SOP: PP031.2
Modified 7/1/15 by MCL

Purification of 19kDa

Materials and Reagents:

1. Cell Wall from *M. tuberculosis* (see SOP: PP008)
2. 2:1 Chloroform:Methanol
3. 10:10:3 Chloroform:Methanol:Water
4. PBS (Gibco, 10010)
5. 32% Triton (see SOP: R001)
6. -20°C Cold Acetone
7. Ambic: 10mM ammonium bicarbonate
8. 5X Sample Buffer (see SOP: SP007)
9. 2D gels: NuPAGE 12% Bis-Tris gel, 1.0mm x 2D well (Invitrogen, NP0346BOX)
10. 20X MOPS SDS Running Buffer (Invitrogen, NP0001)
11. Tris-Caps Buffer: 60mM Tris, 40mM Caps, pH 9.4
12. Lyophilizer flask
13. 50ml Teflon oakridge tubes
14. Foil
15. Glass rod
16. 250ml Polypropylene centrifuge bottle
17. BCA supplies
18. Invitrogen gel runner
19. Power supply
20. Mini whole gel eluter (Bio-Rad, 165-1256)
21. Amicon ultra-4, 10000 MWCO
22. IT-19 Antibody
23. Rocker
24. Vortexer
25. Sorvall centrifuge
26. Air bath
27. 4°C walk-in cold room
28. 37°C water bath
29. -20°C Freezer
30. Chemical fume hood
31. Clinical centrifuge
32. Vacuum Pump
33. Upper filter paper (Bio-Rad, 165-1282)
34. Lower filter paper (Bio-Rad, 165-1283)
35. Sealing tabs (Bio-Rad, 165-1278)
36. Cellophane membrane (Bio-Rad, 165-1276)
37. Roller (Bio-Rad, 165-1279)
38. 1.5ml capless tubes (Bio- Rad, 223-9500 or VWR 89004-290) (note 6)
39. Glass pipet
40. PBS Saturated phenol (keep at 4°C)
41. 30 ml Teflon oakridge centrifuge tubes

Protocol:

1._____ Lyophilize cell wall fraction (see SOP: SP004).

2._____ Distribute dried cell wall into teflon oakridge centrifuge tubes, approximately 1.5g per tube, based on dry weight.

3._____ Add 30 ml 2:1 to each tube.

4._____ Incubate at room temperature, with rocking, for 2 hours. Vortex every 30 min.

5._____ Centrifuge at 27000xg, 15°C, for 1 hr.

6._____ Decant supernatant into a waste bottle (note 1).

7._____ Add 30 ml 2:1 to each tube (enough to fill the tube).

8._____ Repeat incubation, centrifugation, and removal of supernatant as in steps 4-6.

9._____ Cover the tubes with foil and place on the nitrogen bath overnight (note 2).

10._____ Break up the dried pellet with a glass rod.

11._____ Add 30 ml 10:10:3 to each tube.

12._____ Repeat incubation, centrifugation, and removal of supernatant as in steps 4-6.

13._____ Add 30 ml 10:10:3 to each tube and repeat incubation, centrifugation, and removal of supernatant as in steps 4-6.

14._____ Cover tubes with foil and place on the air bath overnight.

15._____ Break up the pellet with a glass rod and place back on the air bath to remove any residual 10:10:3.

16._____ Once completely dry, crush the pellet as much as possible with a glass rod.

17._____ Add 26.25 ml PBS to each tube.

18._____ Stir at room temperature overnight.

19._____ Add 3.75 ml 32% triton X-114 to give a total concentration of 4%.

20._____ Rock at 4°C for at least 2 hr.

21._____ Incubate in 37°C water bath until a biphase forms.

22._____ Warm the centrifuge and rotor to 25°C.

23._____ Centrifuge at 27000xg, 25°C, 30 min.

24._____ Carefully remove the top aqueous layer with a pipet.

25._____ Fill the tubes to 30 ml with PBS to return the triton concentration to 4%.

26._____ Disrupt the pellet in each tube.

27._____ Repeat extraction from step 20-24 (note 3).

28._____ Transfer all triton layers to a 250 ml centrifuge bottle

29._____ Wash the pellets gently with PBS to remove all the triton and add to the triton already collected.

30._____ Add enough cold acetone to the pooled triton layers to fill the centrifuge bottle.

31._____ Incubate at -20°C overnight.

32._____ Centrifuge at 27000xg, 4°C, for 30 min.

33._____ Decant the supernatant and discard as hazardous waste.

34._____ Fill the bottle with cold acetone and dislodge the pellet from the side of the bottle.

35._____ Repeat steps 32-33 (note 4).

36._____ Leave the centrifuge bottle open in the fume hood to dry.

37._____ Resuspend each pellet in 30 ml of PBS. It may be necessary to gently scape the pellet from the side of the centrifuge bottle and to slowly stir on a stir plate. The sample will not go completely into solution.

38._____ Split each 30 ml sample between two 50 ml Teflon oakridge centrifuge tubes.

39._____ Add 15 ml of PBS saturated phenol to each tube and rock at room temperature for 4 hours (note 5).

40._____ Centrifuge at 27,000 xg, 25°C, for 1 hour.

41._____ Remove aqueous (upper) layer without disturbing the interface. Note volume of aqueous layers removed.

42._____ To the phenol layer add a volume of PBS equal to that removed.

43._____ Rock at room temperature for 4 hours, then centrifuge and remove aqueous layers as in steps 40-41.

44._____ Transfer final phenol phase + interface to dialysis tubing. Do not fill tubing more than half full.

45._____ Place in dialysis tank, and dialyze 48-72 hours against running DI water. Occasionally gently knead the tubing (make certain to wear gloves!) to help break up larger chunks of material (note 6).

46._____ Transfer dialysis tubing to MilliQ water, and dialyze at 4°C for 24 hours.

47._____ Recover sample from dialysis tubing by pipetting into a clean sterile plastic container. Rinse the dialysis tubing with MilliQ water to recover particulate material from the dialysis tubing.

48._____ Make a homogeneous suspension of the material by breaking apart large aggregates using a bath sonicator and/or manual breaking using a cell scraper.

49._____ Estimate protein concentration by BCA (see SOP SP003).

50._____ Make 1.5 mg aliquots (note 7).

51._____ Concentrate one of the 1.5 mg aliquots to less than 300 µl on the savant (see SOP: SP005).

52._____ Add 80 µl 5X Sample Buffer and bring the total volume up to 400 µl with water.

53._____ Boil the sample for 5 min.

54._____ Make 700 ml 1X MOPS Buffer.

55._____ Load the sample onto a 2D gel and run at 200V for 50 min. (note 8)

56._____ Soak gel, cellophane and filter paper in Tris-Caps Buffer for 15min, and set up the mini whole gel eluter as described in the eluter manual using Tris-Caps Buffer. (note 9)

57._____ Run the eluter at 100 mA for 30 min.

58._____ Reverse polarity for 15 sec.

59._____ Harvest fractions from the eluter. (note 10)

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

61._____ Pool all fractions containing clean 19kDa.

62._____ Concentrate the pool using amicon ultra-4. (Optional)

63._____ Wash three times with ambic. (Optional)

64._____ Estimate protein concentration by BCA (note 11).

65._____ Run 1 and 2 μg on a gel and blot (developed with IT-19) to confirm purity.

Notes:

1. The supernatant can be saved for purification of other products. If the supernatant is not going to be used for other purposes, dispose of it as hazardous waste.
2. It will be necessary to stab the needle of the air bath through the foil. This prevents any sample from blowing out of the tube once it starts to dry.
3. The 4°C incubation can go overnight for this step.
4. The pellet may be quite loose after the second centrifugation, so be sure to decant carefully.
5. Use caution while working with phenol. Always handle phenol in a chemical fume hood, wearing proper PPE, and only use glass pipets. Phenol is not compatible with all centrifuge tubes (the recommendation here is Teflon). Check chemical compatibilities of all materials before use.
6. Two acetone precipitations have been used as an alternative to the dialysis step.
7. Each aliquot will constitute one run of the mini whole gel eluter. Freeze back any aliquots that you are not ready to use yet.
8. If the whole sample doesn't fit in well, run the gel on 200V for ~1min, then add the rest of sample.
9. Eluter Manual can be found on Bio-Rad website, document 4006086 or 4006106. An alternative buffer system that works well for 19kDa is 0.1M ammonium bicarbonate run at 100 mA for 1 hour. This buffer system allows for direct use and/or lyophilization of the protein without buffer exchange.
10. The eluter harvester does NOT hold capped Eppendorf tubes.
11. Be sure to use appropriate protein standards, either 10 mM ammonium bicarbonate, or if optional steps 62-63 are omitted, Tris-Caps or 0.1M ammonium bicarbonate depending on the buffer system used for elution.