

Purification of His-Tagged Proteins

The following protocol is for the purification of ONE protein. Scale up as needed.

Stock Solutions:

1M Imidazole
7% Glucose (filter-sterilized)

Buffers:

Lysis Buffer

20mM HEPES-KOH pH: 8.0¹
1M NaCl
2mM β -Mercaptoethanol²

Wash Buffer 1

Lysis buffer + 10mM Imidazole

Wash Buffer 2

Lysis buffer + 20mM Imidazole

Elution Buffer

Lysis buffer + 250mM Imidazole

Dialysis Buffer

100mM KCl
0.2mM EDTA
20mM HEPES-KOH pH: 8.0¹
20% Glycerol
1mM DTT³

Other Supplies:

- LB-Ampicillin plates
- Qiagen Nickel-NTA Agarose (stored at 4°C)
- Ampicillin (stored at -20°C)
- 100mM PMSF dissolved in isopropanol (stored at -20°C); use caution when making this!
- cOmplete Protease Inhibitor Cocktail, Roche (stored at 4°C); does not contain EDTA
- 1M DTT (stored at -20°C)
- 0.5M IPTG (stored at -20°C in solution, 4°C as a dry powder)
- 100% Glycerol
- SDS-PAGE supplies
- Bradford Assay Reagents

¹pH HEPES with dry KOH instead of 1M NaOH solution

²Add β -Mercaptoethanol just before use of the lysis buffer to ensure it does not oxidize

³Add the total volume of DTT over the span of three hours (1/3 of the volume of total DTT every hour)

Procedure:

Day 1

1. Inoculate a 5mL LB+antibiotic liquid culture with BL21 cells transformed with your clone in a protein expression vector (i.e., pHAT) and let incubate at 37°C overnight.
2. Make and autoclave 500 mL of 1.5x LB in a 2 L Erlenmeyer flask.

Day 2

1. Amend the 500 mL 1.5x LB with 0.7% glucose and 1X ampicillin.
2. Inoculate this 2 L flask with 2.5 mL of the overnight culture.
3. Incubate and shake the cell culture at 37°C until the $OD_{600} = 0.5-0.6$ (takes approximately three hours, but measure the OD_{600} at least every hour).
4. After the cell culture has reached the appropriate OD_{600} , take a 1 mL aliquot of the suspension and save for later.
 - a. Spin the aliquot down in a centrifuge, decant the LB supernatant, resuspend the pellet with 100ul of ddH₂O and store at -80°C.
5. Add 1 mL of 0.5M IPTG. Induce for 3 hours at 37°C shaking. Take an aliquot of the culture every hour to save for later (step 4a).
6. Spin down the 1 L culture at 4°C, 8000 rpm (10,500xg) for 8 minutes.
7. Remove the supernatant by decanting. You can store the pellet dry in the -80°C short-term until you are ready to do the purification.
8. Assess the results of the induction. Add 100 mL water to your aliquot samples to resuspend. Take a 10-20 µl sample and add 6X SDS-PAGE loading dye directly to this sample. Load onto an SDS-PAGE gel to check for induction. If there was no induction based on the gel, do not proceed with "Day 3" procedures.

Day 3

1. Pre-make the cOmplete Protease Inhibitor Cocktail and PMSF (two tablets). Add 40 µl PMSF, cOmplete Protease Inhibitor Cocktail, and 1mM Imidazole to 40 mL of Lysis Buffer (will later be used to resuspend the pelleted cells).
2. Resuspend the cell pellets (stored at -80°C) in a total of 40 mL Lysis Buffer (resuspend one culture and pour into the next, resuspend, etc.)

3. Sonicate. Pulse for 2-5 seconds, then turn off for 2-5 seconds. Keep cells on ice at all times!
Pulse for 15 minutes total.
4. Transfer lysates to SS34-compatible tubes (be sure tubes are equally loaded). Spin down cells at 11,000 rpm (15,000xg) for 1 hour in the SS34 rotor at 4°C.
5. In the meantime, prepare the Ni-NTA slurry (as follows):
 - a. Let the Ni-NTA Agarose homogenize at room temperature for 5 minutes.
 - b. Gently(!) pipet 1.5 mL of the Ni-NTA Agarose into a 50 mL tube. Centrifuge at 4°C, 500xg for 5 minutes. Remove the supernatant with a pipette (do not disturb the beads), and add up to 15 mL of Lysis Buffer.
 - c. Mix gently by inversion.
 - d. Repeat the centrifugation step and remove the supernatant.
6. Transfer the centrifuged cell lysate supernatant to a cold 50 mL tube. Add up to 20 mL of cleared lysate to the equilibrated Ni-NTA matrix (may need to split into two tubes).
7. Bind using end-over-end rotation (Ferris wheel) on the slowest setting at 4°C for 1 hour.
8. Centrifuge the bound beads at 4°C, 500xg for 5 minutes and CAREFULLY remove the supernatant using a pipette.
9. Add 25 mL of Wash Buffer 1 with a pipette using the CAREFUL technique. Mix gently by inversion then by end-over-end rotation at 4°C for 5 minutes.
10. Pipet off the supernatant without disturbing the beads into a designated container. Measure the OD₂₈₀. Repeat Steps 10-11 until an OD₂₈₀ of zero is reached, then proceed to next step.
11. Wash the beads a final time by adding 25 mL of Wash Buffer 2 with a pipette using the CAREFUL technique. Mix gently by inversion then by end-over-end rotation at 4°C for 5 minutes.
12. Pipet off the supernatant without disturbing the beads into a designated container. Measure the OD₂₈₀ (it should be a small fraction above zero).

The following steps must be conducted in the cold room.

13. Add 10 mL Wash Buffer 2, mix gently by inversion, and transfer to a 10 mL gravity column. Make sure the bottom outlet is capped before loading. Let the beads settle for 10 minutes, or until they are settled evenly.

14. Remove the bottom cap and collect the flow-through for SDS-PAGE analysis later. *Never let the column run dry!* Check the OD₂₈₀ of the flow-through. If it is not close to zero, wash by adding 10 mL of Wash Buffer 2, collecting and saving the wash, then checking the OD₂₈₀. If the wash is not close to zero, repeat this wash until it is.
15. Elute by adding 5 mL Elution Buffer. Put the cap on the column and leave for 5 minutes.
16. Collect ten 500 µL samples of each fraction in 1.5 mL Eppendorf tubes.
17. Estimate the protein concentration by the Bradford Assay. Take a 2.0 µL sample and add 100 µL of Bradford dye. Pool the highest concentrated fractions (the one which are most Bradford blue).
18. Perform a dialysis overnight in the cold room by dialyzing against 1 L of Dialysis Buffer. Place a stir bar in a 1 L graduated cylinder and place dialysis bag (containing protein) in the buffer. Stir overnight in the cold room.
 - a. Add the total volume of DTT required for the Dialysis Buffer over the span of three hours (1/3 of the volume of total DTT every hour).

Day 4

1. Measure the dialyzed protein concentration. Run protein (and flow-through/washes from yesterday) on an SDS-PAGE gel to check for purity.
2. Separate the protein into 10 µL aliquots (do this in the cold room) and store in the -80°C freezer in a separate box. Label the box with the protein name, tag, and concentration.