

SOP: PP010.1
Modified 8-10-07

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. Complete, EDTA-free protease inhibitor tablets (Roche, 11 873 580 001)
5. DNase 30 μ l of a 1 mg/ml stock (-20° C)
6. RNase 30 μ l of a 1 mg/ml stock (-20° C)
7. Ice-cold acetone (-20°C)
8. PBS saturated phenol (keep at 4° C)
9. Sorvall GSA rotor
10. Eight 250 ml centrifuge bottles
11. Ice bucket with ice
12. Plastic pipettes (25 ml and 50 ml)
13. Glass pipettes
14. Sorvall SS34 rotor
15. 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°C in 50 ml of 4% TX-114-PBS (0.5 ml/g of cells) (note 2).
3. ____ Add DNase, RNase, and one Complete tablet.
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° C for fifteen minutes to pellet unbroken cells.
12. ____ Divide supernatant into two equal aliquots and transfer to two 250 ml centrifuge bottles.

13. ____ Rock overnight at 4°C.
14. ____ Centrifuge 27,000 x g (13,000 rpm using GSA rotor), 4°C, 1 hour.
15. ____ Collect supernatants and place at 4° 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°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°C, occasional swirling by hand until partitioning is readily apparent (1-2 hours).
20. ____ Centrifuge at 27,000 x g, 25°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°C overnight.

From this point on, use only glass pipettes.

26. ____ Centrifuge acetone precipitate at 27,000 x g, 4°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°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 4°C 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.