Molecular biology Experiment

LB medium (for *E. coli*)

Tryptone ： 10 g/L
Yeast extract：5 g/L
Sodium chloride：10 g/L
Agar：15 g/L

RMG 2/5(for *Z. mobilis*)

D(+)-Glucose anhydrous：20 g/50g/L
Yeast extract：10 g/L
Potassium dihydrogen phosphate：2 g/L
Agar：15 g/L

Inoculation and preservation of strains

From liquid to liquid: Use the pipette head to absorb bacterial solution into 6 ml with special antibiotics liquid medium.
From liquid to solid: Use the tip of the pipette to streak the plate in special antibiotics solid medium.
Strain preservation: Add 60% glycerol solution and bacterial solution to the frozen storage tube with one to one volume ratio and keep in -80℃ refrigerator.

Strain culture

Culture of *E. coli*

Plate culture: Preserved / cultured strains are inoculated into solid LB medium with special antibiotics, and plates are incubated by inverted in a 37 ℃ incubator overnight
Liquid culture:The strains are inoculated into 6 mL of special antibiotics liquid medium on a constant temperature shaker and grown overnight at 37 ℃, 250rpm.

Culture of *Zymomonas mobilis*

Plate culture： Preserved/ cultured strains are inoculated into solid RMG 2 medium with special antibiotics, and the plate are sealed and placed inverted in a 30℃ incubator for two days.
Liquid culture:The preserved strains are inoculated into 200 μL liquid RMG 5 medium on a constant temperature incubator and incubated for half a day.

Molecular biology Experiment

Preparation of competent cells of *E. coli*

1.Pick a single colony of *E. coli*, incubate in 10 mL LB medium, and incubate at the conditions of 37 ℃, 250 rpm shaking overnight.

2.After incubation,take 1-2% overnight culture media into 500 mL triangular flask containing 100 mL of LB medium and incubated at 37℃ with 250 rpm shaking for 3 hours to make OD_{600nm} to 0.4-0.6. (optimal OD_{600nm}= 0.4-0.6).

3.After incubation,divide into two sterilized ice-cold 50 mL centrifuge tubes to ice bath cells for 30 min.

4.4℃, 4000 rpm for 10 min (after the operation is strictly on ice, 10% glycerol, EP tube and other required material should be pre-cooled). Remove the supernatant , the cells are suspended in 40 mL ice cold 0.1 M $CaCl_2$ for 25 min, centrifuged at 4000 rpm for 10 min, remover the supernatant .

5.Repeat step 4.

6.All cells are re-suspended in pre-cooled 0.1 M $CaCl_2$ containing 15% glycerol.

7.Divide them in EP tubes, 80-100 μL of each EP tube.

8.Pour the liquid nitrogen into each EP tube.

9.Use it immediately for transformation or keep the packed competent cells in a-80℃ refrigerator.

Primer design

Principle of primer design：

1.The primer length is generally between 15 and 30 bp. The primer length is usually 18-27 bp, but should not be greater than 38 bp, because too long will cause its extension temperature is greater than 74 ℃, which is not suitable for the reaction of Taq DNA polymerase.

2.The GC content of the primers is between 40% and 60%: too high or too low GC content is not conducive to trigger the reaction.The Tm value (melting temperature) of the upstream and downstream primers is the effective start temperature of the unwinding temperature of the oligonucleotide, which is generally lower than the Tm value of 5~10℃.

3.Bases should be randomly distributed: primer sequences should not have high similarity within the template, especially sequences with high similarity at the 3′ end, otherwise it is easy to lead to errors

4.There should be no complementary sequence between the primer itself and the primer

5.The 5′ end of the primer can be modified, but not the 3′ end: the 5′ end of the primer determines the length of the PCR product, which has little effect on the amplification specificity.

Polymerase chain reaction

1.PCR reaction

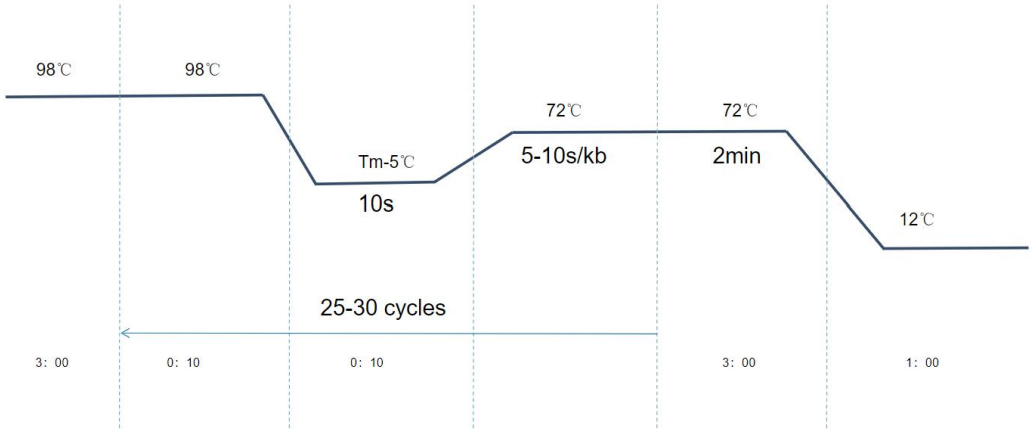
Reagent	10 μL Volume	Concentration
Primer F	0.4 μL	10 μM
Primer R	0.4 μL	10 μM
DNA	0.2 μL	20 ng

DNA pol	5 μL	2×
ddH_2O	Up to 10 μL	

Reagent	50 μL Volume	Concentration
Primer F	2.0 μL	0.4 μM
Primer R	2.0 μL	0.4 μM
DNA	1~3 μL	0.05~1 ng
DNA pol	43~45 μL	1×
ddH_2O	Up to 50 μL	

2.Parameters:

Procedure	Temperature	Time	Cycle
Initial Denaturation	98℃	3 min	1
Denaturation	98℃	10 s	1
Annealing	X ℃	10 s	1
Extension	72℃	5~10 s/kb，X s	25~30
Final Extension	72℃	3 min	1
Hold	12℃	1 min	1



3. Loading samples and running an agarose gel

Pouring a standard 1% agarose gel, add loading buffer to each of DNA samples. Fill gel box with 1×TAE buffer until the gel is covered. Carefully load a molecular weight ladder into the first lane of the gel. Carefully load samples into the additional wells of the gel. Run the gel until the dye line is approximately 75-80% of the way down the gel.

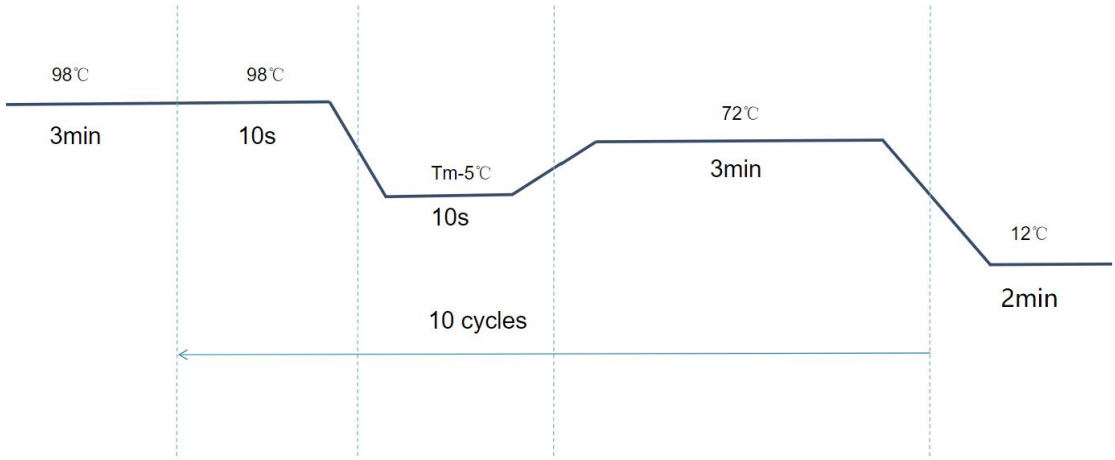
4.Overlap

1)system:50μL

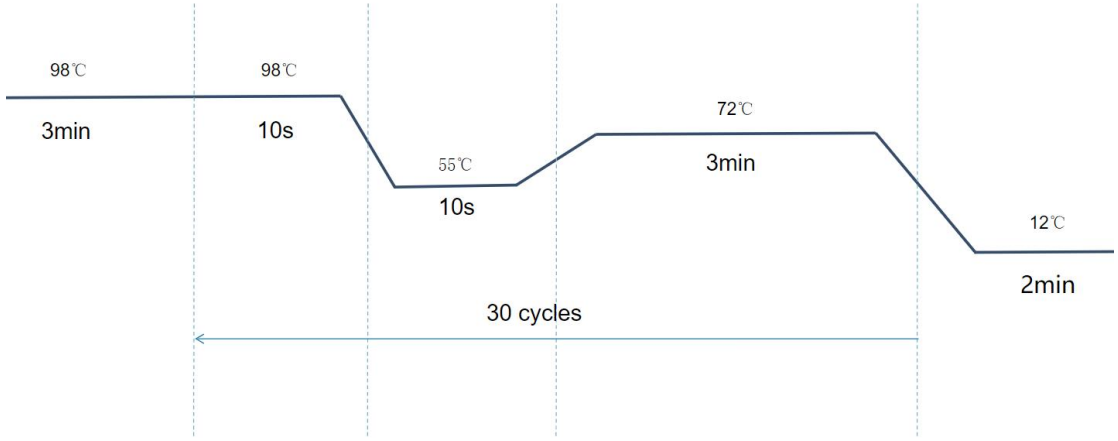
Reagent	50 μL Volume

Prime STAR Max	25 μL
Primer F	2.0 μL
Primer R	2.0 μL
Fragment1	1~2 μL
Fragment2	1~2 μL
ddH_2O	Up to 50 μL

2）Parameters



Add the F/R primers，run again



3）Recovered the product gel

Purify the PCR products

1.Direct recovery of PCR products

Add Buffer GL according to the ratio of PCR stock solution: Buffer GL=1:3 (when PCR stock solution is lower than 50 μL, Buffer GL dosage is 150 μL) and mix well (for example, in 1.5 mL centrifuge tube, 50 μL PCR stock solution is mixed well after adding 150 μL Buffer GL). After the

activation of the adsorption column (step 1 below), add the above mixture directly to the adsorption column and proceed to step 5.

2.DNA recovery in the agar gel

1)Activate the silica gel membrane：Place the adsorption column in the collection tube，add 250 μL BufferBL, centrifuge at 12000rpm for 1min,discard the waste.

2)Under the UV lamp, cut the gel as small as possible, put it into a 2 mL centrifuge tube.

3)Add 500 μL Buffer GL (If the gel is too large, increase the amount of Buffer GL appropriately)；

4)65℃ water bath for 4 to 6 min, mix upside down every 2 to 3 min until the gel is completely melted, the solution is light yellow (if the solution is lilac, add an appropriate amount of Buffer GL to the solution to light yellow);

5)Transfer the solution to the adsorption column,centrifuge at 12000 rpm for 1 min,discard the waste,place the adsorption column back to the empty collection tube.

6)Add 700μL Buffer W2 to the adsorption column (please check whether the specified volume of absolute ethanol has been added), centrifuge at 12,000rpm for 1min, and discard the waste .

7)Repeat step 6 once.

8)Placed the adsorption column back into an empty collecting tube and centrifuged at 12,000 rpm for 2min.

9)Remove the adsorption column, place it in a clean 1.5 mL EP tube, and open it at 65 °C for 4-5 min to dry the left ethanol.Add 30μL of H_2O to the middle of the adsorption membrane, hold for 4-5 min at 65 °C, and centrifuge for 2 min at 12000 rpm.

T5 exonuclease assisted cloning

1.Prepare reaction system(5μL)

F/V:molar ratio of 3:1

T5 Exonulease: 0.5 μL

Buffer: 0.5 μL

ddH_2O: complemented to 5 μL

All regents are mixed and react on the ice for 5 min.

2. Add 50 μL *E. coli* DH5α competent cells and react on the ice 30 min.

3.Heat shock at 42℃ for 45 s and then immediately put in an ice bath for 3 min.

4.Add 1 mL of LB medium and then incubate at 37℃ for 1 hour.

5.Collect cells at 1,2000 rpm for 1 min, remove the supernatant and the cells resuspended with residual medium, then Plate all cells on soild LB medium with special antibiotics.

6.The plates are inverted in a 37 ℃ incubator.

Screening of target strain（colony PCR）

1.Prepare reaction system(10 μL）

Pick a single colony in pcr tubes containing 10 μL H_2O,take 1μL mixed bacterial solution. Primer F/R: 1 μL

T5 Super PCR mix:5 μL

H_2O: up to 10 μL

2.Parameters（refer to PCR）

Plasmid extraction

1.(Optional) Activate the silica gel membrane: place the adsorption column in the collection tube, add 250 μL Buffer BL, centrifuge at 12,000 rpm for 1min, and discard the waste.
2.Take 1 ~ 6 mL of overnight culture solution and centrifuge at 12,000 rpm for 1min, collect the cells and remove the supernatant Precipitation of resuspended bacteria.
3.Add 250 μL Buffer PA (Please check for RNase A and TSINGRed have been added), suspensed cell, vortex until there are no obvious clot.
4.Add 250 μL Buffer PB, gently invert the tube 4-6 times to completely lyse the bacteria (This step requires gentle invert and should not be violently shaken. After mixing, the bacteria should become clear and viscous. If it is not clear, it may be due to the excessive amount of bacteria and insufficient lysis, so the amount of bacteria should be reduced. After using TSINGRed, if the bacteria is fully cracked, the solution should be completely changed from cloudy pink to clear purple; if insufficient, the purple solution is mixed with cloudy pink. At this point, continue to flip up and down and mix until the solution becomes clear purple.)
5.Add 350 μL Buffer PC, gently invert the tube 4-6 times to mix， centrifuge at 12000 rpm for 10min (After using TSINGRed, if the neutralization is sufficient, the solution should completely change from clear purple to light yellow, with white flocculent precipitation.)
6.The supernatant after centrifugation in the previous step is poured into an adsorption column， centrifuge at 12,000 rpm for 1min， place the adsorption column back to the empty collection tube
7.Add 500 μL Buffer PWA to the adsorption column, centrifuge at 12,000 rpm for 1min,discard the liquid.
8.Add 600 μL Buffer PWB to the adsorption column (please check if absolute ethanol has been added), centrifuge at 12000 rpm for 1 min, and discard the liquid in the collection tube.
9.Repeat step 8.
10.Remove the adsorption column, place it in a clean 1.5 mL EP tube, and open it at 65 °C for 4-5 min to dry the left ethanol.Add 30μL of H_2O to the middle of the adsorption membrane, hold for 4-5 min at 65 °C, and centrifuge for 2 min at 12000 rpm.

Preparation of competent cells of *Zymomonas mobilis*

1.Prepare sterile water, RMG 5 medium, and 10% glycerol solution. Streak the *Zymomonas mobilis* soltion on RMG 2 plate, and cultured in 30℃ constant temperature incubator for activation, for 2 days long (pick a single colony for verification if necessary).
2.Pick activated single colonies of *Zymomonas mobilis* into bacterial vials containing about 10 mL of RMG 5 liquid medium and culture for 30℃ overnight (different volumes of RMG 2 can be set to ensure that the next day activated fluid is in logarithmic phase). Transfer an appropriate amount of logarithmic solution into a 500 mL triangle containing 200 mL RMG5 liquid medium to set the initial OD_{600nm} at at 0.025-0.05. 100 rpm, 30℃ until OD_{600nm} is between 0.3 and 0.4.The angles are incubated on a 30℃ shaker at 100 rpm until the OD_{600nm} is between 0.3 and 0.4.
3.collect the cells (25 °C, 4,000 rpm, 10 min) with four 50 mL centrifuge tubes. (50 mL of bacterial solution is collected per centrifuge tube),after centrifugation, carefully discard the liquid in the horizontal flow clean bench.

4.Add 40 mL sterile water to each tube, resuspend and wash the cells, centrifuge (25 °C, 4,000 rpm, 10 min) and discard the supernatant.

4. Add about 5 mL of sterile water to each tube, resuspend the cells, concentrate the 4 tubes in a centrifuge tube, fill with sterile water to 40 mL , resuspend and wash the cells; prepare another centrifuge tube and centrifuge together with it; Centrifuge (25 °C, 4,000 rpm, 10 min)and carefully discard the supernatant in the horizontal flow clean bench.

5. Add 40 mL 10% glycerol to each tube, resuspend and wash the cells, centrifuge (25 °C, 4,000 rpm, 10 min) and discard the supernatant in the horizontal flow clean bench.

6. Repeat step 6.

8.Add 200 μL of 10% glycerol and resuspend the cells,Load 55 μL of bacteria solution per EP tubes.Store in liquid nitrogen after dispensing and transfer to the -80 °C refrigerator.

Electroporation transformation

Electroporation is used to transfer plasmids or fragments into *Z. mobilis*. The steps are as follows:

1.Pre-cooled electric cup, put the competent cell, plasmid on ice.

2.Mix plasmid and competent cell into the cooling electric cup.

3.Carefully dry the outside of the cup and press the start button,the cells and plasmid DNA are electroporated (1.6 KV, 25 μF, and 200 ohms) using a Bio-Rad Gene Pulser.

4.Put the G5 medium into the electric cup, mix, absorb, and repeat several times.

5.The mixed medium is put into the EP tube, plastic-sealed and placed in the 30℃ incubator.

Fermentation of *Zymomonas mobilis*

1.Glycerol preservation bacteria activation: Take the glycerol bacteria from the -80°refrigerator, 100 μL are added to a cryopreservation tube containing 2mL liquid RMG5 culture with special antibiotics , then put it in the incubator to cultivate to turbidity.

2.Transfer to a large system for expanded culture as seed liquid :Add the bacteria from the whole cryopreservation tube to a centrifuge tube containing 20 ml/30 ml liquid RMG 5 with special antibiotics and incubate overnight in a 30°incubator.

3.Prepare medium for fermentation: 40 ml RMG 5 medium，the second day，clean the horizontal flow clean bench and fully irradiate and sterilize with ultraviolet rays.

4.Measure the OD value of seed liquid (seed liquid should be blown evenly):The initial OD value of the control fermentation culture medium is about 0.10. The OD_2 value of seed liquid is measured for one night of culture, and the algorithm is $0.1 \times 40 = OD_2 \cdot x$. x μL is the bacterial solution to be added.Add all media to a certain concentration of the resistance of the corresponding bacteria, and set the tetracycline gradient (generally set the tetracycline gradient for each parallel group).

5.After collecting x ml of seed solution, resuspend it with medium into a triangular flask containing 40 ml of medium of interest (RMG 5) and mix well.

6.Connect the medium label of the seed liquid to mark, suck and beat evenly and take samples, put them in the spectrophotometer to measure the 0h OD value, measure the OD in one bottle, and retained one bottle.

7.Place the fermentation medium in a 30℃-shaker culture.After that: 0h, 3h, 6h, 9h, 12h, 24h, 30h, 36h, 48h (according to the specific situation to adjust the sampling time) sampling OD value, sample retention, generally for 48h fermentation test.

Determination of 3-HB using HPLC

1. Configuring the mobile phase: configure sulfuric acid with a concentration of 5 mol/ ml, and use a filter film with a pore diameter of 0.22 μm to defilter the configured mobile phase.
2. Ultrasonic degassing of the mobile phase: ultrasonic degassing of the mobile phase after filtration 20-30 minutes, and then used after returning to room temperature.
3. Standard product configuration.
4. Sample processing: After high-speed centrifugation, add 500 μL sample into the sample bottle through a 0.22 μm filter with a syringe.
5. Sample analysis: determine isobutanol content using the HPLC system.

Detection by flow cytometry

With a fluorescent tag on the recombinant plasmid, different inducer concentrations can be set, and the optimum inducer concentration can be found according to the fluorescence intensity.
1. Remove the frozen bacteria in the -80℃ refrigerator,dashed on the special antibiotics plates, and culture for activation in a constant-temperature incubator.
2. Pick a single colony in an appropriate volume of liquid medium and culture until the late of logarithmic phase and preserved with 60% glycerol.
3. For growth testing, take glycerol bacteria and inoculate them into the culture medium until the mid-log phase period and then inoculate.
4. The cells were collected using the EP tube and the above cells are inoculated to the liquid medium containing different concentrations of tetracycline in the deep well plate, so that the initial OD_{600nm} is 0.1 with three biological repeats for each concentration. 30℃, 100 rpm for *Zymomonas mobilis*, and 37℃, 250 rpm for *E. coli.*
5. Culture until 8 h and 16 h,then collect cells and preserved.
6. The collected bacterial solution is loaded into 2 mL EP tubes and centrifuged at 12,000 rpm for 1 min, discard the supernatant and cells were resuspended and centrifugation using PBS. Cells were washed three times and finally resuspended to 107 cell number / mL and configured as sample solution.
The ultrasonic cleaning experiment will use the nozzle, and exhaust the air bubbles. Disinfect the working environment and instruments with 75% alcohol
7. The nozzle to be used in the ultrasonic cleaning experiment is designed to expel air bubbles.Disinfect the working environment and instruments with 75% alcohol.
8. Utilize sorting quality control microspheres and adjust them to the optimal value. Set the excitation wavelength to 506 nm and the FITC channel.
9. Adjust the voltage of the deflection electrode plate for the side liquid flow window, setting an appropriate deflection angle to ensure clear fluid stream separation for ease of sorting.
10. After cleaning the tubing with a 0.5% sodium hypochlorite solution for 5 minutes, place the samples on the sample rack and proceed to detect fluorescently labeled cells.

Strain growth and growth curves

1.Take the cryopreserved bacteria and transfer 100 μm into 200 mL medium, and put in a 30℃ incubator overnight to grow the bacteria until mid-log phase.
2.The medium was sterilsterile and divided into 50 mL triangular flasks.
3.Collect the seed liquid and centrifuge to remove the background culture medium. After washing twice with fermentation medium, adjust the initial OD_{600nm} to 0.1 and inoculate it into the

corresponding fermentation medium, placing it on a shaker for cultivation.

4. Sample collection is performed every 3 hours, measuring the OD_{600nm} using a spectrophotometer. Additionally, 1 mL is taken in a sterile EP tube, and after centrifuging to separate the bacterial cells, the supernatant is collected. After passing it through a sterile 0.22 μm filter, it is stored at -20°C. This will be used later for glucose and ethanol analysis.

5. Continue this process until sugar depletion in the sample. After obtaining the data, perform data processing with time on the x-axis and OD600 nm on the y-axis, using GraphPad Prism 8.0 for plotting and conducting significance analysis.

Determination of the glucose and ethanol concentrations

1. Take samples for biosensor testing.

2.Sample concentration range: Ethanol ≤ 0.5 g/L, Glucose ≤ 1 g/L.

3. Diluted samples with a volume greater than 700 μL are transferred into 1.5 mL EP tubes using a pipette and then subjected to machine analysis.

Protein expression in *E. coli* using an IPTG inducible expression system

Materials

- 1mL overnight culture of cells containing ECN cells transformed with pET28-His6-GFP
- 1000× kanamycin stock
- 1M IPTG stock
- 50mL of liquid LB media

1. Add 1000× kanamycin to 50mL of LB so that the final concentration is 1×. Swirl to mix.
2. Dilute the overnight culture 1:100 into 50mL of LB supplemented with 1 × kanamycin. Shake culture at 37 °C. Remove 1mL of sample periodically to check the OD_{600nm} (to measure the concentration of bacteria). See Appendix for Spectrophotometer usage instructions.
3. When the OD_{600nm} reaches between 0.6-1.0 (approximately 2 hours), add IPTG to a final concentration of 500μM. This will induce the expression of the His6-GFP protein.
4. Allow the culture to grow and express the GFP by shaking at 18°C for 16-24 hours. Note that in some cases, lowering the temperature after the induction may help proteins fold.
5. After 16-24 hours, pour the culture into a 50mL conical tube.
6. Pellet cells by spinning in a centrifuge at 4000 g for 10 minutes. Remember to balance the centrifuge!
7. Remove supernatant by pouring into a waste container for liquid media (near the sink). We will bleach the media to ensure any bacteria left in the media are killed before disposing down the drain.
8. Freeze the cell pellet until you're ready to start the protein purification.

Protein Purification

Proteins with a hexa-histidine (His6) affinity tag at either their N- or C-terminus can be purified using nickel-based affinity chromatography. The His6 tag binds to metal ions like nickel (Ni^{2+}), allowing us to separate our His6-GFP from all the other proteins in the cell. You will be given a frozen cell pellet of bacteria cells that were induced to express His6-GFP. After lysing the cells to release the protein, we will first run a nickel column to extract the His6-tagged protein. We will run an SDS-PAGE gel to assess the purity of our His6-GFP protein.

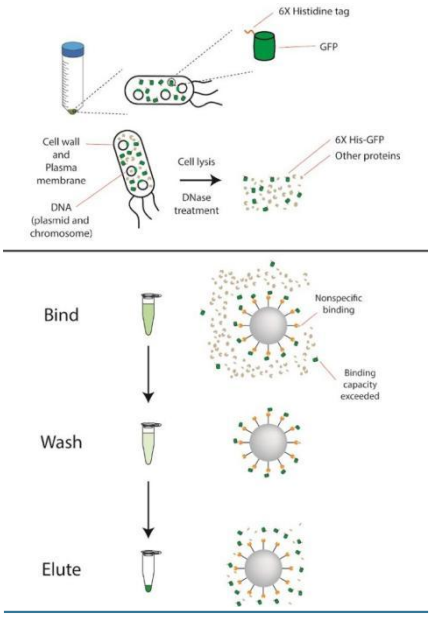


Image courtesy of QB3-Berkeley's Lab Fundamentals Bootcamp instructors.

Bacterial cells containing the His6-GFP protein are pelleted and lysed. Nickel affinity beads specifically bind the His6 tag of the His6-GFP protein, allowing separation from other cellular proteins. The beads are washed and the His6-GFP protein is eluted, yielding a concentrated protein prep.

Materials

- Cell pellet from 100 mL *E. coli* cells expressing His6-GFP
- 200 µL Ni-NTA agarose (in 2mL tube)
- 1.5 mL Microcentrifuge tubes
- 2 mL Microcentrifuge tubes
- 2 mL BPERII lysis buffer
- 1 µl Lysozyme
- 1 µl DNase I enzyme
- 3 mL Wash buffer
- 1 mL Elution buffer
- 1 mL Regeneration buffer

Lysis

1. Add 2 mL of the BPERII lysis buffer to the cell pellet. Resuspend cells by pipetting up and down, making sure that no chunks remain.
2. Add 1 µl of lysozyme and 1µl of DNase I. Lysozyme helps break down bacterial cell walls and DNase I degrades unwanted DNA from bacterial lysis to assist with protein

purification.

3. Incubate at room temperature 15 minutes.

Separate cell debris from lysate

1. Take your lysate and split it into two separate 1.5 mL tubes, 1mL in each.
2. Spin lysate in centrifuge at maximum speed for 5 minutes. While this is spinning, equilibrate your Ni-NTA agarose beads:
3. Add 500 µL water to the 200 µL bead slurry (Ni-NTA Agarose) and pipette gently to mix.
4. Settle the resin by centrifuging at 800 g for 1 minute.
5. Aspirate (remove with a pipette) and discard the supernatant. Be careful to not disturb the bead bed.
6. Add 500 µL of wash buffer to the bead slurry and pipette gently to mix.
7. Settle the resin by centrifuging at 800 g for 1 minute.
8. Aspirate and discard the supernatant. Be careful to not disturb the bead bed.
9. Transfer 50 µL of the supernatant from one of the tubes to a clean 1.5 mL tube and label **"Clarified Lysate" ("CL" for short)**. Set this sample aside. You will run it on a gel for analysis.
10. Carefully remove remaining supernatant from both tubes with a pipette (Avoid disturbing any of the pellets!) and transfer the supernatant to a clean 2mL centrifuge tube.

Purifying His6-GFP Protein

1. Add 100 µL of Wash buffer to your beads. Pipette gently to mix, and then add this entire slurry to the 2mL microcentrifuge tube with your clarified lysate.
2. Incubate at room temperature for at least 10 minutes, inverting the tube every minute or so to resuspend the beads. Longer binding times may result in more effective binding.
3. Settle the resin by centrifuging at 800g for 1 minute.
4. Transfer 1mL of the supernatant to a clean 1.5mL tube and label "**Flow-Through" ("FT" for short)**. Discard the remaining flow-through. Be careful to not disturb the bead bed.
5. Add 1mL of Wash buffer to the bead slurry and invert slowly to mix.
6. Settle the resin by centrifuging at 800g for 1 minute.
7. Collect the supernatant in a clean 1.5mL tube and label "Wash (W)". Be careful to not disturb the bead bed.
8. Repeat steps 12 – 14. (You do not need to save this second wash).
9. Elute by adding 200 µL of Elution buffer to the bead slurry with gentle agitation. Incubate for 1 minute at room temperature.
10. Settle the resin by centrifuging at 800g for 1 minute.
11. Collect the supernatant in a clean 1.5mL tube and label "Elution (E)".
12. Add an additional 200 µL of Elution buffer to the bead slurry with gentle agitation. Incubate for 1 minute at room temperature.
13. Settle the resin by centrifuging at 800g for 1 minute.
14. Combine the supernatant with the previous elution. Save the purified protein at 4°C for future use.

Washing and storing resin (optional)

Centrifuge and discard the supernatant between the following rinses:

1. Rinse resin with remaining Elution buffer.

2. Rinse resin with 1 mL Wash buffer.
3. Rinse resin with 1 mL water.
4. Rinse resin with 1 mL of Regeneration buffer.
5. Rinse resin with 1 mL of water.
6. Centrifuge and discard the supernatant. The resin is now regenerated.
7. Add 200µL 20% EtOH, and resuspend resin.
8. Transfer resin to the indicated 15mL conical. Instructors will collect and save the resin for future use.

Preliminary analysis of fractions

GFP can be detected visually by its green color by eye. You might notice that your purified protein has a yellow-green hue. However, a more sensitive way to assess the presence of GFP can be done using the inherent fluorescent properties of the protein. The SafeImager light overlaps with the excitation wavelength of GFP. Use the SafeImager to detect the presence or absence of GFP in the lysate, flow-through, washes, and elution. The samples will fluoresce if GFP is present.

Buffer Recipes

Wash Buffer

20 mM Tris pH 8.0
500 mM NaCl
20 mM Imidazole

Nickel Column Elution Buffer

1. 20 mM Tris pH 8.0
2. 500 mM NaCl
3. 500 mM Imidazole

Imidazole is used to elute the tagged proteins bound to nickel ions attached to the surface of beads in the chromatography column. An excess of imidazole is passed through the column, which displaces the His6-tag from nickel coordination, freeing the His6-tagged proteins.

Resin Regeneration Buffer

100mM Sodium Acetate, pH 4.5

SDS-PAGE and Coomassie staining

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate and visualize individual proteins from a complex mixture. Dodecylsulfate (SDS) is a detergent that binds to and denatures (unfolds) proteins. Because SDS is negatively charged, each protein will be separated by size, similar to the separation of different DNA fragments in an agarose gel. We will run a gel of our fractions from the nickel column purification to identify the GFP-containing fraction and assess its purity.

Four groups will run their samples from the His6-GFP purification on the same gel. All the samples will also be run in duplicate on another gel – one gel will be stained for TOTAL protein with Coomassie and the other gel will be used for a Western blot to identify a SPECIFIC protein with an antibody.

Materials

- Fractions from purification (lysate, protein elution)
- Prestained protein ladder

- 5x SDS loading buffer
- 1x Running buffer
- Bio-Safe Coomassie Stain
- 1 Gradient gel
- 1 Mini-PROTEAN 1D gel electrophoresis system/power source

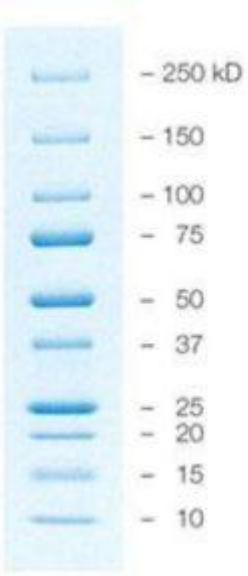
Loading the gel

1. Prepare samples for the gel by mixing 10 µL of the CL, FT, W, and E fractions with 30 µL of Wash buffer and 10µL of 5x SDS loading buffer.
2. Assemble the gel apparatus. Two gels can be run simultaneously in each apparatus. **Remove the sealing tape from the bottom of the gel before loading.**
3. Make 500mL of 1x Running buffer. Pour buffer into the inner chamber of the gel box between the gels so that the wells are completely submerged. Pour the Running buffer into the outer chamber until it is half-full.
4. Carefully load 10 µL of ladder and 20 µL of each prepared sample into the wells of the gel using gel-loading tips. Add the volume slowly and watch to ensure that it sinks to the bottom of the well. Below is a sample of how you and your neighboring group might load your gel. Be sure to record how your gel was loaded.

Lane 1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Protein ladder	GFP CL Gr1	GFP FT Gr1	GFP W Gr1	GFP E Gr1	Protein ladder	GFP CL Gr2	GFP FT Gr2	GFP W Gr2	GFP E Gr2

Running the gel

1. Attach the lid and plug the leads into the power source. Turn on the power to 120 V. Check that bubbles are emerging from the electrode at the bottom of the apparatus.
2. Check the buffer levels in approximately 10 minutes to ensure that the chamber has not leaked. If it has leaked, add more Running buffer.
3. When the dye front has nearly run off the bottom of the gel (this should take at least an hour), turn off the power source and unplug the apparatus.
4. Grab an empty pipette tip box for Coomassie staining.
5. Remove the gel and rinse the entire set-up thoroughly with distilled water.
6. Pry apart the gel casing using the green lever tool. The casing should break into two parts with the gel in the middle. Be careful not to tear the gel, which is very fragile.
7. With the gel sitting on one side of the casing, transfer the gel to the plastic tip box for staining with distilled water.



Bio-Rad Precision Plus protein standards. Image courtesy of Bio-Rad.

Coomassie staining

1. Wash the gel in distilled water on a shaker for 5 minutes.
2. Remove all water from the gel container and add enough Bio-Safe Coomassie Stain to completely cover the gel. Let stain for 1 hour on a shaker. If the protein signal is low, stain overnight.
3. Rinse gels with water. For a more complete destain, add a kimwipe to a corner of the box and leave on a shaker.
4. Analyze gel and record in your notebook:

    - Does your protein run according to its expected molecular weight?
    - Which sample contains your protein? How clean is your sample?