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, Burdick & Jackson HPLC-grade
5. Methanol, Burdick & Jackson HPLC-grade
6. Graduated cylinder, glass, 100 ml
7. Chemical fume hood
8. Magnetic stir bar, large
9. Parafilm
10. Magnetic stir plate
11. Incubator, set at 37°C
12. Round-bottom flask, 2 L, tared (1)
13. Rotary evaporator (Rotovap)
14. Metal spatula
15. Sorvall centrifuge bottles (1 to 6)
16. Sorvall centrifuge
17. Sorvall centrifuge rotor, GSA
18. Glass Pasteur pipet
19. Rubber Pasteur pipet bulb
20. TLC reagents and equipment (see note 1)
21. N2 bath
22. Glass tubes, 16 x 100 mm + PTFE-lined lids
23. Glass syringe (10 ml)
24. Large conical filter paper (VWR funnel #313 Cat. No. 28333-123)
25. TLC plate, silica, glass-backed preparative (20x20 cm, 1 mm silica, F254 fluorescent dye)
26. TLC sheets, silica gel 60, alumina (20x20 cm, F254)
27. TLC tanks (Kontes, large and small)
28. Ruler
29. Pencil
30. Pipet, glass, (5 and 10 ml)
31. Rubber pipet bulb
32. Vortex
33. Benchtop centrifuge
34. Glass tubes, 13 x 100 mm + PTFE-lined lids
35. CDCl3, HPLC-grade (Aldrich 423661-1PAK)
36. CD3OD, HPLC-grade (Aldrich 308811-1PAK)
37. NMR tube
38. 1H NMR machine (see note 2)

Protocol

1. Freeze dry H37Rv γ-irradiated cells by lyophilization (note 3).

2. Weigh dried cells and transfer to a 2.0 L Erlenmeyer flask.

3. Suspend cells in freshly prepared CHCl3/CH3OH (2:1) 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 and foil.
5. Place on magnetic stir plate in the 3rd floor 37°C chamber and stir at least 4 h. If the parafilm begins to bubble, simply remove and retain the foil. Denote on the flask that the contents are γ-irradiated Mtb cells in chloroform/methanol (2:1).

6. Transfer extracted material to a sterile 250 ml Sorvall centrifuge bottle.

7. Working in the hood, filter extract through large conical filter paper, once into a clean beaker, and once during transfer to round-bottom flask.

8. Centrifuge at 27,000 x g, 4°C for 30 minutes.

9. Transfer organic supernatant to 2 L round bottom flask employing a glass funnel.

10. Repeat steps 3-9 2X for a total of 3 extractions, with one of the extractions overnight.

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

12. Dry material in the 2 L round bottom flask on a rotary evaporator and weigh.

13. Re-suspend the extracted material in a minimal volume of 2:1 (note 5).

14. Partition the resuspension into several 16x100 mm tubes in about 200 mg aliquots. Dry down any tubes not to be applied to TLC plates in the next step. At this point it is critical to keep all lipid material containing TDM dry and in 4°C storage.

15. Apply material to preparative TLC plates. Since there is a lot of material to be applied, load 2 cm from bottom of plate (note 6).


17. Use the UV light box on the 3rd floor to visualize the TDM band. Outline with a pencil the upper and lower limits of the band.

18. Working in the hood, use a glass slide to scrape non-specific areas above and below the TDM band. Deposit silica in wet towels for disposal.

19. Working at the bench, wearing a surgical mask to prevent inhalation of the silica, scape the TDM-specific portion by pulling the slide away from body. Use folded foil to transfer to 16x100 mm tubes, filling each tube no more than ¼ full.

20. Add 8 ml of CHCl3/CH3OH (2:1) to each tube and vortex for at least 30 s.

21. Balance and centrifuge at 3,000 rpm, 4°C for 15 minutes.

22. Transfer the organic supernatant to new 16 x 100 mm tubes, one of which is tared (note 8).

23. Dry all tubes under a stream of N2, collating into the tared tube.

24. Repeat steps 20 to 23 twice more.

25. Assay the TDM by 2-dimensional TLC to detect organic compounds and sugar residues (note 9).

26. Take 5 to 10 mg of TDM fraction and transfer to a new 13 x 100 mm tube.
27. Working in the hood, resuspend TDM in 1 ml of CDCl3/CD3OD (2:1, deuterated).
28. Completely dry under a stream of N2.
29. Repeat step 27 once more.
30. Resuspend TDM in 1 ml of deuterated 2:1.
31. Transfer the TDM suspension to a clean NMR tube and analyze by 1H NMR (note 10).
32. Once NMR analysis is complete, transfer TDM suspension from the NMR tube back to the 13 x 100 mm tube.
33. Completely dry under N2, resuspend in 1 ml of 2:1, and dry again.
34. Repeat step 32 once more. Resuspend in 1 ml 2:1 and transfer back to the remainder of TDM. Dry completely.
35. Re-suspend TDM in CHCl3/CH3OH (2:1) and aliquot into new 13 x 100 mm tubes. Label appropriately.
36. Completely dry under of N2 for -80ºC storage.

Notes:

1. See Thin Layer Chromatography, SOP SP033, for a complete list of equipment and reagents.
2. See NMR SOP, SP-046, for a complete list of equipment and reagents.
3. See Lyophilization SOP, SP004.
4. All organic solvents should be used in a chemical fume hood. An exception can be made for using the 37º incubator on the 3rd floor. Make sure to use glass pipets with rubber bulbs for all work with organic solvents.
5. See Preparative Thin Layer Chromatography, SOP SP032, for directions on preparing the material for preparative TLC.
6. 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. Overnight incubation at 4ºC in 2:1 is a more efficient means of resuspension.
7. It is best to run 2 plates per tank, and a maximum of 8 plates per day in 2 separate runs. The best way to clean a tank is to rinse once with MeOH and once with acetone, completely drying in between.
8. The organic supernatant should be passed through a 0.2 μm PTFE syringe filter, attached to a glass 10 ml syringe, prior to placement in the pre-weighed 16 x 100 mm tube. This removes any contaminating silica from the supernatant.
9. Analyze by 2-D TLC, using the solvent system CHCl₃/CH₃OH/H₂O (100:14:0.8) in the first dimension and CHCl₃/CH₃OH/NH₄OH (90:10:1) in the second. The plates should then be developed with charring spray (SOP R011) and α-naphthol spray (R012). If further purification is required, repeat steps 15-29, loading everything on 1 prep plate.

10. See NMR SOP SP-046.

References
