

SOP: PP015

Preparation of LAM, LM, and PIM

Materials and Reagents:

1. 100g γ -irradiated H37Rv Cells
2. 10:10:3 (CHCl₃:CH₃OH:H₂O)
3. 32% Triton X-114 (note 1)
4. Breaking Buffer (note 4)
5. Phosphate Buffered Saline (Gibco)
6. Cold 95% Ethanol
7. Water, endotoxin free
8. Alditol Acetate Reagents
9. Pronase Stock Solution
10. SDS-PAGE Supplies
11. Lyophilizer flask
12. 50ml Teflon Centrifuge Tubes
13. Aluminum Foil
14. 225mL Falcon Tubes
15. Glass Rod
16. 13x100mm Glass Culture Tubes with Screw Caps
17. Slide-A-Lyzer Cassette 3,500 MWCO
18. 15ml Falcon Tubes
19. Lyophilizer
20. Rocker
21. Sorvall Centrifuge
22. Air Bath
23. Probe Sonicator
24. French Press
25. 37°C Incubator or water bath
26. Savant
27. Mettler-Toledo balance

Protocol:

1. ____ Obtain approximately 100g γ -irradiated cells and freeze dry by lyophilization (note 2).
2. ____ Weigh out dry cells into teflon centrifuge tubes, approximately 3g in each tube.
3. ____ Delipidate cells by adding 30ml 10:10:3 and rocking for 2 hours at room temperature, vortexing the cells every 30 minutes.
4. ____ 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.
7. ____ Cover tubes with foil and place on the air bath to dry (see SOP SP031 for use of the air bath). It will be necessary to stab the needle of the air bath through the foil. 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 (note 4).
10. ____ Freeze/Thaw cells three times to ensure complete suspension.

11. ____ Break cells by passing over the French Press 8 times (notes 5 and 6).
12. ____ Perform an acid fast stain on a smear of the broken cells to check for at least 90% breakage.
13. ____ Spin cells at 2000 x g for 10 minutes to precipitate unbroken cells.
14. ____ Distribute the broken cells equally into 2 teflon tubes and fill the tubes the rest of the way with breaking buffer. Ratio of breaking buffer to cells should be 1:1.
15. ____ Rock at 4°C overnight.*
16. ____ Centrifuge at 27,000xg, 4°C for 1 hour.
17. ____ Decant supernatant into new tubes (note 7) and place pellet at 4°C.
18. ____ Place supernatant in 37°C incubator.
19. ____ When supernatant is partitioned, centrifuge at 27,000xg, 25°C for 15 minutes.
20. ____ Remove top aqueous layer and transfer it to the pellet from step 17. Pool detergent layers and store at 4°C.
21. ____ After adding aqueous layer to cell pellets, split between 2 tubes. Add 8.75ml 32% Triton and fill to 50ml with PBS. This will give an 8% solution.
22. ____ Rock at 4°C for 2 hours.
23. ____ Repeat Triton partition (step 16 to current) two more times (note 8).
24. ____ Add cold 95% ethanol to the pooled detergent layers at a 1:10 concentration and leave at -20°C overnight.*
25. ____ Collect precipitate in teflon tubes and centrifuge at 27,000xg, 4°C for 20 min.
26. ____ Decant supernatant (note 9).
27. ____ Once the precipitate is collected in one tube, transfer it to a preweighed 15ml falcon tube.
28. ____ Dry on the savant (note 10).
29. ____ Weigh material and resuspend in endotoxin free water at a concentration of 50mg/ml.
30. ____ Take a 100µg aliquot to perform alditol acetate derivation (note 11).*
31. ____ Run sample on GC to check for excessive glucan contamination (note 12).
32. ____ Add pronase at a ratio of 1:10 v/v (note 13) and incubate at 37°C overnight.*
33. ____ Dialyze the digest for 24 hours in running DI-water using the 3,500 MWCO Slide-A-Lyzer Cassette.*
34. ____ Remove from dialysis and transfer to preweighed 15ml falcon tube. Take a 50µl aliquot to run on gel (note 14) and check that all the protein has been removed (note 15).
35. ____ Dry remaining volume on savant and weigh material (note 16).

* 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 PIM
 4. Breaking Buffer
 - 50ml 32% Triton X-114
 - 140µl 1mg/mL Pepstatin A
 - 100µl 1mg/mL Leupeptin
 - 400µl 1mg/mL PMSF
 - 400µl 0.5M EDTA
 - 150ml PBS
- Immediately before using the French Press, add:
- 300µg DNase
 - 330µg RNase
5. 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).
 6. See SOP SP027 for use of French Press. Use 225ml falcon tubes to collect cells while running through French press. When transferring cells to French press cell, rinse the tube with 2ml 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.
 7. 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.
 8. After the third partition, the aqueous layer can be discarded. The pellet should be saved for preparation of MAGP (see SOP PP011)
 9. 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.
 10. See SOP SP005 for use of the Savant
 11. See SOP SP022 for preparation of Alditol Acetate Derivatives
 12. If glucose concentration is reasonable, proceed with the rest of the protocol. If it is too high, repeat triton extraction, ethanol precipitation, and GC (steps 18-31)
 13. A new stock of pronase should be made for each digestion. Solution made at 10mg/ml.
 14. See SOP for Running of SDS-PAGE Gels. Because the concentration is unknown, it is best to run various amounts of sample in each lane of the gel, ranging from 0.5-20µl
 15. If protein is seen on the gel, perform a pronase digestion as follows:
 - Add 10µl of pronase stock solution (at 10mg/ml) for every 1ml of sample
 - Incubate at 37°C for 1 hour
- Then extract residual pronase with phenol: chloroform: iso-amyl alcohol as follows:
- Add an equal amount of 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)
 - Add an equal amount of 24:1 (chloroform:iso-amyl alcohol)
 - Rock in the fume hood for 10 min
 - Repeat spin
 - Transfer aqueous layer to new tube
 - Freeze at -80°C and lyophilize
16. To continue purification further, see SOP PP016 for separation of LAM, LM, and PIM