

**SOP: PP020**

**Preparation of Purified Ag85 Complex**

**Materials and Reagents:**

1. Culture filtrate proteins (CFP) from *M. tuberculosis* (~300mg)
2. Ammonium bicarbonate
5. MilliQ Water
6. Ammonium sulfate
7. Buffer A: 10 mM KH<sub>2</sub>PO<sub>4</sub> (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% NaN<sub>3</sub>)
12. 15% SDS-PAGE gels
13. 13x100 mm polypropylene culture tubes
14. 10 cc syringe
15. Transfer pipets
16. 150 mL plastic container
17. 10 ml plastic disposable pipets
18. Dialysis tank
19. Dialysis tubing (3,500 Da MWCO)
20. Filter bell funnel with Pall membrane filter (catalog number P/N 66548)
21. Lyophilizer flask
22. Waters HPLC system (high flow)
23. Lyophilizer
24. Waters fraction collector
25. 60 ml Phenyl Sepharose HPLC column
26. Waters injection needle
27. Amicon ultrafiltration system with a 10,000 MWCO membrane (catalog number PLGC07610)
28. High speed centrifuge
29. Centrifuge bottles, 250 ml
30. F16/250 rotor
31. 120 ml Sephadex-75 HPLC size exclusion column
32. Size Exclusion Buffer: PBS (pH7.4), 1mM DTT, 0.1% n-octylthioglucoside
33. Amicon ultra-15 30,000 MWCO centrifugal device
34. 0.2 µm 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<sub>2</sub>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 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 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 buffers A, B, and C 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 method set 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 and 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 every other 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 10 $\mu$ l aliquots and run on a gel (SOP: SP007 and SP012 for silver staining)
28. \_\_\_\_ Pool all the fractions that contain the Ag85 complex (runs at ~30kDa).
29. \_\_\_\_ Amicon to concentrate. Wash three times with 10 mM ammonium bicarbonate to remove any residual buffers (note 7).
30. \_\_\_\_ Determine the protein concentration using the BCA assay.
31. \_\_\_\_ Run a gel of the pooled Ag85 complex to check purity (note 8).
32. \_\_\_\_ Lyophilize the protein.
33. \_\_\_\_ Set up the Sephadex-75 size exclusion column on the waters HPLC.
34. \_\_\_\_ Wash the column in 120 ml water.
35. \_\_\_\_ Equilibrate the column in 120 ml size exclusion buffer.
36. \_\_\_\_ Resuspend the dry sample in approximately 7 ml size exclusion buffer.
37. \_\_\_\_ Filter the protein suspension through a 0.2  $\mu$ m filter.
38. \_\_\_\_ Start up the Empower program and select the S-75 method set (note 9).
39. \_\_\_\_ Inject sample and start fraction collector as in step 21-24 (note 10).
40. \_\_\_\_ Run 10  $\mu$ l of each fraction on a gel.
41. \_\_\_\_ Pool all fractions containing relatively clean Ag85.
42. \_\_\_\_ Concentrate using amicon ultra-15 30,000 MWCO centrifugal device and wash three times with 10mM ambic.
43. \_\_\_\_ Run BCA, gel, and blot using IT-49 antibody, for QC.
44. \_\_\_\_ Make aliquots (default quantity = 0.5 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.
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 = 40 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
½ CV	A→B Gradient	15 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>
		345 min = 5hr 45min

6. This material is used for other purifications (see SOP PP022).
7. See SOP: PP005 for details on how to set up the amicon (steps 1-12). Disregard the use of the 10L amicon reservoir. Check the amicon every 30 minutes. When the sample is concentrated down, turn off the nitrogen and vent the system. Open the lid and add the ambic to wash. After the last wash, remove the sample and rinse the membrane.
8. The Ag85 complex should be approximately 90-95% pure as determined by SDS-PAGE and silver staining. If this level of purity has been achieved, then move on to the QC. If more purification is required, 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 used 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