SOP: PP010.3
Modified 5/8/15 by MCL

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 **or** *M. tuberculosis* Whole Cell Lysate (Begin at protocol starting point specific for starting material)
4. Complete, EDTA-free protease inhibitor tablets (Roche, 11 873 580 001)
5. DNase 30 µl of a 1 mg/ml stock (-20°C)
6. RNase 30 µl of a 1 mg/ml stock (-20°C)
7. Ice-cold acetone (-20°C)
8. PBS saturated phenol (keep at 4°C)
9. Eight 250 ml centrifuge bottles
10. Ice bucket with ice
11. Plastic pipettes (25 ml and 50 ml)
12. Glass pipettes
13. Four 35 ml centrifuge tubes (Teflon)
14. Dialysis tubing (3,500 Da MWCO)
15. Dialysis tank
16. Graduated cylinders (100 ml and 250 ml)
17. Sorvall centrifuge
18. Table top centrifuge

Protocol for 100 g γ-irradiated *M. tuberculosis* cell starting material:

1._____ Thaw γ-irradiated cells overnight at 4°C

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

3._____ Add 50 ml of 4% TX-114-PBS to cells (0.5 ml/g of cells) (note 2).

4._____ Add 1 stock DNase, 1 stock RNase, and one Complete EDTA-free tablet (note 2).

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

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

7._____ 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.

8._____ Place lysate on ice.

9._____ Repeat steps 5-7 until all of the cell suspension has passed through the French press cell.

10._____ Repeat steps 6-9 six more times for a total of seven passes through the French press (note 4).

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

12._____ Centrifuge at 3,000 x g (3000 rpm using table top centrifuge), 4°C for fifteen minutes to pellet unbroken cells.

13._____ Divide supernatant into two equal aliquots and transfer to two 250 ml centrifuge bottles.

Continue on to **Extraction**

Protocol for *M. tuberculosis* Whole Cell Lysate starting material:

1._____ Thaw cells overnight at 4°C.

2._____ Dialyze cells into PBS (note 5) at 4°C with 3 buffer exchanges, each exchange 4-12 hours apart.

3._____ Add 0.125 ml of 32% Triton X-114 (TX-114) (note 1) per 1 ml of PBS-γ-irradiated cells to bring total solution to 4% TX-114-PBS

*Example: 175ml of PBS-γ-irradiated cells after dialysis * 0.125ml 32% TX-114 = 25 ml 32% Triton X-114 to add to solution*

4._____ Dilute a stock of 32% TX-114 solution to 4% using PBS and keep separate for later use.

Continue on to **Extraction**

Extraction

1._____ Rock overnight at 4°C.

2._____ Centrifuge at 27,000 x g, 4°C, for 1 hour.

3._____ Collect supernatants into clean centrifuge bottles and place at 4°C for later use.

4._____ Suspend the pellets in 150 ml of 4% TX-114 and repeat extraction steps 1-3 (note 6).

5._____ Combine the supernatants from the first and second extracts (note 7).

6._____ Centrifuge the combined supernatants at 27,000 x g, 4°C, for 1 hour to remove remaining insoluble material. Transfer the supernatant to a new centrifuge bottle and repeat centrifugation until no visible pellet is obtained.

7._____ Incubate the final clarified supernatant in a 37°C water bath until a partition appears (1-2 hours) (note 8).

8._____ Centrifuge at 27,000 x g, 25°C, 1 hour.

9._____ Using a 50 ml plastic pipet remove the upper (aqueous) phases, making note of the volume being removed. Be sure to remove all of the aqueous material.

10._____ To the TX-114 layer, add a volume of PBS equal to that removed.

11._____ Repeat steps 7-9 twice.

12._____ To final TX-114 (lower) layers, slowly add 9X the volume of ice-cold acetone and place at –20°C overnight.

From this point on, use only glass pipettes.

13._____ Centrifuge acetone precipitate at 27,000 x g, 4°C, for 1 hour.

14._____ Decant the acetone supernatant and dispose of as hazardous waste.

15._____ Wash the precipitated material with ice cold acetone, repeat centrifugation, and decant the acetone supernatant.

16._____ Remove residual acetone by applying a gentle stream of nitrogen to the pellet (note 9) or by leaving the tubes open in the fume hood until dry.

17._____ Suspend each acetone precipitate in 30 ml of PBS (pH 7.4). It may be necessary to gently scape the pellet from the side of the centrifuge bottle and to slowly stir on a stir plate. The sample will not go completely into solution.

18._____ Split each 30 ml sample between two 50 ml Teflon oakridge centrifuge tubes.

19._____ Add 15 ml of PBS saturated phenol to each tube and rock at room temperature for 4 hours (note 10).

20._____ Centrifuge at 27,000 x g, 25°C, for 1 hour.

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

22._____ To the phenol layer add a volume of PBS equal to that removed.

23._____ Rock at room temperature for 4 hours, then centrifuge and remove aqueous layers as in steps 20-21.

24._____ Transfer final phenol phase + interface to rehydrated dialysis tubing. Do not fill tubing more than half full to allow for expansion.

25._____ 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 11).

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

27._____ Recover sample from dialysis tubing by pipetting into a clean sterile plastic container. Rinse the dialysis tubing with MilliQ water to recover particulate material from the dialysis tubing.

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

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

30._____ Run 4 μg on a SDS-PAGE gel (SOP SP007) and silver stain (SP012) (note 12).

31._____ 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. Add 1.0g KH_2PO_4, 63.0g NaCl, and 5.56g Na_2HPO_4 to7L Milli-Q H_2O for each buffer exchange
6. The 4°C incubation can be shortened to 1 hr for the second extraction.
7. Retain the pellets for production of mAGP (SOP PP011).
8. Be sure to balance tubes before incubation as transferring material between tubes after incubation can disrupt the partition. A 50°C water bath can also be used if a partition does not form at 37°C.
9. See SOP SP031 for use of the nitrogen/air bath

10. Use caution while working with phenol. Always handle phenol in a chemical fume hood, wearing proper PPE, and only use glass pipets. Phenol is not compatible with all centrifuge tubes (the recommendation here is Teflon). Check chemical compatibilities of all materials before use.
11. Two acetone precipitations have been used as an alternative to the dialysis step.
12. 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.