

**SOP: PP031****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
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

**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 air 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 biphasic 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 the pellet in ambic.
38. \_\_\_\_\_ Estimate protein concentration by BCA (see SOP: SP0003).
39. \_\_\_\_\_ Make 1.5 mg aliquots (note 5).
40. \_\_\_\_\_ Concentrate one of the 1.5 mg aliquots to less than 300  $\mu$ l on the savant (see SOP: SP005).
41. \_\_\_\_\_ Add 80  $\mu$ l 5X Sample Buffer and bring the total volume up to 400  $\mu$ l with water.
42. \_\_\_\_\_ Boil the sample for 5 min.
43. \_\_\_\_\_ Make 700 ml 1X MOPS Buffer.
44. \_\_\_\_\_ Load the sample onto a 2D gel and run at 200V for 50 min.
45. \_\_\_\_\_ Set up the mini whole gel eluter as described in the eluter manual using Tris-Caps Buffer.
46. \_\_\_\_\_ Run the eluter at 100mA for 30 min.
47. \_\_\_\_\_ Reverse polarity for 15 sec.
48. \_\_\_\_\_ Harvest fractions from the eluter.
49. \_\_\_\_\_ Run 10  $\mu$ l of each fraction on a gel.
50. \_\_\_\_\_ Pool all fractions containing clean 19kDa.
51. \_\_\_\_\_ Concentrate the pool using amicon ultra-4.
52. \_\_\_\_\_ Wash three times with ambic.
53. \_\_\_\_\_ Estimate protein concentration by BCA.
54. \_\_\_\_\_ Run 1 and 2  $\mu$ g 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. Each aliquot will constitute one run of the mini whole gel eluter. Freeze back any aliquots that you are not ready to use yet.