His_6-tagged Sigma factor purification

Adapted from Jule Ollinger, the Goebel lab's notes, Amersham's On-Column Refolding protocol

Purpose: To purify His-tagged sigma factors for in vitro use. This method includes isolation and washing of inclusion bodies, followed by denaturation and refolding of the purified proteins. Sigma factors are generally quite stable after refolding (personal communication, Helmann lab), but alternatively, native purification can be used to if protein activity after refolding is questionable.

The strains carrying His-SigA and SigB are Qiagen's E. coli M15 [pREP4]. They contain a plasmid, pREP4, which is included to silence the Pspac promoter prior to induction. All media and agar must contain kanamycin to select for pREP4.

Furthermore, these strains contain Qiagen's pQE30 vector. This vector contains an ampicillin resistance gene. All media and agar must contain ampicillin to ensure this vector is maintained. See Qiagen website for more details about the strain and vectors.

Procedure:

1. Streak out appropriate *E. coli* strains from frozen on LB plates:
   E. coli M15(pREP4) with pQE30HissigA = FSL B2-273
   E. coli M15(pREP4) with pQE30HissigB = FSL B2-274

2. Inoculate 5 mL LB with appropriate antibiotics w/1 colony and incubate overnight at 37C with shaking, to final concentrations:
   ampicillin 100 ug/ml
   kanamycin 25 ug/ml

3. Transfer 2 ml of overnight culture to 500 ml LB + Amp + Kan in 1 L flask. Incubate at 37C with shaking.

4. Monitor OD_{600}. At OD_{600} ~ 0.7-0.9, remove 1.5 ml as "Pre Induction Control." Spin for 1 min at 10K rpm, and freeze pellet at -20C. To induce protein expression, add 0.5 ml 1 M IPTG at (final = 1 mM IPTG). Grow for 3 hrs.

5. Transfer culture to 2 X 250 ml centrifuge bottles evenly. Centrifuge at 10,000 x g at 4 C for 15 min.

6. Resuspend each pellet in 5 ml culture supernatant. Recombine into 2 15 ml round bottom centrifuge tubes.

7. Centrifuge at 10,000 x g for 15 min at 4 C. Pour off supernatant. Freeze pellets at -80C.

8. **Day of purification:** Thaw cells on ice. Resuspend each pellet in 5 ml lysis buffer (buffer recipes below).

9. Sonicate cells at 60% power for 4 intervals at 20s with 1 min intervals to cool, on ice water. Vortex briefly.

10. Add 0.5 ml 10% Triton X-100 to each tube of cells (1% Triton-X final concentration). Incubate cells on ice for 10 min.

11. Centrifuge tubes at 10,000 x g for 15 min at 4 C. Keep supernatant in labeled tube at 4 C.

12. Resuspend each pellet in 5 ml lysis buffer with 1% Triton X-100, vortex, incubate on ice for 10 min, sonicate at 60% power, for 4 intervals at 20s with 1 min intervals to cool, on ice water. Centrifuge again.

13. Resuspend each pellet in 5 ml lysis buffer WITHOUT Triton and recentrifuge.

14. Resuspend each pellet in 5 ml solubilization buffer (8 M Urea, made fresh that day). Sit at room temp for about 15 min, with occasional vortexing. NOTE: urea at this concentration will freeze. Keep these high concentration solutions off ice but relatively cool.

15. Centrifuge again for 15 min to remove insoluble material.

16. Proceed with supernatant.

Column prep and filtration

Start here if using column for the first time or recharging a used column:

1. Fill syringe with 5 mL dH2O, remove stopper from column and connect syringe to column (1 mL HiTrap Chelating) with drop to drop method (see demo on website: www.gelifesciences.com/aptrix/upp01077.nsf/content/protein_purification~prepacked_columns~hitrap#)

2. Remove snap off end at column outlet.

3. Wash column twice with 5 mL dH20 to wash away EtOH.

4. Load column with 0.5 mL 0.1 M NiSO4 (stock solution already prepared

Start here if using column used 3 times or less:

6. Get buffers from 4C and keep on ice.

7. Equilibrate column with 7 mL solubilization buffer attempting to get flow rate ~1ml/min

8. Apply sample with syringe, save flow through in 2 mL aliquots in 2 ml eppendorf tubes.

9. Wash column with 6 ml 6 M urea buffer. Keep all flow through on ice, labeled!

10. Wash with 6 ml 4 M urea buffer.

11. Wash with 6 ml 2 M urea buffer.

12. Wash with 6 ml 0 M urea buffer.

13. Wash with 6 ml 40 mM imidazole buffer.

14. Wash with 6 ml 100 mM imidazole buffer.

15. Wash with 6 ml 500 mM imidazole buffer, keeping 1 ml aliquots of flow through (to make concentration easier).

16. Check Abs_{280} of all aliquots for protein contents. Choose ~15 highest Abs for analysis. Use buffers as blanks prior to reading appropriate fractions because imidazole has a high Abs230.

17. Check protein comp. of high absorbing aliquots on SDS-PAGE gel: (denaturing; 20 ul sample + 20 ul Laemmli buffer); heat at 70C for 10 min, load 30 ul in gel, run gel at 50 V at start (will drop because current is constant). Include PreInduction control to ensure that the protein of interest is being overexpressed in response to IPTG addition.

12. Store column in 20% EtOH at 4C (long term storage: column should be stripped).

13. Dialyze protein in Tris buffer (20 mM Tris, 100 mM NaCl, 5-10% glycerol, 0.1 mM DTT), using Slide-A-lyzer cassette (use MWCO at least half or less of your protein) in 2 L of buffer. Include spin bar, and place in cold room overnight. Change buffer in the morning, allow equilibration for at least 4 hours.

14. Remove protein, pipette 0.5 ml aliquots, freeze at -80C.

Buffer Recipes:

Make stocks, adjust pH, add Beta-mercaptoethanol (14.3 M stock).

Lysis buffer (20 ml)
20 mM Tris-HCl
0.5 M NaCl
10 mM Imidazole
1 mM 2-mercaptoethanol (1.4 ul)
100 ul Halt Protease Inhibitor
pH 8.0

Solubilization buffer
8 M urea (24 g/50 ml)
20 mM Tris-HCl
1 mM 2-mercaptoethanol (3.5 ul)
0.5 M NaCl
10 mM imidazole
pH 8.0

Wash buffer (50 ml)
20 mM Tris-HCl
0.5 M NaCl
10 mM imidazole
1 mM 2-meEtOH (3.5 ul)
pH 8.0

Elution buffer (20 ml)
20 mM Tris-HCl
0.5 M NaCl
0.5 M Imidazole
1 mM 2-mercaptoethanol (1.4 ul)
pH 8.0

Refolding buffers: Make 10 mL of each:

	6 M	**4 M**	**2 M**
8 M Urea	7.5 ml	5 ml	2.5 ml
0 M Urea (wash)	2.5 ml	5 ml	7.5 ml

	40 mM	**100 mM**
500 mM Imidazole	0.6 ml	1.8 ml
10 mM Imidazole (wash)	9.4 ml	8.2 ml

Troubleshooting:

- Always keep cell lysate and protein cold to limit proteolysis. Better yet, add a protease inhibitor, but make sure its compatible with Nickel columns (eg, EDTA and EGTA free!)

- Always make urea solutions fresh. Urea will degrade over time, and will carbamylate your proteins if old urea solutions are used.
- Never use EDTA or other chelating solutions in your purification, as these will chelate nickel and prevent binding of your protein.
- Glycerol and higher salt concentrations can be used in elution buffers to prevent nonspecific binding of proteins and increase purity.

References:

Amersham Biosciences. Rapid and efficient purification and refolding of a (His)6-tagged recombinant protein produced in E. coli as inclusion bodies. http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/D8D71A01D3D720B6C1257628001CC8EE/$file/18113437AB.pdf. September 1999.

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