NBD Protein Expression and Purification
Thibodeau Lab

Day 1:
Stab glycerol stocks of BL21 cells and grow donor cultures overnight at 37°C in LB with 50 μg/ml kanamycin.

Day 2:
Inoculate expression cultures (generally 1l of LB medium with kanamycin in 2l flask) and grow at 37°C. Once culture starts to become cloudy (~1.5-2 hours), check OD_{600} and continue growing until culture reaches an OD_{600} of ~1.5. Add 1 mM IPTG and place cultures in refrigerated incubator at 15°C. Allow cultures to express overnight.

Prepare buffers for purification.

Lysis (100 mls):
50 mM Tris
150 mM NaCl
2 mM ATP
1 mM β-ME or DTT
10% v/v glycerol
pH 7.6

Binding (400 mls):
50 mM Tris
150 mM NaCl
10% v/v glycerol
pH 7.6

Washing (250 mls):
50 mM Tris
150 mM NaCl
40 mM Imidazole
10% v/v glycerol
pH 7.6

Elution (250 mls):
50 mM Tris
150 mM NaCl
400 mM Imidazole
10% glycerol
pH 7.6

S300 (500 mls):
50 mM Tris
150 mM NaCl
2 mM ATP
1 mM β-ME or DTT
10% v/v glycerol
pH 7.6

2nd Ni-NTA buffer (200 mls):
Binding buffer + 2 mM ATP

Place buffers in refrigerator for cooling overnight. All buffers should be cold for purification. Buffers without ATP, β-ME or DTT can be made in advance. ATP, β-ME and DTT should be added the day of purification.

Day 3:
Harvest cells by centrifugation using the large Sorvall preparative centrifuges. Either 1l or 500ml bottles can be used, depending on culture size and rotor availability. Spin cells at 10,000 G RCF for 15 minutes at 4°C. – the rotor RPM will vary. Pour off the supernatant

and retain the cell pellet. The pellet can be transferred to a 50 ml comical vial and frozen at -80°C.

Cell lysis:

1. Add ATP and reductant to the lysis buffer prior to cell resuspension. Use at least 30 mls of lysis buffer to keep the lysates cool when sonicating.
2. Resuspend cells by pipetting up and down on ice.
3. Sonicate cells using program 2 (30 secs @ 50% power and 50% DC) in an ice bath. Repeat sonication two times with gentle mixing between sonication cycles.
4. Spin cells at 25,000 G RCF for 30 minutes at 4°C in a round bottom tube.
5. Retain the supernatant – this is the soluble protein fraction and will contain the NBD protein.

Ni-NTA purification:

1. Load supernatant into SuperLoop for purification on AKTA equipment. Try to eliminate all air from the lower chamber of the SuperLoop.
2. Add 1 ml of elution buffer with 10 mM ATP and 5 mM DTT to each fraction tube.
3. Run appropriate program – will depend on machine and lysate volume.
4. Pool eluted fractions and concentrate to <5 mls for S300 column

S300:

1. Remove SuperLoop and install 5ml sample loop into injection valve
2. Wash sample loop with at least 10mls of S300 buffer to clear it of residual proteins/buffer from previous purifications
3. Load protein into sample loop
4. Run S300 program (~10.5 hours)

Ulp1 digestion:

1. Collect fractions that appear to contain protein by either UV absorbance and/or BioRad dye assay – will likely be 3-4 fractions (~10 mls)
2. Add Ulp1 to NBD protein and incubate at 4 C for at least an hour. Ulp1 is highly active and should be used at roughly 1:500 dilution after S300 column (10 mls protein = 200 μl Ulp1).

2nd Ni-NTA to remove Ulp1 and Smt3-tag:

1. Equilibrate column with 2nd Ni-NTA buffer.
2. Load sample loop/SuperLoop with digested NBD protein
3. Collect flow through – this will be your NBD protein
4. Wash column and collect eluate – occasionally the NBD will adhere nonspecifically to the column and decrease the yield