Purification of secreted protein from cell culture supernatant using Ni^{2+}NTA chromatography

Harvesting and preparation of supernatant

1. Harvest conditioned medium by centrifugation (~300g 5 min). Transfer sup into new tubes and repeat centrifugation (1500 g 5 min). Repeat with 15000 g 10 min.
2. Optional: Depending on the cell culture medium and the pH at the binding step, you might need to dialyse your conditioned medium. Some cell culture media contain high concentrations of histidine or metal ions (which interfere with IMAC binding) or high concentrations of phosphate buffer (which precipitate at pH 8), e.g. some of the insect cell culture media. Refer to the QIAexpressionist handbook page 76ff (http://www.qiagen.com/literature/render.aspx?id=128) in order to decide whether you need to dialyze or not. Dialyse conditioned medium at 4°C extensively against sodium phosphate phosphate buffer (pH 6.5-7.0) containing 400 mM NaCl. Three buffer changes should be sufficient (each > 18 hours when using large tubing with Ø > 25mm) if the dialysis buffer volume/sample volume ratio is 10. Use a dialysis membrane that has a MWCO which is maximally one third of the size of your protein (e.g. a 30 kDa protein should be dialysed against a membrane with a MWCO of maximally 10 kDa). You might want to include penicillin/streptomycin or other antibiotics in the dialysis buffer to prevent bacterial growth if you are not sure whether you managed to fill the dialysis tubes in a sterile manner.
3. Adjust pH to 8.0 (or the pH of your choice) with 3M Tris (pH 10.5). If your protein binds only weakly to Ni^{2+}NTA, use 0.1 M NaOH instead (Tris competitively weakens the interaction of Ni^{2+} with the histag).
4. Centrifuge at 11000 rpm for 30 min.

Ni^{2+}NTA affinity chromatography

5. Add washed Ni^{2+}NTA Superflow resin (Qiagen). Add ~1 ml 50% resin per 250 ml supernatant. The exact amount necessary depends on the expression levels. Binding capacity is about 10 mg protein/ml resin for Ni^{2+}NTA Superflow resin. If the media volumes are very large and the protein concentration very low, you need to use more resin than actually necessary based on the binding capacity (otherwise you will loose the resin).
6. Incubate with very gentle agitation o/n at 4°C.
7. Harvest resin by centrifugation at 4000 rpm (GSA rotor) for 15 min or simply let settle at 1g for about 1 hour. Carefully remove supernatant. Don't discard but keep for a second round of purification if necessary. Add 1 ml 10% NaN_3 for each 100 ml depleted supernatant to prevent microbial growth. Store at 4°C.
8. Transfer resin into an empty column and attach to Äkta Explorer.
9. Wash with ~10 column volumes binding buffer
10. Wash with 10-50 column volumes wash buffer (until OD_{280nm} shows a stable baseline)[1].
11. Elute with elution buffer.

Exchanging the elution buffer

Since the elution buffer is not ideal for most applications (high salt, imidazole), the eluate needs a buffer exchange. This can be done by:
a) dialysis
b) desalting column (PD-10 on bench or Fast Desalting column on the Äkta Explorer)
c) gel filtration (which has the advantage of further purifying the protein based on its size), e.g. Superdex 75 or 200 10/300 GL (running buffer PBS, flow rate 0.4 ml/min). Sterile filter and aliquot peak fraction.

1 The concentration of imidazole in the washing step needs to be determined individually. Some histagged proteins can already elute at 10 mM! If unsure which washing conditions to use, you can e.g. wash/elute with 5 mM, 20mM and 200mM imidazole and analyze the elution peaks on a gel to identify the peak that contains your protein.

Group 1: Sunday afternoon/evening; group 2: Monday afternoon/evening

Overnight batch binding of histagged proteins to the Ni^{2+}NTA resin

1. Take 100 µl aliquots of the (dialyzed) cell culture supernatant for the SDS-PAGE on Friday.
2. Add the Ni^{2+}NTA resin to the cell culture supernatant and put it to for binding with gentle agitation to 4°C.

Group 1: Monday afternoon; group 2: Tuesday

Purification on the Äkta Explorer FPLC machine

1. Centrifuge the cell culture supernatant to recover the Ni^{2+}NTA resin.
2. Remove supernatant and add NaN_3 to the supernatant (in case it needs to be repurified).
3. Fill an empty column with the resin and attach it to the Äkta Explorer.
4. Wash the column with binding buffer.
5. Elute/Wash the column with elution buffer containing 5% imidazole.
6. Elute/Wash the column with elution buffer containing 20% imidazole.
7. Elute/Wash the column with elution buffer containing 250% imidazole.
8. Take 100 µl aliquots of flowthru, wash and all three elutions for the SDS-PAGE on Friday and store at -20°C.

Both groups: Friday

Running SDS PAGE gels with all fractions (supernatant, flowthru, wash, three eluates) plus one protein marker (7 samples) and silver staining

1. Take 48 µl of the aliquot and add 12 µl 5x Laemmli Standard buffer.
2. Boil for 5 min at 95°C.
3. Load gel.
4. Run gel ~20 min at 12mA and then at 40mA until the blue front reaches the gel bottom.
5. Remove gel and fix for 5-10 minutes in 7,5% acetic acid.
6. Wash gel 3x for 2-5 min in water.
7. Incubate gel for 1 hour in silver solution.
8. Wash gel for 30-60 s in water.
9. Develop gel in developer solution until desired band intensity is reached (30 s to 10 minutes).
10. Stop development by placing gel into 7.5% acetic acid for 5 minutes.
11. Wash gel twice for ~10 minutes with water and once with gel drying solution for ~10 minutes.
12. Dry gel.

Recipes

Dialysis buffer (30 mM sodium phosphate, 400 mM Na_2HPO_4, pH ~6.6)
4.4 g Na_2HPO_4
23.4 g NaCl
water ad 1000 ml, adjust pH to 6.6 with NaH_2PO_4 or HCl (approx. 1 ml conc. HCl).

Binding buffer (50 mM Na_2HPO_4, 400 mM NaCl, 5% glycerol, pH 8.0)
7.1 g Na_2HPO_4,
23.4 g NaCl
57.5 ml (69g) 87% glycerol
water ad 1000 ml, adjust pH with NaH_2PO_4 to 8.0.

Washing buffer
individual mixture of binding and elution buffer, we use two different washing buffers:

a) 5 mM imidazole (98% binding buffer + 2% elution buffer)
b) 20 mM imidazole (92% binding buffer + 8% elution buffer)

Elution buffer (50 mM Na_2HPO_4, 250 mM imidazole, 400 mM NaCl, 5% glycerol, pH 8.0)
7.1 g Na_2HPO_4
17 g imidazole
23.4 g NaCl
57.5 ml (69 g) 87% glycerol
water ad 1000 ml, adjust pH with NaH_2PO_4 to 8.0.

5x Laemmli Standard Buffer (LSB)
3.7 ml 0.5 M Tris HCl, pH 6.8
2.9 ml glycerol 87%
3 ml 20% SDS
2.5 ml β-Mercaptoethanol
0.2 ml 0.5% (w/v) bromophenol blue

SDS-PAGE running buffer (10x)
30.3 g Tris base
144 g glycine
10 g SDS
water ad 1000 ml. Don't adjust pH! Dilute 1:10 for each electrophoresis run.

silver solution
0.3 g AgNO3
200 ml water
307 µl formaldehyde solution (36.5%)

development solution
6 g Na2CO3
200 ml water
307 µl formaldehyde solution (36.5%)
400 µM sodium thiosulfate

gel drying solution
1-2% glycerol
25% ethanol
73-74% water