SOP: PP015.6
Revised 02/27/2017 by JMR

Preparation of LAM, LM, and PIM6

Materials and Reagents:

1. ~100 g γ-irradiated *M. tb* or *M. smegmatis* cells; or ~200 mg of *M.leprae* cells
2. 10:10:3 ($CHCl_3$:CH_3OH:H_2O)
3. 35 ml Teflon Oak Ridge Tubes
4. 32% Triton X-114 (note 1)
5. Breaking Buffer (note 4)
6. JL slants (*M.smeg only*)
7. Phosphate Buffered Saline
8. Cold 95% Ethanol
9. Water, endotoxin free
10. Proteinase K Stock Solution (10 mg/ml in water)
11. Calcium chloride stock solution (100 mM in water)
12. Pronase Stock Solution
13. SDS-PAGE Supplies
14. Lyophilizer flask
15. 250 ml Centrifuge Bottles
16. Aluminum Foil
17. 225, 50, and 15 mL Falcon Tubes
18. Glass Rod
19. Dialysis Tubing, 3,500 MWCO
20. Lyophilizer
21. Rocker
22. Sorvall Centrifuge
23. Air Bath
24. Probe Sonicator (*M.lep.)*
25. French Press *(M.tb. and M.Smeg.)*
26. 37ºC Incubator
27. Savant
28. Mettler-Toledo balance
29. Vortex mixer

Protocol:

1._____ Obtain approximately 100 g γ-irradiated cells (or 200 mg of *M. leprae* cells), thaw, and split between 6 x 35 ml Oak Ridge tubes.

2._____ Freeze dry by lyophilization (note 2). *

3._____ Delipidate cells by filling tubes with 10:10:3 and rocking for 2 hours at room temperature, vortexing the cells every 30 minutes. Cells can be left overnight rocking if necessary.

4._____ Balance tubes then centrifuge at 27,000xg at 15°C for 20 minutes.

5._____ Decant organic supernatant (note 3).

6._____ Repeat delipidation (steps 3-5) two more times, being sure to manually break up the cell pellet before 2 hr rocking.

7._____ Cover tubes with foil and place on the air bath to dry (see SOP SP031 for use of the air bath), stabbing the needle of the air bath through the foil to prevent dried sample from escaping. Allow cells to dry completely (will probably need to be left overnight to dry).*

8._____ Use a glass rod to break up clumps of cells and create a fine powder.

9._____ Add a minimum amount of breaking buffer to get cells into solution (~5 ml per tube), combine all cells into a single 225 ml Falcon tube, and rinse each tube with 5 ml breaking buffer. You should have ~60 ml when done (note 4).

10._____ Freeze/Thaw cells three times to ensure complete suspension (note 5).

11._____ Break cells by passing over the French Press at least 6 times (notes 6 and 7).

12._____ Perform an acid fast stain on a smear of the broken cells to check for at least 90% breakage using 100x magnification. Blue cellular debris indicates broken cells. Pink rods are unbroken cells.

13._____ Spin cells at 2000xg for 10 minutes to precipitate unbroken cells.

14._____ Distribute the broken cells equally into 2-4 teflon oakridge tubes and double the volume with breaking buffer.

15._____ Rock at 4°C overnight.*

16._____ Centrifuge at 27,000xg, 4°C for 1 hour.

17._____ Decant supernatant into new tubes (note 8) and place pellet at 4°C.

18._____ Balance the supernatant tubes and place in 37°C incubator or water bath for at least 2 hours until a visible partition forms (note 9).

19._____ Pre-warm the centrifuge and rotor by running the empty rotor at 27,000xg, 25°C for 30 minutes.

20._____ When supernatant is partitioned, centrifuge at 27,000xg, 25°C for 15 minutes.

21._____ Remove top aqueous layer, being sure to remove all of the aqueous layer. Transfer aqueous layer to the pellet from step 17. Pool detergent layers and store at 4°C until all extractions are complete.

22._____ After adding aqueous layer to cell pellets, split between 2 tubes. To each tube, add 8.75 ml 32% triton and fill to the neck of the tube with PBS (~35ml). This will give an 8% solution.

23._____ Repeat triton partition (step 15 to 22) two more times (note 10). *

24._____ Add cold 95% ethanol (note 11) to the pooled detergent layers at a 1:10 concentration and leave at –20°C overnight. Depending on the volume of your pooled detergent layers, this may require a large (up to 4L) beaker to accommodate volume *

25._____ Collect precipitate into pre-weighed 250 ml polypropylene centrifuge bottles and centrifuge at 27,000xg, 4°C for 1 hour (note 12).

26._____ Decant supernatant.

27._____ Allow pellets to dry in the chemical fume hood. This may take overnight depending on the volume of residual ethanol. *

28._____ Weigh material and resuspend in endotoxin free water at a concentration of 50 mg/ml.

29._____ For each 1 ml of sample, add 10 µl Proteinase K stock solution (final concentration = 0.1 mg/ml), and 50 µl $CaCl_2$ stock solution (final concentration = 5 mM). Incubate at 37°C overnight. *

30._____ Dialyze the digest for 24 hours in running DI-water using the 3,500 MWCO Slide-A-Lyzer Cassette.*

31._____ Remove from dialysis and transfer to a pre-weighed 50 ml falcon tube. Take a 8 µl aliquot to run on gel (note 13) and check that all the protein has been removed (note 14).

32._____ Dry remaining volume by lyophilization and weigh material (note 15).

* These are good places to stop at the end of the day

Notes:

1. See SOP R001 for protocol on how to make 32 % Triton X-114
2. See SOP SP004 for use of Lyophilizer
3. Save organic phase for preparation of PIM1,2, label with lot number of the original cells
4. Breaking Buffer
   50 ml 32% Triton X-114
   4 EDTA-Free Protease Inhibitor Cocktail Tablets
   400 µl 0.5M EDTA
   150 ml PBS
   Immediately before using the French Press, add to the cell suspension:
   300 µg DNase
   330 µg RNase
5. If the starting material was not irradiated and tested for viability, the cells should be tested for viability at this point in the protocol (only applicable for BSL-2 strains such as *M. smegmatis*, BSL-3 strains must be irradiated before starting this protocol). Take 1% of the cell suspension before breaking, streak on LJ slants, and incubate at 37°C for three weeks to confirm non-viability.
6. If you do not have access to a French Press, or have a cell mass lower than 3 g, you can break your cells with a probe sonicator (12 cycles of 60 seconds on and 90 seconds off).
7. See SOP SP027 for use of French Press. Use 225 ml falcon tubes to collect cells while running through French press. When transferring cells to French press cell, rinse the tube with 2 ml breaking buffer, but make sure that the cells remain in a small volume of buffer to obtain maximum breakage. If too much buffer is added and the cells are too thin, causing them to pass through the French Press with ease, freeze cells for a few minutes at –80°C after each pass.
8. If the supernatant is not clear at this point, transfer supernatant to new tubes and repeat centrifugation until clear supernatant is obtained. This may require several centrifugation, be sure to transfer to clean tubes each time.
9. Be sure to balance tubes before incubation as transferring material between tubes after incubation can disrupt the partition.
10. After the third partition, the aqueous layer can be discarded. The pellet should be saved for preparation of MAGP (see SOP PP011)
11. To make 95% cold ethanol: Add 5ml of MilliQ (ETF) H_20 to 95 ml of Absolute Ethanol that has been stored at 20°C.
12. There will be a large volume of ethanol, so it will be necessary to perform several centrifugations, each time adding to the tubes already containing precipitate, until all of the precipitate is collected and reduced into one or two tubes. Oakridge centrifuge tubes may also be used, but will require more rounds of centrifugation to collect the entire volume of ethanol.
13. See SOP for Running of SDS-PAGE Gels.
14. If protein is seen on the gel, perform a pronase digestion as follows:
    - Add 10 µl of pronase stock solution (at 10 mg/ml) for every 1ml of sample
    - Incubate at 37°C for 1 hour

    Then extract residual pronase with phenol: chlorofrom: iso-amyl alcohol as follows:
    - Double your sample volume with 25:24:1 (phenol:chloroform:iso-amyl alcohol)
    - Rock in the fume hood for 30 minutes
    - Centrifuge at 12,000xg at 15°C for 30 min
    - Transfer aqueous layer to new tube (discard bottom organic layer into a container for hazardous waste disposal)
    - Double the volume with 24:1 (chloroform:iso-amyl alcohol)
    - Rock in the fume hood for 10 min
    - Repeat spin
    - Transfer aqueous layer to new tube
    - Dialyze the digest for 24 hours in running DI-water using the 3,500 MWCO Slide-A-Lyzer Cassette.*
    - Freeze at –80°C and lyophilize

15. To continue purification further, see SOP PP016 for separation of LAM, LM, and PIM