SOP: PP051

Subcellular Fraction of *M. leprae*

Materials and Reagents

1. ~200 mg *M. leprae* whole cells (see SOP: PP050)
2. 50 ml Oakridge centrifuge tubes
3. Centrifuge
4. Sonicator
5. Ice and Ice bucket
6. DNase
7. Rnase
8. Timer
9. PBS
10. Protease Inhibitor (Roche #04693132001)
11. Centrifuge tubes
12. Ultra Centrifuge (75,000 xg capabilities)
13. 10% SDS
14. Glass slide
15. Microscope
16. Acid fast stain reagents
17. Balance
18. 15 ml falcon tube
19. Cryovials
20. Glass homogenizer and glass rod
21. BCA reagents
22. Plate reader
23. Microscope
24. Small spatula
25. 10, 20, 200, 1000 µl tips & pipets

Protocol:

Whole Cell Sonicate (Day 1)

1._____ Transfer ~200 mg of *M. leprae* cells to one 50 ml centrifuge tubes and centrifuge for 10,000 xg for 10 min at 4°C.

2._____ Decant supernatant and resuspend pellet in 5 ml PBS w/ Protease Inhibitor. Place centrifuge tube in a beaker of ice before placing in the sonicator.

3._____ Lyse cells on ice with a sonicator on a setting of 60sec ON/60sec OFF/5 cycles. Do ~30 rounds (of 5 cycles each) or until ~95% cell breakage.

4._____ Make a slide and perform an acid fact stain to confirm cell breakage of >95%. Continue sonicating if whole cells are still visible (note 1).

5._____ If whole cell sonicate is the desired final product, proceed to QC analysis. If not, store the sonicate at 4°C overnight and continue with separation.

Separation of Cell wall from Cytosol and Membrane (Day 2)

1._____ Add 10 µg DNase and RNase each to the tube. Incubate on ice for 60 min, inverting occasionally to mix.

2._____ Centrifuge at 27,000 xg for 30min at 4°C.

3._____ Decant supernatant into a sterile 15 ml falcon tube and place on ice. Supernant contains the *M. leprae* cytosol (MLSA) and *M. leprae* membrane (MLMA).

4._____ Resuspend pellet with 2 ml PBS with Protease Inhibitor and sonicate for an additional 10 cycles.

5._____ Repeat centrifuge at 27,000 xg for 30 min at 4°C.

6._____ Add the supernatant to the tube with the supernatant from Step 3. The pellet contains *M. leprae* cell wall (MLCwA) and *M. leprae* mAGP (note 2).

Separation of MLSA and MLMA (Day 3)

1._____ Divide the pooled supernatant evenly into ultracentrifuge tubes and balance to within 10 mg.

2._____ Centrifuge at 75,000 xg for 2 hr at 4°C.

3._____ Pour all the supernatants into one 15 ml falcon tube labeled MLSA.

4._____ Scrape the pellets with a small spatula and pool into a small glass homogenizer.

5._____ Homogenize the pooled pellets with a glass rod adding 1-2 ml PBS.

6._____ Redistribute the homogenate into the same centrifuge tubes and repeat centrifugation as in Step 2.

7._____ Pool the supernatants into the 15 ml falcon tube labeled MLSA in Step 3. This is the *M. leprae* cytosol (MLSA).

8._____ Pool together the pellet in a cryovial and resuspend it in 1-2 ml of PBS. This is the *M. leprae* membrane (MLMA)

Separation of *M. leprae* soluble cell wall (MLCwA) and *M. leprae* mAGP (Day 3 cont.)

1._____ While the MLSA and MLMA are centrifuging, add PBS to the pellet from Day 2 to bring the total volume to 5 ml and add 1 ml 10% SDS, for a final concentration of 2% SDS. Vortex to mix (the SDS will cause a lot of bubbling, that's okay).

2._____ Place in a 56°C water bath for 1 hr. Then centrifuge at 27,000 xg for 30 min at 4°C.

3._____ Decant the supernatant into a sterile 15 ml falcon tube labeled MLCwA and place on ice.

4._____ Resuspend the pellet with 2 ml PBS and 400 µl 10% SDS. Vortex to mix and place back in 56°C water bath for another hour.

5._____ Centrifuge at 27,000 xg for 30min at 4°C.

6._____ Decant the supernatant into the same tube in Step 3.

7._____ Repeat Steps 4-6 again.

8._____ Preform a SDS Removal Protocol (SP019) on the supernatant to remove residual SDS. This is the *M. leprae* MLCwA.

9._____ Resuspend pellet with ~35 ml cold 100% acetone (the pellet will not go into solution).

10._____ Place at -20°C for 8-16 hours.

11._____ Centrifuge at 27,000 xg for 30min at 4°C. Discard supernatant as hazardous waste.

12._____ Repeat steps 9-12 two more times. Dry pellet in the hood overnight or in a nitrogen bath. This is the *M. leprae* mAGP.

Quality Control Analysis

1._____ Obtain a picture of a stained slide of WCS to include on the QC sheet.

2._____ Perform a BCA on WCS, MLSA, MLMA, and MLCwA, using 1:10, 1:20 and 1:50 dilutions.

3._____ Based on the BCA run a gel with 2 µg for silver stain (see SOP: SP007 and SP012) and 4 µg for western blot (see SOP: SP011). The following chart shows for proper antibody for each product and recombinant protein to be used as positive control:

Product	1° Ab	Dilution	Rec protein
MLSA	CS-01	1:20	GroES
MLMA	CS-38, CS-41	1:20, 1:10	MMP-I, Bacterioferin
MLCwA	CS-44	1:20	N/A

4._____ Aliquot products into 0.25 mg/vial, freeze and lyophilize. Store in -80°C.

5._____ For mAGP, perform a GC Analysis using Preparation of Alditol Acetate Derivatives (see SOP: SPO22) for purity and quantification.

6._____ Aliquot into 2 mg/vial.

Notes:

1. If >95% breakage is not achieved by the end of the day, tube can be placed in 4°C overnight and continue with sonication the next morning.
2. This is a good stopping point for the day cover the pellet with 2 ml PBS and store in 4°C along with the supernatant.