SOP: PP023.6
Modified 2/27/2017 by MCL

Preparation of Purified 16kDa (HspX)

Materials and Reagents:

1. 100g γ-irradiated *M. tuberculosis* whole cells
2. PBS (pH 7.4)
3. N-octylthioglucoside
4. 8M urea
5. High speed centrifuge with appropriate rotors
6. Centrifuge tubes
7. Dialysis tubing (6,000-8,000 MWCO)
8. Ammonium Bicarbonate (ambic)
9. DTT
10. Endotoxin-free (ET-free) water
11. BCA kit
12. Lyophilizer and lyophilizer flasks
13. 3-10 pharmalytes (Invitrogen®)
14. 4-7 pharmalytes (Invitrogen®)
15. Rotofor® cell
16. Anode and Cathode membranes
17. Power-pack
18. 4-12% Bis-Tris protein gels
19. Blotting supplies for Western blots
20. Sephadex-75 column (HPLC)
21. HPLC
22. 10 ml and 25 ml syringes
23. Injection needle for HPLC
24. Fraction collector
25. 5 ml tubes polypropylene tubes
26. 225 ml, 50ml and 15ml falcon tubes

Procedure:

1._____ Break cells as described in SOP: SP027.

2._____ Add an equal vol (~ 150 ml) of PBS-EDTA buffer to the lysate and mix.

3._____ Centrifuge the lysate 3,000 rpm, 4°C in the table top centrifuge for 15 minutes.

4._____ Collect the supernatant

5._____ Centrifuge the supernatant at 40,000 x g, 4°C for 1 hour

6._____ Reserve the supernatant in a clean 225 ml falcon tube. (Keep at -20°C until final protein has been QC'd)

7._____ Resuspend and combine the pellets in 100-150 ml of PBS containing 1% N-octylthioglucoside

8._____ Stir at room temperature until pellets are completely in solution, usually 2-4 hours. This may also go over night

9._____ Centrifuge at 27,000 x g, 25°C for 1 hour

10._____ Reserve the supernatant and dialyze against 10mM ambic, with at least 3 exchanges at 4-12 hours per exchange (note 4).

11._____ Perform a BCA (SOP: SP003) assay and lyophilize in ~ 50 mg aliquots for the rotofor.

12._____ Incubate the anode and cathode membranes in 50 ml of the appropriate buffers overnight.

13._____ At the same time, resuspend one of the aliquots in 50 ml of rotofor buffer. Stir overnight at room temp. If it doesn't go into solution overnight, it may be necessary to sonicate with light heat

14._____ Assemble the rotofor following the manufacturer's instructions. Be sure to put the membranes and their buffers on the correct side (note 3)

15._____ Fill the rotofor with water and run at 5W for 20 minutes to be sure it's not leaking

16._____ Remove the water and add the entire 50 ml of sample to the rotofor. Run at 6W for 12 minutes, then run at 12W, taking note of the volts every 15 minutes. The run will be finished when the volts begin to stabilize, about 4 hours (note 5).

17._____ Once finished, harvest the fractions by vacuum pump and store at 4°C (note 6).

18._____ Run 10 µl of each fraction on a gel and silver stain (SOP: SP012). A CS-49 western blot (SOP: SP011) can be performed to help decide which fractions to pool.

19._____ Pool the cleanest fractions containing 16kDa; pool the rest of the fractions containing 16kDa. (note 7)

20._____ Prepare 6000-8000 MWCO membranes by soaking in ET-free water and add each pool to a separate tube.

21._____ Dialyze against dialysis buffer at room temp to remove all of the urea for at least 4 hours. Exchange the buffer twice more at 4°C for 4-16 hours each (note 8).

22._____ Remove from dialysis and perform a BCA. Run 2 µg on a silver stained gel and CS-49 western blot to verify.

23._____ Lyophilize the sample

24._____ Hook up the sephadex-75 column to the HPLC (note 9)

25._____ Prime line B with water and line A with SE buffer.

26._____ Wash the column with 200 ml of HPLC-grade ET-free water starting at 0.5 ml/min, then gradually increasing to 1.5 ml/min

27._____ Equilibrate the column in 200 ml of SE buffer

28._____ Resuspend the sample in no more than 6 ml of SE buffer, but at a concentration that's no more than 15 mg/ml.

29._____ Sonicate with heat to get as much into solution as possible, then centrifuge at 3,000 RPM for 10 minutes to remove any insoluble material.

30._____ Filter the supernatant through a 0.2 µm filter.

31._____ Open Empower and chose the "16kDa clean-up" program (note 10)

32._____ Draw up the protein in a syringe with an injection needle and get rid of all bubbles.

33._____ Move the lever on the HPLC to "load." Click "inject" on the computer and inject the sample.

34._____ Collect the fractions as noted and store at 4°C once finished

35._____ Run 10 µl of each fraction on gel and silver stain.

36._____ Pool the fractions containing clean 16kDa. If they don't look clean, pool them and run the pool over the sephadex again.

37._____ Dialyze the clean fractions against 10mM ambic at room temp for the first exchange to prevent urea from precipitating; then place a 4°C and exchange at least 3 times with 4-16 hours per exchange.

38._____ Perform a BCA assay to determine protein concentration. If it falls off the curve, lyophilize the sample and resuspend in a small volume (~1 ml) of 10 mM ambic.

39._____ Run 1 µg on a gel and silver stain and perform a CS-49 western blot for QC

40._____ Make 100 µg aliquots, lyophilize, and store at -80°C

Notes:

1. To make 50ml (one aliquot) of buffer for rotofor:

1.6%	4-7 pharmalytes:	2 ml
0.4%	3-10 pharmalytes	0.5 ml
1%	N-octylthioglucoside	0.5 g
2mM	DTT	15.4 mg
7.25M	8M deionized urea	45.3 ml

   QS to 50 ml with ET-free water
2. To make 1 liter of SE buffer:

3M	8M deionized urea	375 ml
20mM	Tris-Hcl	3.152 g
0.15M	NaCl	8.76 g
0.1%	N-octylthioglucoside	1 g

   pH = 7.6
   QS to 1 liter with ET-free water and filter through a 0.45 µm filter
3. Anode buffer: 0.1M H_3PO_4
   Cation membrane goes in Anode buffer
   Cathode buffer: 0.1M NaOH
   Anion membrane goes in Cathode buffer
   Follow instructions on membrane bags- red label=red dot on machine, black label= black dot on machine
4. The pellets from this spin can be saved for 19kDa and 38kDa preps by electro-elution. Resuspend the pellets in 100 ml of 5M urea and stir at room temp for at least 1 hour until completely in solution. Centrifuge at 27,000 x g, 25°C, 1 hr. Reserve the supernatant and dialyze into 10mM ammonium bicarbonate and lyophilize until ready to use.
5. The volts will be going up as time goes on. Every prep will have different voltages and total time to run. The volts should stabilize for at least 30 minutes
6. The urea will crash out of solution, but once placed at room temp for dialysis, it should go back into solution.
7. The fractions will not be clean on a gel, but look for the most abundant 16kDa band. Continue forward with the clean pool. The dirty pool will need to go over the rotofor again. Proceed with dialysis (steps 20-23), then repeat rotofor (starting at step 12).
8. The urea must be "step-down dialyzed" out of the solution, and the first exchange should include NaCl in order to help remove pharmalytes. Urea dialysis buffers are as follows:
   7 liter each in ET-free water
      Dialysis #1: 4 M Urea, 1 M NaCl, 10 mM ammonium bicarbonate
      Dialysis #2: 2 M Urea, 10 mM ammonium bicarbonate
      Dialysis #3: 10 mM ammonium bicarbonate
9. 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. Be sure to use the column specified for 16kDa clean-up only.
10. The program should be run as follows at 1.5 ml/min in isocratic mode:
    20 min wash
    60 min run (collect 30 x 2 min fractions)
    20 min wash
    Collect the pre- and post-column washes in 225 ml falcon tubes