SOP: PP035.5
Modified 11-29-16 MCL

Purification of native GroES

Materials and Reagents:

1. Supernatant from 40% ammonium sulfate cut from native Ag85/Mpt32 prep (see SOP:PP020 or PP021)
2. 3500 MWCO dialysis tubing
3. 10 mM ammonium bicarbonate
4. Buffer A: 0.1% TFA in B&J Water
5. Buffer B: 80% acetonitrile, 0.1% TFA in B&J water
6. 4-12% Bis-Tris protein gels (Invitrogen NP0323BOX)
7. 5x loading buffer (see SOP: SP007)
8. MES running buffer (Invitrogen NP002)
9. 20 mL C18 reverse-phase HPLC column
10. HPLC instrument with software
11. Fraction collector
12. Steriflip filter, 0.2 µm
13. Injection needle
14. 10 mL syringe
15. BCA supplies (see SOP:PP003)
16. SA-12 Mouse monoclonal antibody (see SOP:PP011 for western blotting)
17. α-Mpt64 Rabbit polyclonal antibody (see SOP:PP011 for western blotting)
18. Silver staining material (see SOP:PP007 for running and PP012 for staining of gels)
19. SimplyBlue Safe Stain
20. 8 mL polypropylene collection tubes
21. Savant
22. Lyophilizer

Protocol:

1._____ Add a stir bar to the sample, place on a stir plate and begin stirring. Slowly add ammonium sulfate to 50% saturation (note 1).

2._____ Stir at room temperature until ammonium sulfate is completely dissolved, then transfer sample to 4°C and stir for 4-16 hr.

3._____ Remove stir bar and centrifuge the CFP/ammonium sulfate solution at 10,000 x g, 4°C for 30 minutes.

4._____ Prepare 7 L of dialysis buffer (10mM ammonium bicarbonate).

5._____ Decant the supernatant into a clean container (note 2). The pellet is the 50% ammonium sulfate cut.

6._____ Suspend the protein pellet in approximately 25-30 ml of dialysis buffer and pipet it into the dialysis tubing. Close the dialysis tubing and place the tube into the dialysis tank.

7._____ Dialyze at 4°C for 4-16 hours.

8._____ Exchange the dialysis buffer (7 L) two times for 4-16 hours each.

9._____ Collect the protein solution from the dialysis tubing and rinse the dialysis tubing with a minimal volume of fresh 10 mM ammonium bicarbonate. Place the protein solution along with the rinse in a sample tube.

10._____ Determine the protein concentration using the BCA assay (see SOP SP003).

11._____ Lyophilize the protein (see SOP SP004).

12._____ Suspend the lyophilized protein in 90% buffer A:10% buffer B so that the final protein concentration is between 1.5 and 2.0 mg/ml. About 40-60 mg total per prep.

13._____ Filter the protein suspension through a 0.2μm steriflip filter

14._____ Connect a 20 mL C18 reverse HPLC column to the HPLC system (note 3).

15._____ Prime line B in buffer B and line A in buffer A.

16._____ Equilibrate the column with 90% buffer A:10% buffer B at 1 ml/min for at least 2 column volumes (CV)

17._____ Open the correct HPLC run in Empower (Large C18 GroES Gradient program)

The program should be as follows at 1ml/min:

30 min (1.5 CV) wait:	90% buffer A/ 10% buffer B
10 min (0.5 CV) gradient:	90%/10% A/B → 65%/35% A/B
20 min (1 CV) hold:	65%/35% A/B
60 min (3 CV) gradient:	65%/35% A/B → 40%/60% A/B
30 min (1.5 CV) hold:	40%/60% A/B
10 min (0.5 CV) gradient:	40%/60% A/B → 100% B
30 min (1.5 CV) hold:	100% B
10 min (0.5 CV) gradient:	100% B → 90%/10% A/B
20 min (1 CV) hold:	90%/10% A/B

(220 min total = 3 hr 40 min)

18._____ Program fraction collector for 30 min void, followed by 90 x 2 minute fractions

19._____ Draw 10 ml of the filtered protein solution into a 10 ml syringe. Free the syringe of any bubbles by gently tapping it on a hard surface (the bubbles should move to the surface). Expel the bubbles by pushing up on the plunger. Attach the Waters injection needle and expel some of the liquid through the needle. This is to make sure that there are not any air bubbles preceding the liquid.

20._____ Move the HPLC injection lever to "load", insert the needle into the injection lever and expel the liquid by pushing on the plunger. After all the liquid has been dispensed, remove the needle from the injection lever, move the lever to "inject".

21._____ If more injections are required, wait 11 minutes, then repeat injection (steps 18-19). Repeat as many times as necessary to inject all material, being sure to collect the flow through from the injection and wash.

22._____ Before the final injection, click on the inject icon on the computer. Be sure that the program and fraction collector start once the injector lever is switched to the "inject" position.

23._____ Upon completion of the run, move the fractions to a test tube rack and store the fractions at 4°C. Store the C18 column in 50% methanol.

24._____ Remove 6 µl from each fraction and place into a 0.65 mL microcentrifuge tube.

25._____ Dry on the Savant to remove the ACN.

26._____ Resuspend each fraction in 10 µl of 10 mM ambic.

27._____ Run each fraction on a gel and stain with SimplyBlue Safe Stain (see SOP:SP007 for running of gels) (note 4).

28._____ Determine which fractions have GroES and run SA-12 and α-Mpt64 western blots on those fractions to confirm which fractions contain GroES without Mpt64 contamination (see SOP:PP011 for western blotting).

29._____ Determine which fractions to pool and dry them down completely on the Savant to remove any ACN (note 5).

30._____ Resuspend each fraction in about 0.5 mL 10mM ambic and pool them together, washing the tubes several times to ensure that all of the protein is retrieved.

31._____ Run a BCA to determine protein concentration and run on a gel and SA-12 and α-Mpt64 western blots to verify the pool (note 6).

32._____ Final QC includes gel, SA-12 and α-Mpt64 western blot (with CFP or pure protein as positive control). Make 0.5 mg aliquots. Freeze, lyophilize and store at -80°C

Notes:

1. Determine the appropriate amount of ammonium sulfate using the calculator at http://www.encorbio.com/protocols/AM-SO4.htm. Be sure to calculate based on a starting concentration of 40%.
2. The supernatant can be immediately used for a 70% ammonium sulfate cut, or saved at -20°C (see SOP SP024).
3. Before using the HPLC and Empower HPLC program, read the HPLC SOP:SP025 or have lab personnel trained in the use of the HPLC assist you in setting up the liquid chromatography of GroES. Be sure that all buffer bottles, filters, and fractions tubes are compatible with acetonitrile use.
4. Silver stain can also be used (see SOP:SP012), however low molecular weight proteins such as GroES can be seen more clearly using SimplyBlue.
5. Fractions containing relatively clean Mpt64 can also be pooled and saved for cleanup.
6. At this point, the pool may be clean enough for QC. If it is not, lyophilize the pool and start at step 12 to clean-up (a 4 mL C18 column is also available for small-scale cleanup using adjusted gradient times based on the column volumes listed).