

PP023.1
Modified June 14, 2006

Preparation of Purified 16 kDa

Materials and Reagents:

1. 100 g γ -irradiated *M. tuberculosis* whole cells
2. PBS (pH 7.4)
3. N-octylthioglucoside
4. 8M Urea
5. Beckman CS-6R centrifuge
6. Ultrafuge (with rotor)
7. Centrifuge tubes
8. High speed centrifuge
9. F16/250 rotor
10. Dialysis tubing (3500 MWCO)
11. Ammonium Bicarbonate
12. Sodium Azide
13. DTT
14. Endotoxin-free Water
15. BCA reagents
16. Lyophilizer
17. Lyophilizer flasks
18. 3-10 pharmalytes
19. 4-6.5 pharmalytes
20. NP40
21. Rotofor cell
22. Power pack
23. 15% SDS-PAGE gels
24. Blotting supplies
25. Sephadex-75 size exclusion column (HPLC)
26. Waters fraction collector
27. 13x100 mm polypropylene culture tubes
28. 10 cc syringe
29. Waters injection needle
30. Rotofor Buffer: 7.25 M urea, 0.4% 3-10 pharmalytes, 1.6% 4-7 pharmalytes, 1% N-octylthioglucoside, 2mM DTT (note 1)
30. SE buffer: 3M Urea, 20mM Tris, 0.15M NaCl, 0.1% n-octylthioglucoside, 0.02% NaN₃, pH 7.6 (filtered)
31. Amicon Ultra-15 with 10 MWCO filter

Procedure:

1. ____ Break cells as described in SOP PP007 for production of whole cell lysate.
2. ____ Centrifuge the lysate at 40,000 x g, 4°C for 30 minutes.
3. ____ Reserve the supernatant in a clean container.
4. ____ Combine all of the pellets and resuspend in 100 ml of PBS containing 1% N-octylthioglucoside by sonication.
5. ____ Stir for 1 hr at room temperature and spin at 27,000 x g for 1 hour.
6. ____ Reserve the supernatant from the above spin and resuspend the pellet in 100 ml of 5M urea. Stir for 1 hour at room temperature. Spin at 27,000 x g for 1 hour.
7. ____ Reserve the supernatant.

8. ____ Dialyze the reserved supernatants into Ambic and perform a BCA protein determination assay on them.
9. ____ Lyophilize the supernatant from the detergent fraction in 100 mg aliquots; lyophilize all of the 5M Urea soluble material (note 2).
10. ____ Resuspend one of the aliquots in Rotofor Buffer and rock at 4°C overnight.
11. ____ Sonicate with heat to get the urea back into solution.
12. ____ Put the Rotofor together, add water to the chamber and run at 5W for 20 minutes to make sure that it doesn't leak.
13. ____ Add the sample and run at 6W for 12 minutes, then at 12W taking note of the volts every 15 minutes until it's finished (it's finished when the volts stabilize for 30 minutes).
14. ____ When the run is finished, harvest the fractions and run gels (note 3).
15. ____ Pool the fractions containing the 16 kDa and transfer to 3500 MWCO dialysis tubing. Place in a dialysis tank containing 7L of dialysis buffer (note 4).
16. ____ Dialyze at room temperature for several hours (this is to allow the urea to dialyze without crashing out again) then transfer to 4°C for 4-12 hours.
17. ____ Change dialysis into fresh buffer and dialyze for 4-12 hours.
18. ____ Change dialysis buffer to 10mM ammonium bicarbonate and dialyze for 4-12 hours.
19. ____ Remove the pool from dialysis.
20. ____ Estimate protein concentration by BCA and run 3 µg on a gel. Lyophilize the sample.
21. ____ Resuspend the proteins in the smallest amount of the size exclusion (SE) buffer as possible but no more than 6 ml, with the concentration being no more than 15 mg/ml.
22. ____ Sonicate the sample to get as much material in solution as possible, then centrifuge at 3000 rpm, for 10 minutes to remove the remaining insoluble material.
23. ____ Filter the supernatant before loading on the HPLC.
24. ____ Hook the Sephadex-75 column up to the high flow HPLC system (note 6).
25. ____ Put 300 ml of filtered water through the column, starting with a flow rate of 0.25 ml/min and gradually increasing the flow rate to 1 ml/min.
26. ____ Prime line A with the buffer filtered SE buffer and run 200 ml of it through the column at 1.5 ml/min.
27. ____ Start the empower HPLC program, choose the S-75 method set and enter 55 minutes for data collection. Then click on prepare.
28. ____ Using a 10 ml syringe, draw up the filtered proteins. 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 aren't any air bubbles preceding the liquid.

29. ____ Move the 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” and at the same time, click on inject on the computer screen.
30. ____ Start the fraction collector which should be set for 20 minute wait, then 30 fractions at 2 min/fraction.
31. ____ Once the run is over, remove the tube holder from the fraction collector and remove 10 μ l aliquots from every other fraction for analysis by SDS-PAGE.
32. ____ Pool all of the fractions containing the 16 kDa.
33. ____ Once all the fractions containing the 16 kDa have been collected, use and Amicon ultra-15 to concentrate and wash three times with 10mM ammonium bicarbonate to remove any residual buffers.
34. ____ Once the purified 16 kDa is obtained, do a BCA and run a gel and a blot (develop using CS-49) for QC.
35. ____ Make aliquots of 100 and 250 μ g.

Notes:

1. Rotofor Buffer:

| | |
|---------------------------------|---------|
| 4-7 Zoomlytes (40% stock soln) | 2 ml |
| 3-10 Zoomlytes (40% stock soln) | 0.5 ml |
| n-octylthioglucoside | 0.5 g |
| DTT | 15.4 mg |
| 8 M Urea | 45.3 ml |
| QS to 50 ml with water | |

If the buffer is made ahead of time, do not add the DTT until ready to use.

2. The 5M Urea soluble material will be used for purification of the 38 and 19kDa proteins by preparative electro-elution.

3. If fractions need to be stored for any amount of time, they can be stored at 4°C. This will, however, cause the urea to crash out of solution. If this happens, sonicate the samples with heat to get the urea back into solution.

4. Dialysis Buffer:

| | |
|------------------------------------|--------|
| 10mM ammonium bicarbonate | 5.53 g |
| 0.02% sodium azide | 1.4 g |
| 1mM DTT | 1.07 g |
| QS to 7L with endotoxin-free water | |

5. Before using the Waters HPLC, read SOP SP025 or ask lab personnel trained in the use of the HPLC how to use the equipment.