant chelating type) at 4°C, 1000g for 10s, dispose of preservation solution, add 0.5mL non-denaturing lysis buffer to balance gel, centrifuge at 4°C, 1000g for 10s, dispose of supernatant, repeat 1-2 times, dispose of supernatant, add 4mL lysis buffer, set on shaker for 60min.
   Notice: BeyoGoldTM His-tag Purification Resin (Reduction-resistant chelating type) can be used without being balanced, but the production rate of proteins would be reduced by 5-20%.
7. Transfer the mixture of lysate and BeyoGoldTM His-tag Purification Resin (Reduction-resistant chelating type) to an affinity chromatography column provided in the test kit.

Notice: It is also feasible to add 1mL evenly mixed BeyoGoldTM His-tag Purification Resin (Reduction-resistant chelating type) to an affinity chromatography column, add 0.5mL non-denaturing lysis buffer to balance 2-3 times, add 4mL bacteria lysate supernatant, then collect solution and load to column 3-5 times to fully bind with target protein. Mixing before loading is more complex to operate, but more beneficial for His tag recombinant protein to bind with the nickel column, especially when the His tag is blocked by the protein itself or when protein concentration is very low.

8. Open lid at the bottom of the purification column, collect 20μL of the liquid dripping out from the column for future verification.
9. Wash column 5 times, adding 0.5-1mL non-denaturing wash buffer, collect 20μL of the solution that passes through the column each time for future verification. Bradford method (P0006) can be used to quickly evaluate the protein concentration in the wash and elute solutions in order to further determine times of wash and elution.

Notice: If protein concentration is low, wash for 2-3 more times.

10. Elute target protein 6-10 times, adding 0.5mL non-denaturing buffer each time. Collect the elution solution each time into different centrifuge tubes, collected elution solution is the purified His tag protein sample.

· Large scale His tag protein purification in denaturing condition

1. Collect bacteria cells, add 80mL denaturing lysis buffer to every liter of bacteria, resuspend bacteria.
2. Ultrasonic lysis bacteria on ice at 200-300W, 2s per duration, 2s between each time of lysis, process 15-30min in total.

   Notice: Process of ultrasonic lysis should be further moderated according to type of ultrasonic
   lysis equipment.
3. (Optional) If solution is viscous after ultrasonic lysis, use syringe to repeatedly aspirate and inject solution and dissect viscous genome DNA.
4. Centrifuge at 4°C, 10000g for 20-30min, collect supernatant and set in ice bath or on ice. Extract and preserve 20μL supernatant for future verification.

   Notice: The supernatant must be clear, that is, not contain anything insoluble, in order to process the next step of purification. Inoluble matter would severely affect resulting protein purity.
5. Centrifuge 1mL evenly mixed 50% BeyoGoldTM His-tag Purification Resin (Reduction-resistant chelating type) at 4°C, 1000g for 10s, dispose of preservation solution, add one column volume of denaturing lysis buffer to balance gel, centrifuge at 4°C, 1000g for 10s, dispose of supernatant, repeat 1-2 times, dispose of supernatant.

6. Add 4mL bacteria lysate supernatant to every 0.5mL gel, mix BeyoGoldTM His-tag Purification Resin (Reduction-resistant chelating type) and bacteria lysate supernatant, set on shaker at 4°C for 60min.
7. Transfer the mixture of lysate and BeyoGoldTM His-tag Purification Resin (Reduction-resistant chelating type) to an affinity chromatography column.

   Notice: It is also feasible to add 1 column volume of denaturing lysis buffer to bacteria lysate supernatant, then collect solution and load to column 3-5 times to fully bind with target protein.Mixing before loading is more complex to operate, but more beneficial for His tag recombinant protein to bind with the nickel column.
8. Open lid at the bottom of the purification column, collect 20μL of the liquid dripping out from the column for future verification.
9. H. Wash column 5 times, adding 0.5-1mL lysate, collect 20μL of the solution that passes through the column each time for future verification. Bradford method (P0006) can be used to quickly evaluate the protein concentration in the wash and elute solutions in order to further determine times of wash and elution.

   Notice: If protein concentration is low, wash for 2-3 more times.
10. Wash column 5 times again, adding 0.5-1mL denaturing wash buffer, collect 20μL of the solution that passes through the column each time for future verification.

    Notice: If protein concentration is low, wash for 2-3 more times.
11. Elute target protein 6-10 times, adding 0.5mL denaturing buffer each time. Collect the elution solution each time into different centrifuge tubes, collected elution solution is the purified His tag protein sample.