SOP: PP008.4
Modified 02-27-17 by KE

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 -20ºC)
5. RNase: 1 mg/ml stock (stored at -20ºC)
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. 32TI 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 4ºC (note 1).

2._____ Make breaking buffer by adding 1 stock of 30 µl DNase, 1 stock of 30 µl RNase, and one Complete, EDTA-free tablet to every 50 ml of PBS-EDTA buffer (notes 2).

3._____ Add 1 ml of breaking buffer to every 2 g of thawed bacterial cells. (50 ml of breaking buffer to the 100 g 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 3).

6._____ Place French Press cell in French Press, collect lysate as it is forced out of the cell with a constant pressure of 1000 PSI 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 4).

10._____ Add an equal volume of breaking buffer (approximately 150 ml) with Complete, EDTA-free tablet, DNAse, and RNAse 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 15 min, 4ºC to

remove unbroken cells.

12._____ Decant the supernatant into clean 40 ml polypropylene centrifuge tubes. Discard cell pellet (note 5).

13._____ Centrifuge the 3,000 x g supernatant (whole cell lysate) at 27,000 x g for 1 hour, 4ºC.

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 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 32TI rotor) for 4 hr, 4ºC.

20._____ Collect the supernatant in clean 40 ml ultra centrifuge tubes. Save all pellets, as these are the membrane fraction.

21._____ Gently wash the 100,000 x g pellets (membrane) with breaking buffer without DNase or RNase. (note 7)

22._____ Repeat ultracentrifugation as in step 19.

23._____ Collect the final 100,000 x g supernatant (cytosol) in 50 ml conical tubes. Save and wash membrane pellets.

24._____ Suspend the washed 100,000 x g pellets in 10 mM ammonium bicarbonate and pool together (approximately 80 ml total).

25._____ Suspend each 27,000 x g cell wall pellet from step 17 in 10 mM ammonium bicarbonate (approximately 20 ml each) and pool together.

26._____ Place the cell wall, membrane, and cytosol preparations in 3500 MWCO dialysis tubing (allow tubing to sit in water for 30 minutes before adding sample) and dialyze against 10 mM ammonium bicarbonate for 24 hr at 4ºC with three changes of buffer.

27._____ After dialysis, collect the fractions in 50 ml conical tubes.

28._____ Remove a small aliquot (100 µl) of each preparation for QC.

29._____ Estimate the protein concentrations by BCA (note 8).

30._____ Run 4 µg of each fraction on a gel and silver stain (note 9).

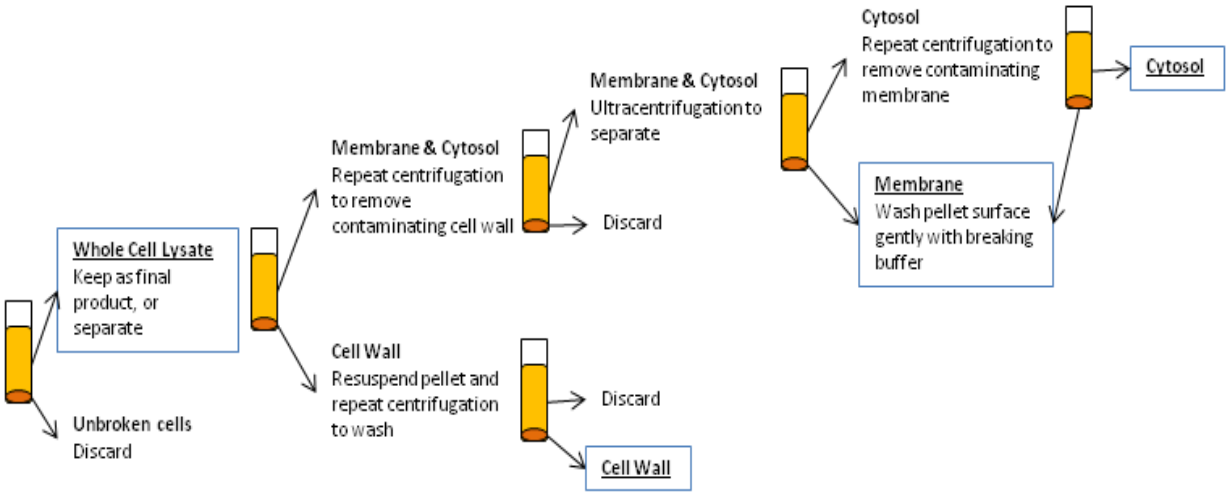
31._____ Aliquot fractions (default quantity is 1 mg) and store at -80ºC

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. 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. 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. See SOP: SP027 for use of the french press.
4. 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.
5. The supernatant is whole cell lysate. If this is one of the desired products, some or all of the lysate can be reserved and completed as described in SOP PP007.
6. Centrifuge tubes must be completely full and must be balanced to within 20 mg using the Mettler-Toledo electronic balance.
7. It is important to wash the membrane pellets immediately, as they are much more likely to be disrupted after storage. 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. For BCA use SP003.
9. See SOP SP007 for running gels and SP012 for silver staining.



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