Subcellular Fraction Production Manual

Version 1
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SOP: PP005

Preparation of CFPs and Low MW CFPs

Materials and Reagents:

1. Harvested CFP from *M. tuberculosis* (see SOP PP003)
2. 90% Isopropyl alcohol
3. 70% Ethanol
4. Dialysis buffer (10 mM Ammonium bicarbonate, 1 mM DTT, 0.02% NaN_3)
5. 10 mM ammonium bicarbonate
6. Endotoxin-free MilliQ water
7. 350 ml Amicon ultrafiltration unit
8. 10,000 MWCO membrane (Millipore catalog number PLGC07610)
   or YM3 membrane (Millipore catalog number 13442AM) (note 1)
9. Amicon tubing (2)
10. Amicon ultrafiltration unit housing
11. 10 liter Amicon reservoir
12. Compressed N_2 cylinder
13. Magnetic stir plate
14. Dialysis tank
15. Dialysis tubing (3,500 MWCO)
16. ZapCaps (0.2 μm)
17. 25 ml Pipettes
18. 225 ml conical tube
19. 150 ml plastic container

Protocol: (note 2)

1. _____ Obtain a clean 10 liter amicon reservoir, cover all openings with aluminum foil, and autoclave on a fast exhaust cycle.

2. _____ Obtain a clean 350 ml amicon ultrafiltration unit, and tubing for connection to the amicon reservoir.

3. _____ Rinse all tubing and components of the amicon ultrafiltration unit with 70% ethanol, and allow to air dry.

4. _____ Equilibrate the 10k MWCO (or YM3) amicon membrane, with the shiny side down, in 90% isopropanol for 10 minutes, then in MilliQ water for 30 minutes.

5. _____ Assemble the amicon ultrafiltration unit with the 10k MWCO (or YM3) amicon membrane (note 3).

6. _____ Fill the 10 liter amicon reservoir with the *M. tuberculosis* CFP, securely replace the lid to the reservoir, and connect the nitrogen line to the input port.

7. _____ Connect one end of amicon tubing to the output port of the amicon reservoir and the other end to the input port on the top (lid) of the amicon ultrafiltration unit.

8. _____ Place the amicon ultrafiltration unit in the housing and close the vent on the back of the lid.

9. _____ Place the amicon ultrafiltration unit and housing on a magnetic stir plate and turn the stir plate on.

10. _____ Ensure the tubing connected to the output port on the base of the amicon ultrafiltration unit is placed in a receptacle to collect the ultrafiltrate.

11. _____ Turn on the nitrogen gas (note 4).

12. _____ Check the amicon ultrafiltration unit to ensure the stirrer is turning, and that there is no leakage around the lid, base, or tubing connections. Also check to see that the CFP ultrafiltrate is slowly flowing from the outlet port at the base of the ultrafiltration unit (note 5).

13. _____ Check the amicon ultrafiltration unit and reservoir daily and discard ultrafiltrate as needed (note 6).

14. _____ When the CFP in the reservoir becomes low or empty turn off the nitrogen and release pressure by opening the vent valve on the top of the reservoir. If required add more CFP of the same lot to the reservoir, close the vent valve and repeat steps 11 to 13. Repeat until all the CFP is concentrated.

15. _____ When the volume of CFP in the amicon ultrafiltration unit is reduced to ~50 ml turn off the nitrogen gas, and vent the system.

16. _____ Remove the amicon ultrafiltration unit from its housing, disconnect all tubing, and remove the lid of the ultrafiltration unit.

17. _____ Transfer the concentrated filtrate to a 225 ml conical bottle.

18. _____ Wash the membrane and ultrafiltration unit two times with ~ 10 ml of 10 mM ammonium bicarbonate and add the washes to the concentrated filtrate.

19. _____ Prepare 3,500 MWCO dialysis tubing by boiling in endotoxin-free MilliQ H_20.

20. _____ Prepare 7 L of dialysis buffer in a dialysis tank.

21. _____ Add the concentrated CFP to the dialysis tubing. Close the dialysis tubing and place in the dialysis buffer.

22. _____ Dialyze at 4^{o}C for 4 to 12 hours.

23. _____ Change the dialysis buffer (7 L) and dialyze at 4^{o}C for 4 to 12 hours.

24. _____ Change the dialysis buffer to 7 L of 10 mM ammonium bicarbonate and dialyze at 4^{o}C for 4 to 12 hours.

25. _____ Collect the protein solution from the dialysis tubing and rinse the dialysis tubing with a minimal volume (~5 ml) of fresh 10 mM ammonium bicarbonate.

26. _____ Filter sterlize the dialyzed CFP using a 0.2 μm ZapCap and collect in a sterile 150 ml plastic container.

27. _____ Estimate protein concentration using a BCA assay (see SOP SP003).

28. _____ Run 4-5 μg of protein on a QC gel (see SOP SP007) and blot (see SOP SP011). Develop the blot using the QC antibodies SA12, CS18, IT49, IT23, CS44, IT41, or for Low MW CFP, use α-CFP10, α-ESAT6, SA12, IT49, IT23.

29. _____ Aliquot the CFP (default aliquot = 1 mg) and store at -80°C, or lyophilize (see SOP SP004).

Notes:

1. For low MW CFP, use the YM3 membrane and only use 2-4L of crude CFP for the starting material.

2. Amicon ultrafiltration must be performed at 4°C.
3. It is important that all O-rings are properly seated to prevent leakage. Also the membrane should be placed in the base of the ultrafiltration unit so that the shiny side of the membrane is facing up.
3. Failure to properly connect and setup the amicon ultrafiltration unit will result in significant loss of CFP and the operator being sprayed once pressure is applied to the amicon unit.
4. The nitrogen pressure should read no greater than 60 psi on the regulator.
5. If a leak is found or the output flow is too great, shut off the flow of nitrogen, release pressure from the reservoir, and correct the problem. Then start at step 7 and repeat startup procedures.
6. It generally takes 10 to 14 days to concentrate one 21 liter batch of CFP. The reservoir will empty in about 2-3 days depending on the concentration of the CFP.

References:

Sonnenberg, M. G., and J. T. Belisle. 1997. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect Immun 65:4515-24.

SOP: PP007

Preparation of Whole Cell Lysate

Materials and Reagents:

1. 100 g γ-irradiated *M. tuberculosis* whole cells
2. PBS (pH 7.4) with 1 mM EDTA
3. Pepstatin: 3mg/ml stock in ethanol (stored at -20°C)
4. Leupeptin: 1 mg/ml stock in ethanol (stored at -20°C)
5. PMSF: 100mM stock in isopropanol (stored at -20°C)
6. DNase: 1 mg/ml stock (stored at -20ºC)
7. RNase: 1 mg/ml stock (stored at -20ºC)
8. Ice bucket
9. Ice
10. French Press
11. French Press cell
12. 225 ml conical falcon tube
13. Vortexer
14. Table top centrifuge
15. Plastic pipets (25 and 50 ml)
16. Graduated cylinders (100 and 250 ml)
17. Beaker (200 ml)

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 23.3 µl Pepstatin stock, 50 µl Leupeptin stock, 200 µl PMSF stock, 60 µl DNase stock and 60 µl RNase stock per 100 ml of PBS-EDTA buffer. (Note: the $t_{½}$ of PMSF in aqueous solution at pH 7 is 100 minutes. It may be necessary to add more PMSF before completion of the protocol.) (note 2)

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 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. _____ To the lysate add an equal vol (~ 150 ml) of PBS-EDTA buffer and mix.

11. _____ Centrifuge the lysate 3,000 rpm, 4°C in the table top centrifuge for 15 minutes.

12. _____ Collect the supernatant, this is the whole cell lysate.

13. _____ Estimate protein concentration by BCA (note 5).

14. _____ Remove a small aliquot (100 μl) for QC.

15. _____ Run 4 μg on a gel and silver stain (note 6).

16. _____ Aliquot (default quantity is 10 mg) and store the whole cell lysate at -80°C

Notes:

1. This protocol deals with highly labile proteins and proteases that are found in *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 inhibitors, 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 whole cell lysate will not be completely in solution, so be sure that it is a uniform suspension before performing the BCA and all other QC procedures.
6. See SOP SP007 for running gels and SP012 for silver staining.

SOP: PP008

Subcellular Fractionation Protocol

Materials and Reagents:

1. 100g γ-irradiated *M. tuberculosis* whole cells
2. PBS (pH 7.4) with 1mM EDTA
3. Pepstatin: 3mg/ml stock in ethanol (stored at -20°C)
4. Leupeptin: 1 mg/ml stock in ethanol (stored at -20°C)
5. PMSF: 100mM stock in isopropanol (stored at -20°C)
6. DNase: 1 mg/ml stock (stored at -20ºC)
7. RNase: 1 mg/ml stock (stored at -20ºC)
8. Ammonium bicarbonate
9. Ice
10. Ice bucket
11. Sterile 50 ml conical tubes
12. Sterile 40 ml Oak Ridge centrifuge tube (polypropylene)
13. 40 ml ultra centrifuge tubes
14. High speed centrifuge
15. Ultra centrifuge
16. SS-34 Rotor
17. SW-28 Rotor
18. Vortexer
19. 50 ml plastic disposable pipets
20. 10 ml plastic disposable pipets
21. Dialysis tank
22. Dialysis tubing (3,500 MW cut off)
23. Mettler-Toledo electronic balance
24. 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 23.3 µl Pepstatin stock, 50 µl Leupeptin stock, 200 µl PMSF stock, 60 µl DNase stock and 60 µl RNase stock per 100 ml of PBS-EDTA buffer (Note: the $t_{½}$ of PMSF in aqueous solution at pH=7 is 100 minutes. It may be necessary to add more PMSF before completion of the protocol) (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, 4ºC 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, 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 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, 4ºC.

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 4ºC 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 inhibitors, 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.

SOP: PP010

Extraction of TX-114 Proteins/Lipoprotein Pool Protocol

Materials and Reagents: (per 100 g of irradiated cells)

1. PBS (pH 7.4)
2. 32% Triton (note 1)
3. 100 g γ-irradiated *M. tuberculosis* cells
4. Pepstatin 11.7 µl of a 3 mg/ml stock in ethanol (-20^{o} C)
5. Leupeptin 25 µl of a 1 mg/ml stock in ethanol (-20^{o} C)
6. PMSF 100 µl of a 100 mM stock in isopropanol (-20^{o} C)
7. DNase 60 µl of a 1 mg/ml stock (-20^{o} C)
8. RNase 60 µl of a 1 mg/ml stock (-20^{o} C)
9. Ice-cold acetone (-20^{o}C)
10. PBS saturated phenol (keep at 4^{o} C)
11. Sorvall GSA rotor
12. Eight 250 ml centrifuge bottles
13. Ice bucket with ice
14. Plastic pipettes (25 ml and 50 ml)
15. Glass pipettes
16. Sorvall SS34 rotor
17. Four 35 ml centrifuge tubes (Teflon)
16. Dialysis tubing (3,500 Da MWCO)
17. Dialysis tank
18. Graduated cylinders (100 ml and 250 ml)
19. Sorvall centrifuge
20. Table top centrifuge

Protocol:

1. _____ Dilute 32% Triton X-114 (TX-114) solution to 4% using PBS.

2. _____ Thaw γ-irradiated cells overnight at 4^{o}C in 50 ml of 4% TX-114-PBS (0.5 ml/g of cells) (note 2).

3. _____ Add DNase, RNase, pepstatin, leupeptin, and PMSF.

4. _____ Create a homogeneous suspension of bacterial cell by vortexing 30 seconds and putting on ice.

5. _____ Place 40 to 45 ml of cell suspension in French Press cell (note 3).

6. _____ Place French Press cell on French Press, collect lysate as it is forced out of the cell at a constant pressure of 1,000 PSI as measured by the gauge on the French Press.

7. _____ Place lysate on ice.

8. _____ Repeat steps 5-7 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 six times (note 4).

10. _____ Add an equal volume of the 4% TX-114-PBS solution (approximately 150 ml).

11. _____ Centrifuge at 3,000 x g (3000 rpm using table top centrifuge), 4^{o} C for fifteen minutes to pellet unbroken cells.

12. _____ Divide supernatant into two equal aliguots and transfer to two 250 ml centrifuge bottles.

13. _____ Rock overnight at 4^{o}C.

14. _____ Centrifuge 27,000 x g (13,000 rpm using GSA rotor)), 4^{o}C, 1 hour.

15. _____ Collect supernatants and place at 4^{o} C for later use.

16. _____ Suspend the pellets in 150 ml of 4% TX-114-PBS solution and repeat steps 13-15 (note 5).

17. _____ Combine the supernatants from the first and second extracts (note 6).

18. _____ Centrifuge the combined supernatants at 27,000 x g, 4^{o}C, 1 hour to remove remaining particulates. Transfer the supernatant to a new centrifuge bottle and repeat centrifugation two times.

19. _____ Incubate the final clarified supernatant at 37^{o}C, occasional swirling by hand until partitioning is readily apparent (1-2 hours).

20. _____ Centrifuge at 27,000 x g, 25^{o}C, 1 hour.

21. _____ Using a 50 ml plastic pipet remove the upper (aqueous) phases.

22. _____ Determine the volume of the TX-114 layers. The TX-114 layers will have a detergent concentration of approximately 12%.

23. _____ Add appropriate volume of PBS to detergent layers to bring concentration of TX-114 to 4% (note 7).

24. _____ Repeat steps 19-23 two more times.

25. _____ To final detergent (lower) layers slowly add 9 volumes of ice-cold acetone and place at –20^{o}C overnight.

From this point on, use only glass pipettes.

26. _____ Centrifuge acetone precipitate at 27,000 x g, 4^{o}C, 1 hour.

27. _____ Decant the acetone supernatant into a waste disposal container.

28. _____ Wash the precipitated material with about 150 ml of ice cold acetone, repeat centrifugation and decant the acetone supernatant.

29. _____ Remove residual acetone by applying a gentle stream of nitrogen to the pellet (note 8).

30. _____ Suspend each acetone precipitate in 30 ml of PBS (pH 7.4). Sonicating in an icebath or adding a stir bar and slowly stirring will help. The sample will not go completely into solution.

31. _____ Transfer half of the sample to one 35 ml centrifuge tube and the other half to a second tube. (You will have four tubes at this point)

32. _____ Add 15 ml of PBS saturated phenol to each tube and rock at room temperature for 4 hours (note 9).

33. _____ Centrifuge at 27,000 x g, 25^{o}C, for 1 hour using the SS34 rotor (15,000 rpm).

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

35. _____ To the phenol layer add a volume of PBS equal to that removed in step 34.

36. _____ Rock at room temperature for 4 hours, then centrifuge and remove aqueous layers as in steps 33 and 34.

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

38. _____ 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 10).

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

40. _____ Recover sample from dialysis tubing by pipetting into a clean sterile plastic container. It may be necessary to rinse the dialysis tubing with MilliQ water to recover particulate material that is stuck to the dialysis tubing.

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

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

43. _____ Run 4 μg on a SDS-PAGE gel and silver stain (see SOP SP007 and SP012) (note 11).

44. _____ Aliquot (default quantity is 1 mg) and dry by lyophilization (see SOP SP004).

Notes:

1. See SOP R001 for preparation of 32% Triton.
2. For cell weights other than 100 g, scale all reagent amounts up or down as appropriate.
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 4ºC incubation can be shortened to 1 hr for the second extraction.
6. Retain the pellets for production of mAGP (SOP PP011).
7. As an example if the detergent layer is 10 ml add 20 ml of PBS (pH 7.4) to obtain a final detergent concentration of 4%.
8. See SOP SP031 for use of the nitrogen/air bath
9. Phenol is dangerous and will burn the skin. Phenol will also melt plastic pipettes. Use caution and always work in the fume hood.
10. Two acetone precipitations have been used as an alternative to the dialysis step.
11. The final product varies from batch to batch. Predominant antigens to look for on the gel are: PhoS1 (38 kDa), and the 19 kDa lipoprotein.

References:

Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard. 1988. Identification and localization of integral membrane proteins of virulent Treponema pallidum subsp. pallidum by phase partitioning with the nonionic detergent triton X-114. Infect Immun 56:490-8.

SOP: PP025

Production of SDS Soluble Cell Wall Proteins SOP

Materials and Regents:
1. *M. tuberculosis* cell wall (note 1)
2. Phosphate Buffered Saline (note 2)
3. 2% SDS/PBS solution (note 3)
4. Oakridge centrifuge tubes, 50 ml, sterile (4)
5. Platform rocker
6. Cold room, 4^{o}C
7. Sorvall RC5-B centrifuge
8. Sorvall rotor, SS-34
9. Electric pipettor
10. Pipette, 25 ml

Protocol:
1. _____ Transfer cell wall preparation to a sterile 50 ml Oakridge centrifuge tube.

2. _____ Using a 25ml pipette and electric pipettor, add PBS to cell wall (note 5).

3. _____ Cap tube and place on platform rocker in 4^{o}C cold room for one hour.

4. _____ Using Sorvall RC5-B centrifuge and SS-34 rotor, centrifuge cell wall/PBS mixture at 27,000 x g 4^{o}C, for 30 minutes.

5. _____ Discard supernatant and keep pellet.

6. _____ Using a 25ml pipette and electric pipettor , add 2% SDS/PBS solution to pellet (note 5).

7. _____ Cap tube and place on platform rocker at room temperature for two to four hours.

8. _____ Using Sorvall RC5-B centrifuge and SS-34 rotor, centrifuge cell wall/PBS mixture at 27,000 x g, 4^{o}C, for 30 minutes.

9. _____ Using a 25ml pipette and electric pipettor, transfer supernatant equally between three sterile 50 ml Oakridge centrifuge tubes.

10 _____ Begin SOP to remove SDS (note 4).

Notes:
1. Material is produced according to SOP PP008.
2. PBS is made according to SOP R001 or is bought-in.
3. 2% SDS/PBS is made as follows: 2 g SDS per 100 ml of PBS.
4. SDS is removed according to SOP SP019.
5. Add at least 30 ml of PBS/ 2% SDS solution in order to have enough volume in Oakridge tube for subsequent centrifugation steps.

Reference:
Hirschfield, G.R., *et al. J. Bacterio*l. **172:**1005, 1990.

SOP: R001

Preparation of 32% Triton X-114

Materials and Reagents:

1. 1 Liter bottle
2. PBS (Phosphate Buffered Saline) 1X, pH 7.4 (Gibco cat# 10010-023)
3. Triton® X-114 (Sigma cat# X114-1L)
4. Stir Bar
5. Stir Plate
6. 4°C refrigerator
7. 37°C Water Bath
8. Serological Pipets
9. Pipetaid

Protocol:

1. _____ In a 1 liter bottle combine 150 ml of Triton® X-114 and 150 ml of PBS.

2. _____ Place on stir plate and mix thoroughly. Several hours is recommended.

3. _____ Transfer mixture to 4°C and let stand until the mixture is clear (note 1).

4. _____ Transfer the mixture to a 37°C water bath and incubate until a biphase occurs (note 2).

5. _____ Carefully remove the top layer of the biphase and discard (note 3).

6. _____ Add an equal volume of PBS to the Triton® layer that was not removed (note 4).

7. _____ Return to the stir plate at room temperature and mix until the solution is clear.

8. _____ Return mixture to the 37°C water bath and incubate until the second biphase occurs (note5).

9. _____ Repeat steps 5-8 once more for a total of 3 biphases.

10. _____ On the third time, remove as much of the top layer as possible, then transfer the remaining Triton® to a stir plate to recombine any liquid not removed (note 6).

11. _____ Store at 4°C until use.

Notes:

1. Overnight incubation is recommended. If the mixture is not mixed well enough it will never go completely clear. If this happens remix and incubate again.
2. If the biphase does not occur after several hours of incubation, continue to step 6 (which would be to double the volume with PBS). The biphase may be too little to see; therefore, the liquid would not be able to be pulled off regardless.
3. Be carefully to not disrupt the Triton® layer.
4. For example: from 300 ml of starting material, after the 1st biphase 50 ml is removed from the top layer, then 250 ml of PBS will be added back to the Triton® layer.
5. A biphase will definitely occur from this step forward so do not proceed until a biphase occurs. The volume of the biphase layer will greatly increase on the 2nd and 3rd pass.
6. This will yield about 400-450 ml of 32% Triton® X-114.

SOP:SP019

Removal of SDS by Paired-ion Extraction Protocol

Materials and Reagents:

1. Acetone
2. Glacial acetic acid
3. Triethylamine (note 1)
4. Burdick and Jackson H_2O
5. Lyophilized sample in 35 ml centrifuge tube(s)
6. 35 ml centrifuge tubes
7. SS34 rotor
8. High speed centrifuge
9. 150 ml flask with screw-top lid
10. Stirbar
11. Stirplate
12. Air bath
13. -20°C freezer
14. Glass pipets (5 and 10 ml)
15. Graduated cylinder (100 ml)

Protocol:

1. _____ In fume hood, add 85 ml of acetone, 5 ml of glacial acetic acid, 5 ml of triethylamine, 5 ml water to a 150 ml flask containing a stir bar (note 2).

2. _____ Place cap on flask and stir on stir plate for 2 minutes to thoroughly mix the solvents.

3. _____ Pipet solvent to each tube containing lyophilized sample (note 3).

4. _____ Cap centrifuge tube(s) and vortex vigorously to disperse the sample in the solvent.

5. _____ Place at -20^{o}C for 4 to 16 hours.

6. _____ Centrifuge at 27,000 x g (15,000 rpm), 4^{o}C, 30 min.

7. _____ Pour off supernatant, making certain not to lose the pellet (notes 4 and 5).

8. _____ Repeat steps 1-7.

9. _____ Add 30-35 ml of ice-cold acetone to the pellet(s) from step 8.

10. _____ Vortex vigorously to disperse pellet(s) in the acetone.

11. _____ Place at -20^{o}C for at least four hours.

12. _____ Centrifuge 27,000x g, 4^{o}C, 30 min..

13. _____ Pour off supernatant. This should remove residual triethylamine.

14. _____ Place tube with pellet in air bath and pass a gentle stream of N_2 over the pellet to volatize residual acetone (note 6).

15. _____ Add the buffer of your choice (usually 10 mM ammonium bicarbonate) to the pellet.

16. _____ Sonicate, or gently vortex to suspend the pellet.

16._____ Perform BCA assay and SDS-PAGE on the extracted sample.
17._____ Aliquot resuspended pellets for long term storage in cryovials suitable for –80^{o}C freezer (note 7).

QC Procedures:
1. SDS-PAGE
2. SDS concentration determination

Notes:
1. The triethylamine used needs to be fresh.
2. Only use glass pipets.
3. A general rule of thumb is to add 1 ml of extraction solvent for every 1 ml of sample that was lyophilized. If your sample is not completely dry, the final concentration of water cannot be greater than 5%.
4. It is okay to leave some solvent in the tube at this point. The important thing is to not lose your sample pellet.
5. Discard of all solvents containing acetone as hazardous waste.
6. See SOP SP031 for use of the N_2 Bath.
7. Default quantity shipped by the TB Contract is 1.0 mg.

References:
William H. Konigsberg and Lou Henderson. 1983. Removal of Sodium Dodecyl Sulfate from Proteins by Ion-Pair Extraction. Methods in Enzymology. 91: 254-258.

SOP: SP027

Operation of French Press

Materials and Reagents:

1. Whole cells (note 1)
2. Breaking buffer (note 2)
3. Ice bucket
4. Ice
5. Glycerol
6. French Press cell (note 3)
7. French Press cell stand
8. Falcon centifuge bottle, 225 ml
9. Lysol I.C. solution, 10%
10. Ethanol solution, 70%
11. Paper towels

Protocol:

1. _____ Suspend cells in breaking buffer at a concentration of 0.5 ml/gram cells (note 4).

2. _____ Place suspended cells on ice in ice bucket.

3. _____ Use a small amount of glycerol to lubricate French Press cylinder and bottom.

4. _____ Attach bottom of French Press to French Press cell and place unit on French Press stand.

5. _____ Attach Pressure Release knob and spout to bottom, making sure knob is closed completely.

6. _____ Place French Press cylinder into unit until reaching “max fill” mark.

7. _____ Turn unit upside-down, place on French Press stand, and remove bottom from unit.

8. _____ Add cell suspension to French Press unit, leaving enough room to attach bottom to the unit.

9. _____ Attach bottom to unit.

10. _____ Turn complete unit right-side up and place in French Press (note 5).

11. _____ Turn French Press on, and move lever to Up/High setting (Med setting if using the mini-cell).

12. _____ Using Pressure Release knob, keep pressure on cell between 1000 and 1500 while collecting eluent into the 225ml Falcon centrifuge bottle (note 6).

13. _____ When French Press cell is empty, turn machine lever to down.

14. _____ Place eluent collection bottle on ice in ice bucket, and carefully remove cell from the French Press.

15. _____ Repeat steps 7 to 14 until cell suspension has been passed through the French Press cell a total of six times.

16. _____ Check breakage by acid fast staining (note 7).

17. _____ When finished, thoroughly clean French Press using Lysol I.C. solution followed by 70% ethanol.

18. _____ Thoroughly clean French Press cell by completely disassembling unit, and washing each part with Lysol I.C. solution, distilled water, then 70% ethanol.

19. _____ Let all parts completely air dry prior to storage.

Notes:

1. *M. tuberculosis* cells must have been γ-irradiated according to SOP PP004 prior to breaking. If *M. tuberculosis* cells are live, protocol MUST be completed under BioSafety Level III conditions in room 101D at BHRB
2. Breaking buffer is made according to SOP PP007, PP008 or PP015, depending on your needs.
3. French Press cell unit contains the following parts: French Press cell, cylinder, bottom, pressure release valve, and spout. The French Press Mini-Cell will also need a silicone bead on the pressure release valve.
4. Cell solution should be viscous in order for proper breaking to occur.
5. Care should be taken to hold onto the unit bottom, otherwise gravity may cause loss of sample due to bottom removal. When the cell is placed in the French Press, be sure that the unit is flush against the bottom pegs, and be sure to turn the cylinder handle perpendicular to the bracing bar.
6. Care should be taken to point mouth of bottle away from eyes and head, as small air pockets inside the French Press cell may cause violent eruption of eluent when expelled.
7. Cells should be more than 90% broken. See SOP SP035 for Acid-Fast staining.

Reference:

Thermo IEC Operation Manual OMFA 078A Revision 0

SOP: SP030

Percent SDS Determination in an Aqueous Solution

Materials and Reagents:

1. Sample to assay
2. Standards for curve (note 1)
3. Pipetman, P-10
4. Pipet tips, 10 µl
5. Eppendorf tube, 0.65 ml
6. Pipetman, P-200
7. Pipet tips, 200 µl
8. Methylene blue solution (note 2)
9. Glass capillary pipettor, 50 µl
10. Glass pipet, 50 µl
11. Chloroform, HPLC-grade
12. Vortexer
13. Eppendorf centrifuge
14. 96 well plate
15. Plate reader, with 655 nm filter

Protocol:

1. _____ Using P-10 pipetman and tip, add 10µl of sample or standard to a 0.65 ml eppendorf tube.

2. _____ Using P-200 pipetman and tip, add 100µl of methylene blue solution to each eppendorf tube.

3. _____ Using a 50 µl glass capillary pipettor, add 20 µl of chloroform to each eppendorf tube.

4. _____ Close caps on all tubes and vortex vigorously.

5. _____ Centrifuge at 3000 x g, room temperature for 5 minutes.

6. _____ Using P-200 pipetman and tip, remove 100 µl of aqueous (top) layer from each sample and standard and transfer to 96-well plate.

7. _____ Put plate in plate reader and read absorbance at 655 nm.

8. _____ Plot square root of SDS standards against square root of O.D.655 correlation coefficient and graph residuals linear regression. Interpolate sample SDS by squaring percent SDS on graph.

Notes:

1. Standards to use: Appearance of standard:

0.1% SDS	$CHCl_3$ layer is darker blue and clear, no visible granules. Only a slight color in aqueous layer.
0.05% SDS	$CHCl_3$ is dark blue and clear, no granules. Aqueous layer very light blue.
0.01% SDS	Similar to 0.05% SDS.
0.005% SDS	Similar to 0.01% SDS.
0.0025% SDS	Aqueous phase a bit more color, both layers are clear.
0.001% SDS	Aqueous phase a bit more color, both layers are clear.
0.00075% SDS	Aqueous layer getting darker blue, CHCl3 getting lighter, both clear.
0.0005% SDS	$CHCl_3$ layer is very light aqua in color and clear. Aqueous layer is blue.
0.00025% SDS	Both layers are about the same color, light aqua. Both clear.
0.0001% SDS	$CHCl_3$ layer is almost colorless, aqueous layer back to original color.
0.00005% SDS	$CHCl_3$ layer is colorless and clear.

2. To make methylene blue solution: 12 mg/L in 0.01 M HCl with 0.02% NaN_3.

Reference:

Mukerjee. Percent SDS Determination in an Aqueous Solution. Analytical Chemistry. (28) 1956.