SOP: PP029.3
Updated 2/27/17

Purification of Trehalose Dimycolate (TDM)

Materials and Reagents

1. H37Rv γ-irradiated whole cells, 50 to 150 mg (wet weight)
2. Mettler-Toledo balance
3. Erlenmeyer flask, 2.0 L
4. Chloroform, HPLC-grade
5. Methanol, HPLC-grade
6. Graduated cylinder, glass, 100 ml
7. Chemical fume hood
8. Glass bottle, 1L
9. Magnetic stir bar, large
10. Magnetic stir plate
11. Incubator, set at 37°C
12. Round-bottom flask, 1 L
13. Round-bottom flask, 250 mL
14. Rotary evaporator (Rotovap)
15. Metal spatula
16. Glass Pasteur pipet
17. Rubber Pasteur pipet bulb
18. TLC reagents and equipment (see note 1)
19. 45 mL Teflon Oakridge tubes (4-6)
20. N_2 bath
21. Glass tubes, 13 x 100 mm + PTFE-lined lids
22. Large conical filter paper (VWR funnel #28310-172)
23. Small conical filter cones (Whatman #1202-125)
24. TLC plate, silica, glass-backed preparative (20x20 cm, Merck 1.05715.0001)
25. TLC sheets, silica gel 60, alumina (20x20 cm, Merck 1.05548.0001)
26. TLC tanks (Kontes, large and small)
27. Pipet, glass, (5 and 10 ml)
28. Rubber pipet bulb
29. Vortex
30. Benchtop centrifuge

Protocol

1._____ Freeze dry H37Rv γ-irradiated cells by lyophilization (note 2).

2._____ Weigh dried cells and transfer to a 1 L bottle.

3._____ Suspend cells in freshly prepared $CHCl_3/CH_3OH$ (2:1) at a concentration of 30 ml/g of cells (note 3).

4._____ Working in the hood, add a large magnetic stir bar and cap; allow extraction to go overnight.

5._____ Filter extract through large conical filter paper. Return cells to bottle, and extract again, 3-4 hrs, with half the volume of 2:1 used the first time.

6._____ Let cells air dry in a chemical fume hood. Label as “delipidated in 2:1” and save for future use.

7._____ Dry combined extract in the 1 L round bottom flask on a rotary evaporator and weigh.

8._____ Re-suspend the extracted material in a minimal volume of 2:1 (note 4).

9._____ Apply total lipid to preparative TLC plates, loading 1.5 cm from bottom of plate (note 5).

10._____ Run preparative TLC plates in solvent system 100/14/0.8 $CHCl_3/CH_3OH/H_2O$ (note 6).

11._____ Use the UV light box to visualize the TDM band with shortwave UV. Outline with a pencil the upper and lower limits of the band.

12._____ Working in the hood, use a glass slide to scrape non-specific area below the TDM band. Deposit silica in ziplock bags for eventual disposal as hazardous waste.

13._____ Scrape the TDM-specific portion using the glass slide method. Use folded foil to transfer silica to two Teflon Oakridge tubes.

14._____ Add 10 ml of 2:1 ($CHCl_3/CH_3OH$) to each tube, vortex, balance, and centrifuge at 3,000 rpm for 15 minutes – temperature doesn't matter.

15._____ Pour supernatant through small filter cones for capture in 250 mL round-bottom flask (note 7).

16._____ Repeat extraction once more and filter into same flask.

17._____ Dry via rotary evaporation. Resuspend in 3-4 mL 2:1, transfer to 13 x 100 mm tube, and dry on N_2 bath, using water bath heat at 37°C if desired.

18._____ Repeat steps 9-17 until 24 plates have been ran.

19._____ Resuspend each batch of dried, crude TDM in 1.0 mL 2:1 per 4 plates, and evaluate on analytical TLC with 100/14/0.8 $CHCl_3/CH_3OH/H_2O$ system.

20._____ Perform weight analysis on extracts requiring further clean-up.

21._____ Load clean-up plates with no more than 5 mg per plate, combining crude TDM extracts.

22._____ Follow steps 10-16, except perform 3-fold extraction. Resuspend in 1.0-2.0 ml 2:1, transfer to a tared glass tube or vial, dry, and weigh.

23._____ Evaluate 50 µg with 2D TLC on 10 x 10 cm analytical TLC sheets, using 100/14/0.8 $CHCl_3/CH_3OH/H_2O$ in the 1^{st} dimension (left to right), and 80/20/2 in the 2^{nd} dimension (bottom to top). Run each dimension until solvent reaches 1.0 cm from edge.

24._____ Submit for MALDI-TOF analysis at a concentration of 1.0 mg/ml. Request positive mode and DHB matrix (note 9).

25._____ Dry aliquots of 0.25 mg on N_2 bath, apply BEI labels if available, and store in the desiccator.

26._____ Complete QC sheet, listing total weight obtained. Small quantities may be retained to use as reference material for the next lot.

Notes:

1. See Thin Layer Chromatography, SOP SP033, for a complete list of equipment and reagents.

2. See Lyophilization SOP, SP004.

3. All organic solvents should be used in a chemical fume hood. *Make sure to use glass pipets with rubber bulbs for all work with organic solvents.*

4. See Preparative Thin Layer Chromatography, SOP SP032, for directions on preparing the material for preparative TLC.

5. See SOP SP032 for directions on loading a preparative TLC plate. If the lipid material was previously dried, resuspend in a small volume of 2:1. Apply no more than 25 mg per plate. Load 25-50 µg TDM control at edge of first few plates.

6. It is best to run 2 plates per tank, and a maximum of 8 plates per day. Run until solvent attains scratch line 1 cm from the top. At least one plate should have TDM control loaded at the edge, ~25 ug, to confirm which band is TDM.

7. This removes any contaminating silica from the supernatant.

8. The plates should then be visualized with charring spray (SOP R011) and α-napthol spray (R012).

9. Should see cluster of peaks from 2700-2900 m/z, separated by 14 amu. Obtain spectra from 2100-3050 m/z, including an expanded view of 2700-2900 with major peaks labelled.

References:

Slayden, RA and Barry 3rd, CE (2001). Analysis of the Lipids of *Mycobacterium tuberculosis*. *Mycobacterium tuberculosis Protocols* (Parish T and Stoker, NG ed), Humana Press Inc, Towata NJ, pp 229-246.

Besra, GS (1998). Preparation of Cell-Wall Fractions from Mycobacteria. *Methods in Molecular Biology, Volume 101: Mycobacteria Protocols* (Parish T and Stoker, NG ed), Humana Press Inc, Towata NJ, pp 91-107.