Per Widlund's TACC3 purification protocol v. 30.03.2009
(adapted from Tim's GST fusion protocol)

Transformation – Day 1

1. Transform vector into Rosetta (DE3) cells (contains pRARE plasmid). Select on LB plates containing100ug/mL Kanamycin and 25ug/mL Chloramphenicol.

Expression – Day 2/3

2. Inoculate a 20mL LB-Kan/Chlor starter culture and incubate shaking overnight – 37C 180rpm. Book separate incubator (for 18C) for the next morning.

3. Set incubator to 18C
4. Prepare 1 liter of LB medium containing Kan/Chlor. Transer 500mL of medium to a 2.8L Fernbach flask. Inoculate with 15mL of the overnight culture.
5. Shake for about 2.5 hours at 37C (OD600~1.0) at 180rpm. Store the remaining 500mL at 4C.
6. Add the cold 500mL LB to the culture
7. Shake for 20min at 18C
8. Take 1mL sample for SDS-PAGE – spin and freeze pellet.
9. Add 200uL of 1M IPTG to the culture (0.2mM final)
10. Shake overnight at 18C (18 hours). Book JLA8.1000 rotor for the next morning.

Harvest – Day 4

11. Take 1mL sample for SDS-PAGE – spin and freeze pellet.
12. Centrifuge culture at 5,000rpm for 10min at 4C in JLA 8.1000 rotor.
13. Resuspend bacteria in 30mL of cold lysis buffer. Freeze suspension in LN2 and store at -80C, or continue with purification.

Lysis and clarification – Day 4

14. Thaw cell suspension if necessary. Add protease inhibitors:
    a. 1 complete tablet (roche)
    b. 40uL 10mg/mL pepstatin (10ug/mL final)
    c. 40uL 10mg/mL PMSF (10ug/mL final)
15. Add 0.5mg/ml lysozyme. Incubate on ice for 30min
16. Sonicate 30 sec at 50% amplitude on ice.
17. Load 6-8 tubes in MLA-80 rotor. Spin in Utracentrifuge for 15min at 40,000rpm at 4C.
18. Pool supernatant and add imidazole (from 1M stock) to 10mM final.

Purification – Day 4

19. Perform first purification with a 5mL Nickel column on a peristaltic pump:
    a. Wash with 5-10 CV of water at 5ml/min
        i. Strip column if necessary
            1. -10 CV of 50mM EDTA, pH 8.0
            2. -8 CV water
            3. -1 CV of 100mM NiCl2
            4. -8 CV water
    b. Equilibrate in 5CV 3% buffer B/97% buffer A at 5ml/min
    c. Load lysate at 1-2ml/min
    d. Wash with 5CV of 3% buffer B/97% buffer A at 5ml/min
    e. Wash with 5CV of 3% buffer B/97% buffer A high salt at 5ml/min
    f. Wash with 5CV of 10% buffer B/90% buffer A at 5ml/min
    g. Elute with 15ml of 100% buffer B at 5ml/min – collect 1mL fractions
    h. Wash with 10CV water
    i. Run 5CV 20% Ethanol for storage.
20. Check for peak protein fractions with using a quick Bradford assay (1-3ul in 500ul 5xdiluted Bradford reagent
21. Check for quality of purification by running positive fractions along with samples of crude lysate, cleared lysate, flow-through and all wash steps on SDS-PAGE.
22. Pool peak fractions
23. Adjust salt concentration by diluting 1:8 with 25mM Tris, pH 7.5
24. Perform second purification with a 1mL HiTrap MonoQ column
    a. Wash with 5-10 CV of water at 5ml/min
    b. Charge column with 5CV 25mM Tris, 1M KCl, pH7.5
    c. Equilibrate in 5CV 25 mM Tris, 75mM KCl ,pH 7.5 at 5ml/min
    d. Load lysate at 1 ml/min
    e. Wash with 5CV 25 mM Tris, 75mM KCl ,pH 7.5 at 5ml/min
    f. Wash with 5CV 25 mM Tris, 150mM KCl ,pH 7.5 at 5ml/min
    g. Elute with 5CV 25 mM Tris, 300mM KCl ,pH 7.5 at 5ml/min
    h. Wash with 5CV 25mM Tris, 1M KCl, pH7.5
    i. Wash with 10CV water
    j. Run 5CV 20% Ethanol for storage.
25. Determine concentration:
    a. Take 5uL of each fraction and add 5uL 8M Urea. Take A280 with nano-drop using Urea/size exlusion buffer mix as a blank. Be careful to remove all traces of urea when finished.
26. Pool peak fractions. Add 80% glycerol to 10% final (140ul for every 1mL) and add 100mM DTT to 1mM final (11ul for every 1mL). Adjust concentration determination accordingly.
27. Snap freeze aliquots in LN2 and store at -80C.

Media
LB or TB medium
30mg/ml kanamycin in water
15mg/ml chloramphenicol in ethanol

Protease Inhibitors
Complete protease inhibitor tablets (Roche)
10mg/ml pepstatin in DMSO
10mg/ml PMSF in ethanol

Lysis buffer

- 50mM Tris-HCl pH7.2
- 500mM NaCl
- 0.1% TritonX-100
- 5% glycerol

Nickel column buffers/solutions
Buffer A

- 25mM Tris-HCl pH 8.0 (3.03g for 1L)
- 300mM NaCl (17.53g for 1L)
- 20% glycerol

Buffer B:

- As above, but with 300mM imidazole (20.4g for 1L)

High Salt Wash

- 1.5mL Buffer B
- 48.5mL Buffer A
- 3.0g NaCl

Buffers for anion exchange (stock)
Nanopure water
20% ethanol
1M Tris pH 7.5 (40x)
3M KCl

100mM DTT
80% glycerol