SOP: PP058
1/4/22

Total Lipid Enrichment

Materials and Reagents:

1. *M. tuberculosis* γ-irradiated whole cells, 50 to 200 g (wet weight)
2. Lyophilizer
3. Erlenmeyer flask, 1800 ml
4. Chloroform, HPLC-grade
5. Methanol, HPLC-grade
6. Glass column with 250-500 ml reservoir
7. Magnetic stir bar, large
8. Magnetic stir plate
9. 13 x 100 mm glass tubes
10. Filter paper 413 (VWR, Cat 28310-172)
11. Graduated cylinder, 1000 ml
12. Separatory funnel
13. Water, HPLC-grade
14. Round bottom flasks
15. Rotovap
16. Silica TLC plate, aluminum backed
17. Glass TLC tank, small
18. Pasteur pipets
19. Capillary bores, 10 µl
20. Capillary pipettor, 10 µl
21. Glass tubes with PTFE-lined lids, 13 x 100 mm
22. Capillary bores, 100 µl
23. Capillary pipettor, 100 µl
24. N_2 bath

Protocol

1._____ Lyophilize the *M. tuberculosis* γ-irradiated whole cells (note 1).

2._____ Obtain Folch-washed total lipid (note 2).

3._____ Resuspend 250-500 mg total lipid in 5 ml $CHCl_3$ (note 3).

4._____ Set up glass column in fume hood in a ring stand with 2 clamps. Rinse column with 2:1 $CHCl_3/CH_3OH$, then $CHCl_3$, making sure the stopcock doesn't leak.

5._____ Place a small cotton plug at the bottom of the glass column to prevent silica from flowing out. Add 200-250 ml $CHCl_3$ to 30 g silica in a large Erlenmeyer flask, swirling gently to create a silica suspension. Gently pour about $1/4^{th}$ of this slurry into the glass column (note 4).

6._____ Place a large, clean Erlenmeyer flask to capture chloroform from the column.

7._____ Open the glass column valve to allow chloroform to flow through, enabling the silica column to begin to stack. Stop the flow after about half of the silica has condensed.

8._____ Remove the glass rod with forceps, then gently swirl the remainder of the silica/chloroform mixture in the flask. Slowly pour the rest of the slurry.

9._____ Open the stopcock valve to allow the remainder of the silica to stack (note 5).

10._____ Keep a small volume of chloroform at the top of the stacked column. Just before adding the 5 ml chloroform suspension of total lipid, drain the excess chloroform until it goes into the column.

11._____ Centrifuge the 5 ml $CHCl_3$ suspension 5 min at 3.5 K rpm, and pull off any aqueous precipitates residing at the top with a Pasteur pipet.

12._____ Slowly add the suspension using a Pasteur pipet, reaching down inside the glass column as far as possible, minimizing the total lipid drying before it reaches the top of the column.

13._____ Open the stopcock and allow the suspension to enter the column, then turn off.

14._____ Chase suspension into column further with 5 ml CHCl3, taking care as before to keep the integrity of the top of the column intact.

15._____ Prepare 3 column volumes of $CHCl_3$ to be passaged through column as the "flowthrough" (note 6).

16._____ While chloroform wash is passaging through, prepare 5%, 6%, and 10% CH_3OH in $CHCl_3$ solutions, making at least 250 ml available (note 7).

17._____ Collect the first 50 ml of the 5% MeOH in chloroform solution as part of the chloroform wash (note 8).

18._____ Swap out the flowthrough flask with chloroform wash for the flask labelled 5% MeOH in CHCl3, and collect 3 CV worth of that solvent, repeating the overlapping scheme described in step 17 (note 9).

19._____ Work through the 6%, 10%, and 33% MeOH in $CHCl_3$ washes of the column (note 10).

20._____ Evaluate 10 ul of flowthrough (FT), 5%, 6%, and 10% MeOH fractions on analytical TLC for TDM and SL (note 11).

21._____ Evaluate 10 ul FT, 10%, and 33% MeOH fractions for enrichment for $PIM_{1,2}$ and TMM (note 12).

Notes

1. See SOP SP004 for use of the lyophilizer.
2. See SOP PP018.2
3. Dry down in a round-bottom flask. Use water bath sonication to resuspend, transfer to 13 x 100 mm tube, and then centrifuge the tube 5 min at 3.5K rpm to see if any aqueous precipitates form *at the top* of the suspension. This may be more of an issue at the 500 mg level. Remove unwanted precipitates from the top with a Pasteur pipet.
4. Use a long glass rod to hold cotton plug in place. Fit a glass funnel to the top of the glass column before pouring the silica slurry.
5. Rinse the Erlenmeyer with chloroform and add this to the building column. Do this at least twice. Rinse the glass funnel of silica with chloroform as well. Once the funnel is clear, rinse the upper reservoir of the glass column with chloroform by adding via pipet, using a swirling motion around the opening of the column.
6. May use the chloroform that has already been ran through the column during stacking. The column is equilibrated in $CHCl_3$, so this is a basic wash step to rinse out highly nonpolar compounds which are not of interest. Use a 500 ml round bottom flask to capture each of these fractions. All fractions will be evaluated on analytical TLC to confirm this. One column volume (CV) is equivalent to 30 g silica divided by 0.45 g/ml silica, or 66.67 ml. Three CV = 200 ml.
7. Will need that bit of excess over the 200 ml to be passaged to resuspend each fraction in the appropriate solvent once they are dried on the rotovap.
8. Take care when adding all solvents to the column to do so slowly at first. It takes roughly 1 CV of the newly added solvent to actually elute as that particular percentage of MeOH in chloroform; undercutting at 50 ml rather than the actual CV of 66.67 ml should help to prevent spillover into the previous fraction.
9. Begin drying each fraction on rotovap. Resuspend each of these with 5 or 6 ml appropriate solvent, e.g. $CHCl_3$ only for the chloroform flowthrough, and transfer to a 13 x 100 mm tube.

10. The 33% MeOH fraction is simply 2:1 $CHCl_3/CH_3OH$. Add an additional 66.7 ml of 2:1 to be passaged such that the total collected is closer to 3 CV.
11. Often the 5% retains TDM along with non-polar material, and can be ran on a prep plate to obtain relatively clean TDM. The 6% MeOH fraction will often have both TDM and SL, which can be obtained crudely for further purification via SepPak C18 columns. Run in 100/14/0.8 $CHCl_3$/ CH_3OH/ H_2O and include SL/TDM controls.

Reference

Minnikin D. E. In Bacterial Cell Surface Techniques (I. C. Hancock and I. R. Paxton, eds.) John Wiley & Sons, New York. Pp 125-135, 1988.