

SOP: PP029a

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. CDCl₂, 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).
16. _____ Run preparative TLC plates in solvent system CHCl₃/CH₃OH/H₂O (80:20:2). Use the 2 large Kontes tanks labeled for “TDM only.” (note 7).
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, scrape 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 -80°C 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 37° 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 4°C 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 $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (100:14:0.8) in the first dimension and $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}$ (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

- 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.