

SOP: PP008.1
Modified 6-20-06

Subcellular Fractionation Protocol

Materials and Reagents:

1. 100g γ -irradiated *M. tuberculosis* whole cells
2. PBS (pH 7.4) with 1mM EDTA
3. Complete, EDTA-free protease inhibitor tablets (Roche, 11 873 580 001)
4. DNase: 1 mg/ml stock (stored at -20°C)
5. RNase: 1 mg/ml stock (stored at -20°C)
6. Ammonium bicarbonate
7. Ice
8. Ice bucket
9. Sterile 50 ml conical tubes
10. Sterile 40 ml Oak Ridge centrifuge tube (polypropylene)
11. 40 ml ultra centrifuge tubes
12. High speed centrifuge
13. Ultra centrifuge
14. SS-34 Rotor
15. SW-28 Rotor
16. Vortexer
17. 50 ml plastic disposable pipets
18. 10 ml plastic disposable pipets
19. Dialysis tank
20. Dialysis tubing (3,500 MW cut off)
21. Mettler-Toledo electronic balance
22. Table top centrifuge

Protocol:

1. ____ Thaw 100 g of γ -irradiated *M. tuberculosis* cells. Thawing of the bacterial pellet should be performed at 4°C (note 1).
2. ____ Make breaking buffer by adding one Complete, EDTA-free tablet to 50 ml of PBS-EDTA buffer (notes 2 and note 3).
3. ____ Add 50 ml of breaking buffer to the thawed bacterial cells.
4. ____ Create a homogeneous suspension of bacterial cells by vortexing for 30 sec and place on ice.
5. ____ Place 40 to 45 ml of cell suspension in French Press cell (note 4).
6. ____ Place French Press cell in French Press, collect lysate as it is forced out of the cell with a constant pressure of 1000 as measured by the gauge on the French Press.
7. ____ Place the lysate on ice.
8. ____ Repeat Steps 5 to 7 until all of the cell suspension has passed through the French Press cell.
9. ____ Repeat steps 5-8 five more times. Thus the total volume of cells should go through the French Press cell 6 times (note 5).
10. ____ Add an equal volume of breaking buffer (approximately 150 ml) to the cell lysate, and mix by stirring with a plastic pipet.
11. ____ Centrifuge the cell suspension at 3,000 x g (3000 rpm using table top centrifuge) for 5 min, 4°C to remove unbroken cells.

12. ____ Decant the supernatant into clean 40 ml polypropylene centrifuge tubes. Discard cell pellet.
13. ____ Centrifuge the 3,000 x g supernatant at 27,000 x g for 1 hour, 4°C.
14. ____ Decant the supernatant (cytosol and membrane) into clean 40 ml polypropylene centrifuge tubes.
15. ____ Suspend each pellet (cell wall) in breaking buffer without DNase or RNase (30 ml) and form a homogeneous suspension by vortexing.
16. ____ Centrifuge the cytosol and membrane supernatant and the resuspended cell wall pellet as in step 13 for 20 min to remove residual contaminating material.
17. ____ Decant and discard the supernatant from the cell wall pellet and save the pellet back for QC. Collect the cytosol and membrane supernatant in 40 ml ultra centrifuge tubes and discard the pellet.
18. ____ Balance the ultra centrifuge tubes very precisely (note 6).
19. ____ Centrifuge the supernatant at 100,000 x g (27,000 rpm using the SW28 rotor) for 4 hr, 4°C.
20. ____ Collect the supernatant in clean 40 ml ultra centrifuge tubes and repeat step 19. Save all pellets, as these are the membrane fraction.
21. ____ Collect the final 100,000 x g supernatant in 50 ml conical tubes.
22. ____ Gently wash the 100,000 x g pellets (membrane) with breaking buffer without DNase or RNase. (note 7)
23. ____ Suspend the washed 100,000 x g pellets in 10 mM ammonium bicarbonate and pool together (approximately 80 ml total).
24. ____ Suspend each 27,000 x g cell wall pellet from step 17 in 10 mM ammonium bicarbonate (approximately 20 ml each) and pool together.
25. ____ Place the cell wall, membrane, and cytosol preparations in 3500 MWCO dialysis tubing and dialyze against 10 mM ammonium bicarbonate for 24 hr at 4°C with three changes of buffer.
26. ____ After dialysis, collect the fractions in 50 ml conical tubes.
27. ____ Estimate the protein concentrations by BCA (note 8).
28. ____ Remove a small aliquot (100 µl) of each preparation for QC.
29. ____ Run 4 µg of each fraction on a gel and silver stain (note 9).
30. ____ Aliquot fractions (default quantity is 1 mg) and store at -80°C

Notes:

1. This fractionation scheme deals with highly labile proteins and proteases that are found in differing fractions of *Mycobacterium tuberculosis* strains. It is important to keep the samples cool at all times, storing them for short periods of time at 4°C (or on ice) and for long periods of time at -80°C.
2. The DNase and RNase need only be kept in the breaking buffer until the 27,000xg centrifugation (step 13) where the cell wall is fractionated from the cell membrane/cytosol fraction. After this point, use buffer without DNase and RNase.
3. It is important to keep the buffer cool (in a refrigerator or on ice) after the addition of the protease

inhibitor tablet, DNase, and RNase.

4. See SOP: SP027 for use of the french press.

5. At this point the efficiency of cell lysis should be checked by acid fast staining and microscopy (see SOP SP035). At least 90% of the cells should be lysed.

6. Centrifuge tubes must be completely full and must be balanced to within 100 mg using the Mettler-Toledo electronic balance.

7. If the pellets are disrupted during washing, the centrifuge tubes must be filled with breaking buffer and centrifuged as in step 19.

8. The cell wall will not be completely in solution, so be sure that it is a uniform suspension before performing the BCA and all other QC procedures.

9. See SOP SP007 for running gels and SP012 for silver staining.

References:

Hirschfield, G. R., M. McNeil, and P. J. Brennan. 1990. Peptidoglycan-associated polypeptides of *Mycobacterium tuberculosis*. *J Bacteriol* 172:1005-13.

Lee, B. Y., S. A. Hefta, and P. J. Brennan. 1992. Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis*. *Infect Immun* 60:2066-74.