

SOP: PP030

Purification of Trehalose Monomycolate (TMM)

Materials and Reagents:

1. H37Rv γ -irradiated whole cells, 50 to 150 mg (wet weight)
2. Mettler-Toledo balance
3. Erlenmeyer flask, 1.8L
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, 1 L (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 (as many as pools and bands)
23. 16 mm lids, PTFE-lined (as many as tubes)
24. TLC plate, silica, glass-backed preparative
25. TLC tank, large
26. Ruler
27. Pencil
28. Pipet, glass, 10 ml
29. Rubber pipet bulb
30. Vortex
31. Benchtop centrifuge
32. Glass tubes, 13 x 100 mm (1 + number of aliquots)
33. 13 mm lids, PTFE-lined (as many as tubes)
34. CDCl₂, HPLC-grade (Supelco)
35. CD₃OD, HPLC-grade (Supelco)
36. NMR tube
37. ¹H NMR machine (see note 2)

Protocol:

1. _____ Freeze dry H37Rv γ -irradiated cells by lyophilization (see note 3).
2. _____ Weigh dried cells and transfer to a 1.8 liter Erlenmeyer flask.
3. _____ Suspend cells in CHCl₃/CH₃OH (2:1) at a concentration of 30 ml/g of cells (see note 4).
4. _____ Add a large magnetic stir bar and cover mouth of flask with parafilm.
5. _____ Place on magnetic stir plate in a 37°C incubator and stir overnight.
6. _____ Transfer extracted material to a sterile 250 ml Sorvall centrifuge bottles.
7. _____ Centrifuge at 27,000 x g, 4°C for 30 minutes.
8. _____ Transfer organic supernatant to 1 L round bottom flask.

9. ___ Let cells air dry in a chemical fume hood; save for future use.
10. ___ Dry material on a rotary evaporator and weigh.
11. ___ Re-suspend the extracted material in a minimal volume of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1) (see note 5).
12. ___ Apply material to preparative TLC plates (see note 6).
13. ___ Run preparative TLC plates in solvent system $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}$ (80:20:2) (see note 7).
14. ___ The major fraction closest to the origin is TMM; extract TMM from preparative TLC plates (see note 8).
15. ___ Dry silica under a stream of N_2 .
16. ___ Add 8 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1) to each tube and vortex vigorously.
17. ___ Centrifuge at 3,000 x g, 4°C for 15 minutes.
18. ___ Transfer the organic supernatant to new, pre-weighed 16 x 100 mm tubes (see note 9).
19. ___ Dry under a stream of N_2 .
20. ___ Repeat steps 16 to 19 twice more.
21. ___ Assay all fractions by TLC; use solvent system $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}$ (80:20:2) and develop with charring spray and α -naphthol spray (see notes 10 and 11).
22. ___ Take 5 to 10 mg of TMM fraction and transfer to a new 13 x 100 mm tube.
23. ___ Re-suspend TMM in 1 ml of $\text{CDCl}_3/\text{CD}_3\text{OD}$ (2:1).
24. ___ Completely dry under a stream of N_2 .
25. ___ Repeat steps 24 and 25 once more.
26. ___ Re-suspend TMM in 1 ml of $\text{CDCl}_3/\text{CD}_3\text{OD}$ (2:1).
27. ___ Transfer the TMM suspension to a clean NMR tube and analyze by ^1H NMR (see note 12).
28. ___ Once NMR analysis is complete, transfer TMM suspension from the NMR tube back to the 13 x 100 mm tube.
29. ___ Completely dry under a stream of N_2 .
30. ___ Re-suspend in 1 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1).
31. ___ Completely dry under a stream of N_2 .
32. ___ Repeat steps 30 and 31 once more.
33. ___ Re-suspend in 1 ml of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1).
34. ___ Transfer from the 13 x 100 mm tube and combine with remainder of TMM.
35. ___ Completely dry under a stream of N_2 .
36. ___ Re-suspend TMM in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1) and aliquot into new 13 x 100 mm tubes.
37. ___ Completely dry under a stream of N_2 .

Notes:

1. See Thin Layer Chromatography, SOP SP033, for a complete list of equipment and reagents.
2. See NMR SOP, SP-XXX, 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.
5. See Preparative Thin Layer Chromatography, SOP SP032, for directions on preparing the material for preparative TLC.
6. See Preparative Thin Layer Chromatography, SOP SP032, for directions on loading a preparative TLC plate.
7. See Preparative Thin Layer Chromatography, SOP SP032, for directions on running a preparative TLC plate.
8. See Preparative Thin Layer Chromatography, SOP SP032, for directions on extracting lipids from a preparative TLC plate.
9. 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.
10. The TMM may also be analyzed 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 dimension. The plates should then be developed with charring spray and α -naphthol spray as previously described.

11. The charring spray (SOP R011) will detect any organic compound and α -naphthol spray (R012) will detect any glycans.
12. See NMR SOP SP-XXX.

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.