

SOP: PP026.1

Purification of PIMs

Materials and Reagents

1. H37Rv γ -irradiated whole cells, 50 to 150 mg (wet weight)
2. Erlenmeyer flask, 1.8L Chloroform, Burdick & Jackson HPLC-grade
3. Methanol, chloroform, Burdick & Jackson HPLC-grade
4. Acetone, Fisher ACS grade
5. Graduated cylinder, glass 100 ml
6. Chemical fume hood.
7. Magnetic stir plate
8. Whatman chromatography paper, 17CHR
9. Buchner funnel
10. Round-bottom flasks, 1 L , 250 ml
11. Rotary evaporator
12. Metal spatula
13. Sorvall centrifuge bottles, compatible with organic solvents
14. Pasteur pipets
15. PIM standard
16. TLC equipment (Note 1)
17. Glass tubes, 16 and 13 x 100 mm + caps
18. Preparative TLC plates, silica gel 60, 20 x 20 cm, glass-backed
19. Aluminum-backed 10 x 10 cm TLC sheets
19. TLC tank, large
20. Glass pipets, 10 ml
21. Rubber pipete bulb
22. Vortex
23. Reagents and equipment for alditol-acetate derivation (Note 2)

Protocol

1. ____ Freeze dry H37Rv γ -irradiated cells by lyophilization (Note 3). If starting with 10:10:3 extract from LAM preparation, allow to evaporate down to 100-200 ml, then skip to step 11.
2. ____ Weigh dried cells and transfer to a 1.8 liter Erlenmeyer flask.
3. ____ Suspend cells in 10:10:3 (CHCl₃:CH₃OH:H₂O) at a concentration of 30 ml/g of cells (Note 4).
4. ____ Add a large magnetic stir bar and cover mouth of flask with parafilm.
5. ____ Place on magnetic stir plate at room temperature and stir overnight.
6. ____ Place an appropriate amount of acetone in a large beaker and store overnight at -20°C.
7. ____ If starting with irradiated cells, cut a round piece of filter paper and fold into a funnel shape, place inside a Buchner funnel, and filter the cell extract into a beaker (Note 5).
8. ____ Let cells air dry in chemical fume hood.
9. ____ Transfer organic supernatant to 1 L round bottom flask.
10. ____ Dry material on a rotary evaporator until the volume is approximately 5 to 10 ml. Alternatively, calculate the amount of acetone required to make the 10:10:3 extract 10-20% of the total volume once added.

11. ____ Using a glass pipet and pipet bulb, add concentrated lipid drop-wise to cold acetone.
12. ____ Place trituration at -20°C overnight.
13. ____ Transfer entire volume of trituration to sterile 250 ml Sorvall centrifuge bottles compatible with organic solvents. One may also spin iteratively in several Teflon Oakridge tubes.
14. ____ Centrifuge at $20,000 \times g$ at 4°C for 30 minutes.
15. ____ Working in the hood, decant supernatant into a preweighed container, if saving, or a beaker for evaporation.
16. ____ The acetone-insoluble material can be resuspended in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1) in centrifuge bottles or tubes and transferred to an appropriate round-bottom flask to reduce volume via rotary evaporation. Minimize the time that 2:1 sits in the bottles.
17. ____ Apply the extract to preparative TLC plates in the hood and run in solvent system 60:30:6 $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (Note 7).
18. ____ Extract PIMs from preparative TLC plates (Note 8).
19. ____ Add at least 5 volumes (relative to silica volume) of 40% CH_3OH in CHCl_3 to each tube and, ensuring that caps are tight, vortex each for just a few seconds.
20. ____ Centrifuge at 3,000 rpm at 4°C for 15 minutes.
21. ____ Transfer the organic supernatant to 10 ml glass syringe with PTFE filter disk attached, and pass through to a fresh round-bottom flask for eventual evaporation (Note 9).
22. ____ Repeat steps 19 to 21 twice more for a total of 3 extractions.
23. ____ Resuspend the lipid in a small volume 2:1 and transfer to preweighed glass tube for drying and weight determination.
24. ____ Along with a phosphatidyl inositol (PI) standard, assay all fractions by TLC using solvent system 60:30:6 again (Note 10).
25. ____ Once the cleaned PIM fractions have been confirmed with TLC, prepare for alditol acetate conversion. This will require three 25-50 ug aliquots of PIM samples in 13 x 100 mm tubes, prerinsed with acetone (Note 2).
28. ____ Make alditol derivatives of sugar standards, sample, one PIM control, and also a reagent control.
29. ____ Conduct GC. Once PIM samples have been confirmed by GC, transfer to 13x100 mm tubes in 0.5 and 1.0 mg aliquots.

Notes

- (1) See Thin Layer Chromatography, SOP SP033, for a complete list of equipment and reagents.
- (2) See Preparation of Alditol-Acetate Derivatives, SP022.
- (3) See Lyophilization SOP, SP004.
- (4) All organic solvents should be used in a chemical fume hood.
- (5) The cells will adhere to the filter paper; save for future use. Note the volume of the filtered organic extract.
- (6) It may be necessary to resuspend the acetone insoluble material in 10:10:3 in order to transfer all it from the Sorvall centrifuge bottles. Use as little solvent as possible and dry under a stream of N₂.
- (7) Ensure that the total lipids are well resuspended by holding in waterbath sonicator for few minutes. See also Preparative Thin Layer Chromatography (SOP SP032) for other directions.
- (8) Stain edges of total lipid on TLC plates, and control PIM as well, to discern the PIM-specific band. Mark PIM-specific area on prep plate with pencil, visualizing under short-wave UV light. Scrape away silica above and below PIM-specific area with glass slide, working in hood. Carefully scrape PIM area and distribute to Teflon tubes or glass tubes with PTFE-lined caps.
- (9) Two syringes can be set up in the hood to expedite this process. Excess filtered extract can be collected into an Erlenmeyer, prerinsed with organic solvent, and stoppered with a glass cap for eventual transport for rotovaporation.
- (10) Run on 3 10x10 mm aluminum-backed plates along with PIM standard. Develop with α -naphthol and charring spray, and if a second band is *not* visualized under the main PIM band with charring spray, proceed to stain with Dittmer-Lester reagent as well. Likely, a second band will indicate PI contamination, not visualized by α -naphthol, in which case the lipid should be reran on preparative plates for clean-up. Follow the regular protocol for subsequent extraction and recheck for purity via TLC.

References

Brennan, P. J. and C. E. Ballou. *Journal of Biological Chemistry*. 1967. 242:3046.
Khoo, K.-H., *et. al.* *Glycobiology*. 1995. 5:117.

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