Batch purification of his-tagged *E.coli* proteins with Ni-Sepharose

Buffers (closely following Hoang & Ferre-d'Amare, Cell 2001)

Buffer A: 20 mM Tris-HCl pH 8.1, 400 mM KCl, 5% (v/v) glycerol, 1 mM β-mercaptoethanol, 0.5 mM PMSF, 30 mM imidazole (~ 500 mL)

Buffer B: 20 mM Tris-HCl pH 8.1, 400 mM KCl, 5% (v/v) glycerol, 1 mM β-mercaptoethanol, 500 mM imidazole (~ 200 mL)

Preparation of the Ni Sepharose slurry

1. gently shake the bottle to create a homogeneous medium, remove 5 ml slurry from bottle using a blue tip whose outmost tip has been cut off to create a larger opening (or a serological pipette) and transfer slurry in 50 ml falcon tube, label the tube with Ni-Sepharose, Date, your initials and the protein (TruB)
2. spin the Ni-NTA sepharose column at 500g for 2 min to sediment the resin. Remove the supernatant.
3. wash the resin with 3 volumes of sterile water (MilliQ). (Gently mix the solid phase with the liquid to create slurry. Spin at 500g for 5min)
4. wash the resin with 6 times of Buffer A (6x4mL=~ 25 mL), leave 3 ml Buffer A on the resin to obtain a 50% slurry
5. divide into 4 (or less) falcon tubes **ONLY** if you have a large volume of lysate (> 50 mL).

Cell opening

1. Add PMSF and β-mercaptoethanol to buffer A
2. Resuspend the frozen cell pellet in Buffer A [approx. 5ml/g(cell)] in a small (100 mL) beaker and thaw cells by stirring the mixture slowly on ice (takes about 30 – 60 min)
3. Add lysozyme to a final concentration of 1mg/ml and incubate the cell suspension on ice for 30 minutes (resuspend the lysozyme in ~ 1 mL buffer A before addition).
4. Add sodium deoxycholate [12.5mg/g(cell)]
5. Continue stirring mixture on ice for 30 minutes.
6. Open cells using a sonicator while sample is on ice: at least 10 minutes for 1 minute, short pause in-between, shake beaker, intensity level 6, duty cycle at 60%
7. Centrifuge cell lysate for 30 minutes at 30 000xg at 4°C in a JA-25.5 rotor
8. Take the A_{280} reading of the cell lysate to monitor cell opening (in comparison to previous/future preps)
9. remove 50 µL of cell lysate for SDS-PAGE analysis (lysate before binding)
10. store pellet for later analysis

Purification by Ni-Sepharose slurry (Batch procedure)

11. Apply the cleared cell lysate to washed Ni-sepharose evenly and gently mix thoroughly. Incubate for 60 minutes inverting periodically to bind protein to the resin at room temperature.
    ALL FURTHER STEPS ARE PERFORMED USING COLD BUFFERS (4°C)
12. spin the slurry at 500g for 5 min, remove the supernatant and store it at 4 °C. Take 50 µL sample and store at 4 °C as well (lysate after binding).

13. wash the resin 6 times with a Buffer A (6x4mL=~ 25 mL). Pool the washes, store them at 4 ºC and take 50 µL sample – also to be stored at 4C.
14. Add β-mercaptoethanol to Buffer B.
15. Elute the protein 8 times using 90% resin volume of Buffer B (~ 1.2 mL). Incubate with elution buffer (Buffer B) for 5 minutes on ice. Spin for 5 min at 500g. Save each elution in a 15 mL tube, store at 4 ºC and take a 50 µL sample from each elution step.
16. Run an SDS-PAGE of all samples (15 µL of elutions; 3 µL of lysate before and after binding to Ni^{2+} sepharose) to ensure proper protein purification (same or next day – cast gel in between!).
17. The column material can be stored as 50% slurry in Buffer C for 1 day before it needs to be regenerated (see below).

Regeneration of Ni-Sepharose

18. Wash resin 5 more times with 20 ml of Buffer B (centrifuge at 500xg for 2 min). Check last wash on gel for presence of protein.
19. Wash resin 3 times with 40 ml MilliQ H_2O.
20. Wash resin once with 40 ml of 20% ethanol (in H_2O). Leave 1.5 ml 20% ethanol on resin to obtain a 50% slurry for storage at 4C. Note: leave slurry in clearly labeled tube such that your colleagues can track for which purification this particularly batch of resin has been used!

Concentration

1. Rinse Vivaspin MWCO 10,000 with Storage buffer (2 ml) and centrifuge at 4000xg for 10 min.
2. Pool elutions after having discussed the SDS-PAGE with your supervisor.
3. remove buffer from Vivaspin and add pooled elutions.
4. Centrifuge at 4000xg for about 15 min.
5. Keep centrifuging: regularly check for speed of concentrating, add more elutions to the top of the Vivaspin, remove filtrate from bottom of the Vivaspin, pool with other filtrate and store at 4C
6. concentrate your elutions to a volume of about 1-2 ml – check carefully for the presence of precipitate!

Optional: Re-buffering if Superdex 75 purification is omitted

7. *dilute sample 1:10 by adding 20 ml Storage Buffer and concentrate again repeat step 29 for 1 or 2 more times (total dilution ~ 1:500)*
8. *aliquot a 1.0 mL sample (for later superdex purification) and a few 50 µL samples in tubes labeled with protein name on top and prep number, your initials and date on site.*
9. *shock freeze aliquots in liquid nitrogen and store at -80C*

Storage Buffer: 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 1 mM BMe, 0.5 mM EDTA, 5 mM MgCl2, 20% (v/v) glycerol