PP057
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Separation of Soluble Filtrates, including CFP, for Native Protein Starting Material

Materials and Reagents:

1. Soluble protein sample (such as CFP)
2. Ammonium sulfate
3. Dialysis tubing, 3.5 kDa MWCO
4. Dialysis tank (7 L)
5. Dialysis buffer #1: 10 mM ammonium bicarbonate, 1 mM DTT
6. Dialysis buffer #2: 10 mM ammonium bicarbonate
7. SDS-PAGE gel
8. Bicinchoninic acid (BCA) protein assay kit
9. High speed centrifuge bottles
10. High speed centrifuge and rotor
11. Freeze-dryer and flask

Protocol:

1._____ Determine the volume of your soluble protein mixture. Go to http://www.encorbio.com/protocols/AM-SO4.htm, an online calculator, and enter in the starting volume, temperature (which will be 4°C), desired ammonium sulfate percentage of 40%, and starting ammonium sulfate percentage 0%. The website will calculate the amount of ammonium sulfate that will need to be added (note 1).

2._____ Add a stir bar to the protein solution, place it on a stir plate, and begin stirring. Gradually add the ammonium sulfate crystals and stir at room temperature until completely dissolved. Transfer sample to 4°C (note 2).

3._____ Incubate the sample at 4°C for 4 - 16 h; be sure sample is stirring at all times (note 3).

4._____ Remove the stir bar. Divide the protein solution equally among centrifuge bottles and balance (note 4). Centrifuge at 10,000 x g, 4 °C for 30 min.

5._____ Decant the supernatant into a clean container. Store the pellet at 4°C. This pellet is the 40% ammonium sulfate cut (note 5).

6._____ Determine the volume of the supernatant and repeat the online calculation as in step 1, entering the new volume, desired ammonium sulfate percentage of 50%, and starting ammonium sulfate percentage 40%.

7._____ Repeat steps 2 through 5. This pellet is the 50% ammonium sulfate cut (note 6).

8._____ Repeat the calculation from step 6 using a desired ammonium sulfate percentage of 70% and starting ammonium sulfate percentage of 50%, then repeat steps 2 through 5 to obtain the 70% ammonium sulfate cut (note 7).

9._____ Resuspend each pellet separately in 25-30 mL 10 mM ammonium bicarbonate.

10._____ Prepare dialysis tubing and add sample.

11._____ Place in 7 L dialysis buffer #1 and dialyze at 4°C for 4 - 16 h.

12._____ Change to fresh dialysis buffer #1 and dialyze at 4°C for another 4 - 16 h.

13._____ Change to dialysis buffer #2 (containing no DTT) and dialyze at 4°C for 4 - 16 h.

14._____ Remove the samples from their dialysis membranes and transfer to clean sample tubes.

15._____ Quantitate the amount of protein by BCA (see SOP SP003). Based on the assay results, run 3 - 4 µg of each sample on SDS-PAGE gel to visualize protein bands (note 8).

16._____ Freeze and lyophilize the samples (see SOP SP004).

17._____ Store at -80°C for long-term storage.

Notes:

1. Any ammonium sulfate percentage may be used depending on your needs, however, many *M. tuberculosis* CFPs are differentiated for purification by beginning with a 40% ammonium sulfate cut of CFP followed by a 50% ammonium sulfate cut and subsequent 70% ammonium sulfate cut, hence why this example is used.
2. The solution may begin to become cloudy as protein precipitates out of solution, but it should be easy to differentiate between this and undissolved ammonium sulfate because the ammonium sulfate will appear as crystals that settle to the bottom of the container.
3. A shaker or rocker may also be used if a stir plate is unavailable for incubation.
4. Polypropylene, polycarbonate, or Teflon tubes can all be used for ammonium sulfate precipitation. Note that some materials allow for better adhesion of the pellet to the bottle. Polycarbonate bottles may require additional care to not disrupt the pellet after centrifugation. Potential solutions may include faster centrifugation (up to 27,000 xg if the bottles are rated for higher speeds), longer centrifugation (up to 1 hr), and/or decreased braking speed.
5. See SOPs PP020 (Ag85 Complex), PP021 (Ag85 A, B, C), and PP022 (Mpt32) for purification of proteins from the 40% ammonium sulfate cut.
6. See SOP PP035 for purification of GroES from the 50% ammonium sulfate cut.
7. See SOP PP024 for purification of 38kDa (PstS1) from the 70% ammonium sulfate cut. There should be little to no protein left in the 70% supernatant; the supernatant is generally discarded once it is confirmed that the protocol was successfully implemented.
8. See SOP SP007 for running SDS PAGE gels. Gels can be visualized by silver stain (SOP SP012), Simply Blue SafeStain, or by western blot (SOP SP011) developed using antibody specific to the protein of interest.