

**SOP: PP021**

**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  $\text{KH}_2\text{PO}_4$  (pH 7.2), 1 mM EDTA, 1 mM DTT,
8. Buffer B: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT,
9. Buffer C: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT, 50% ethylene glycol (v/v),
10. 70% ethanol
11. Dialysis buffer (10 mM Ammonium bicarbonate, 1 mM DTT, 0.02%  $\text{NaN}_3$ )
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. 0.2 $\mu\text{m}$  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  $\text{H}_2\text{O}$ .
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. \_\_\_\_ Lyophilize 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 10 $\mu$ l 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  $\mu$ l 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. \_\_\_\_ Concentrate pools using an Amicon ultra 10 kDa MWCO membrane.
34. \_\_\_\_ Fill the amicon with the protein pool and spin at 3,000 rpm.
35. \_\_\_\_ If there is still some of the protein pool left, add that to the amicon and repeat step 34.
36. \_\_\_\_ Once all of the protein pool has gone through the amicon, add 10 mM ammonium bicarbonate and spin at 3,000 rpm. Repeat this step two more times to make sure the buffer is completely exchanged.
37. \_\_\_\_ Collect the protein from the amicon using 10 mM ammonium bicarbonate.
38. \_\_\_\_ Run a gel of the pooled components to check purity (note 7).
39. \_\_\_\_ Lyophilize the protein.
40. \_\_\_\_ Set up the Sephadex-75 size exclusion column on the waters HPLC.
41. \_\_\_\_ Wash the column in water.
42. \_\_\_\_ Equilibrate the column in size exclusion buffer.
43. \_\_\_\_ Resuspend the dry sample in approximately 7 ml size exclusion buffer.
44. \_\_\_\_ Filter the protein suspension through a 0.2  $\mu$ m filter.
45. \_\_\_\_ Start up the Empower program and select the S-75 method set (note 8).
46. \_\_\_\_ Inject sample and start fraction collector as in step 21-24 (note 9).
47. \_\_\_\_ Run 10  $\mu$ l of each fraction on a gel.
48. \_\_\_\_ Pool all fractions containing relatively clean Ag85.

49. \_\_\_\_\_ Concentrate using amicon ultra-15 30,000 MWCO centrifugal device and wash three times with 10mM ambic.
50. \_\_\_\_\_ Run BCA, gel and blot using IT-49 for QC.
51. \_\_\_\_\_ Make aliquots (default quantity = 0.25 mg) and store at -80°C.

**Notes:**

1. Calculate the appropriate amount of ammonium sulfate using the equation: (767g ammonium sulfate/L)(desired %)(volume in liters). 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.
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	<u>60 min</u>
		360 min = 6hr

6. This material is used for other purifications (see SOP PP022).
7. 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.
8. 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
9. Unlike the phenyl sepharose column, only one injection is done for the size column.

**References:**

Belisle J.T., V.D. Vissa, T. Sievert, K. Takayama, P.J. Brennan, and G.S. Besra. 1977. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* **276**: 1420-1422