Preparation of Purified Ag85 Individual Components (a, b, c)

Materials and Reagents:
1. Culture filtrate proteins (CFP) from *M. tuberculosis* (~300mg)
2. Ammonium bicarbonate
3. Dithiothreitol
4. Sodium azide
5. MilliQ Water
6. Ammonium sulfate
7. Buffer A: 10 mM KH$_2$PO$_4$ (pH 7.2), 1 mM EDTA, 1 mM DTT, 0.02% NaN$_3$
8. Buffer B: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT, 0.02% NaN$_3$
9. Buffer C: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT, 50% ethylene glycol (v/v), 0.2% NaN$_3$
10. 70% ethanol
11. Dialysis buffer (10 mM Ammonium bicarbonate, 1 mM DTT)
12. Trypsin modified sequencing grade
13. 15 % SDS-PAGE gels
14. 13x100 mm polypropylene culture tubes
15. 10 cc syringe
16. Transfer pipettes
17. 150 ml plastic container
18. 10 ml plastic disposable pipettes
19. Deplasticized tubes
20. Dialysis tank
21. Dialysis tubing (3,500 Da MWCO)
22. Filter bell funnel with Pall membrane filter (catalog number P/N 66548)
23. Lyophilizer flask
24. Waters HPLC system (high flow)
25. Lyophilizer
26. Waters fraction collector
27. 60 ml Phenyl Sepharose HPLC column
28. Waters injection needle
29. Amicon ultrafiltration system with a 10,000 MWCO membrane (catalog number PLGC07610)
30. High speed centrifuge
31. 250 ml, Centrifuge bottles
32. F16/250 rotor
33. ESI ion trap mass spectrometer
34. 120 ml Sephadex-75 HPLC size exclusion column
35. Size Exclusion Buffer: PBS (pH7.4), 1mM DTT, 0.1% n-octylthioglucoside
36. 0.2µm acrodisc syringe filter

Protocol:
1. _____ Thaw the CFP at 4°C overnight.

2. _____ Pour the thawed CFP into a centrifuge bottle and slowly add ammonium sulfate while stirring to 40% saturation (note 1).

3. _____ Centrifuge the CFP/ammonium sulfate solution at 27,000 x g, 4°C for 1 hour.

4. _____ While the centrifuge is running boil the dialysis tubing in MilliQ H$_2$O.

5. _____ Make 7 L of dialysis buffer in a dialysis tank.
6. _____ From the centrifuged material, collect the supernatant and store at -20°C for use in other purifications (see SOP: PP024). Suspend the protein pellet in approximately 25-30 ml of dialysis buffer and pipette it into the dialysis tubing. Close the dialysis tubing and place the tube into the dialysis tank.

7. _____ Dialyze at 4°C for 4 to 12 hours.

8. _____ Change the dialysis buffer (7 L) and dialyze at 4°C for 4 to 12 hours.

9. _____ Change the dialysis buffer to 7 L of 10 mM ammonium bicarbonate and dialyze at 4°C for 4 to 12 hours.

10. _____ 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 clean 150 ml plastic container.

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

12. _____ Lyophlilize the dialyzed protein (see SOP SP004).

13. _____ Suspend the lyophilized protein in buffer A so that the final protein concentration is between 1.5 and 2.0 mg/ml.

14. _____ Filter the protein suspension through a 0.2µm acrodisc filter.

15. _____ Filter all of the buffers using the pall filter bell and 0.45µm filters (make sure the filter bell has been cleaned and there is a new filter for each buffer).

16. _____ Connect the 60 ml Phenyl Sepharose HPLC column to the High flow HPLC system (notes 2 and 3).

17. _____ Wash the Phenyl Sepharose column with 60 ml of filtered water, at a flow rate of 2.0 ml/min, to remove the ethanol.

18. _____ Prime line C with buffer C, prime line B with buffer B, prime line A with buffer A (note 4).

19. _____ Equilibrate the Phenyl Sepharose column with 60 ml of buffer A.

20. _____ Start the Empower HPLC program, select the Phenyl Sepharose program and set up the chromatography run (note 5).

21. _____ 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.

22. _____ 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”.

23. _____ If more injections are required, wait 6 minutes, then repeat injection (steps 21-22). Repeat as many times as necessary to inject all material, being sure to collect the flow through from the injection and wash (note 6).
24. _____ On the final injection, click on the inject icon on the computer and start the fraction collector.

25. _____ Upon completion of the run, remove the tube holder from the fraction collector and remove 10µl from each fraction and place in a 0.65 ml eppendorf tube for analysis by SDS-PAGE.

26. _____ Place the fractions from the fraction collector tray into a test tube rack and store at 4°C.

27. _____ Add 2 µl of 5X running buffer to the aliquots and run on a gel (SOP: SP007 and SP013 for coomassie staining).

28. _____ Cut the Ag85 spots from every other fraction and place in deplasticized tubes.

29. _____ Follow the SOP for modified in-gel digestion (see SOP SP021).

30. _____ Prep the samples for analysis by ES-MS-MS (see SOP SP027).

31. _____ Once the MS and MS/MS data are collected, analyze this data using the Sequest software. This should tell you in which fractions contain the individual components (A, B, and C) of the Ag85 complex.

32. _____ Pool individual components according to the MS/MS data and SDS-PAGE results.

33. _____ Place pools in 3,500 MWCO dialysis tubing and place in dialysis buffer (note 7).

34. _____ Dialyze for 24 hours at 4°C with three exchanges. On the last exchange, dialyze with ammonium bicarbonate only (no DTT).

35. _____ Run a gel of the pooled components to check purity (note 8).

36. _____ Lyophilize the protein.

37. _____ Set up the Sephadex-75 size exclusion column on the waters HPLC.

38. _____ Wash the column in water.

39. _____ Equilibrate the column in size exclusion buffer.

40. _____ Resuspend the dry sample in approximately 7 ml size exclusion buffer.

41. _____ Filter the protein suspension through a 0.2 µm filter.

42. _____ Start up the Empower program and select the S-75 method set (note 9).

43. _____ Inject sample and start fraction collector as in step 21-24 (note 10).

44. _____ Run 10 µl of each fraction on a gel.

45. _____ Pool all fractions containing relatively clean Ag85.

46. _____ Concentrate using amicon ultra-15 30,000 MWCO centrifugal device and wash three times with 10mM ambic.

47. _____ Run BCA, gel and blot using IT-49 for QC.
48. _____ Make aliquots (default quantity = 0.25 mg) 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. Stir at room temperature until the ammonium sulfate goes into solution and then stir at 4°C for at least an hour, can go overnight. Make sure that the ammonium sulfate is completely dissolved before proceeding.
2. 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 the Ag85.
3. A 20 ml Phenyl Sepharose column is also available for smaller samples (less than 200 mg of protein). If using this column, adjust the times in the program listed in note 5 to accommodate the necessary volumes.
4. This order is best so that the main line is in buffer A for the start of the column. Buffers A, B, and C and all samples containing them must be disposed of as hazardous waste, due to NaN3 content.
5. The Waters 600 HPLC pump can also be programmed manually. The run parameters are as follows:
   - Flow rate = 2 ml/min
   - Fractions = 45 x 3 min fractions, starting at the A→B gradient
   - Column capacity = 600 mg protein
   - Column Volume (CV) = 60 ml
   - 5 CV Injection/Buffer A Wash 150 min
   - 1 CV A→B Gradient 30 min
   - 1 CV 100% B 30 min
   - ½ CV B→C Gradient 15 min
   - 2 CV 100% C 60 min
   - ½ CV C→A Gradient 15 min
   - 2 CV 100% A 60 min
   - 360 min = 6hr
6. This material is used for other purifications (see SOP PP022).
7. The first exchange of dialysis buffer must be disposed of as hazardous waste due to NaN3 content in the HPLC buffers. For this reason, the volume of the dialysis buffer should be kept to ~10X the sample volume in order to keep hazardous waste volumes to a minimum while still ensuring efficient exchange.
8. The Ag85 components should be approximately 90-95% pure as determined by Mass Spec, SDS-PAGE and silver staining. If more separation of the components is required (for example, if A didn’t separate from C), repeat liquid chromatography with the Phenyl Sepharose column following the previously described procedure. If there is contamination from other proteins, continue on with the remaining steps of the SOP.
9. The program for the Sephadex-75 column is as follows:
   - Flow Rate = 1.5 ml/min
   - Fraction program = 20 minute wait
   - 30 x 2 min fractions
   - 30 minute wash
10. Unlike the phenyl sepharose column, only one injection is done for the size column.

References: