SOP: PP060.2
Last updated 5/8/2023 BM

Purification of mGLP

Materials and Reagents

1. ~100 g γ-irradiated H37Rv *M. tuberculosis* cells
2. 10:10:3 ($CHCl_3$:CH_3OH:H_2O)
3. 35 mL Teflon Oak Ridge tubes
4. Lyophilizer
5. 500 mL bottle
6. Chloroform, HPLC grade
7. Methanol, HPLC grade
8. MilliQ water
9. Magnetic stir bar
10. Magnetic stir plate
11. Glass tubes, 13 x 100 mm + Teflon caps
12. N2 bath
13. Light Sep-Pak C18 reverse phase columns (Waters)
14. Glass syringe, 10 mL
15. Glass vials, 2 mLs + Teflon caps
16. Glass pipets (5, 10 mL)
17. Rubber pipet bulb
18. Amicon® Ultra-15 Centrifugal Filter Unit, 10 kDa
19. Microsep™ Advance Centrifugal Devices with Omega Membrane 3K
20. Pasteur pipettes with bulbs
21. Acetonitrile, HPLC grade
22. Water bath sonicator

Protocol

1._____ Obtain approximately 100 g γ-irradiated H37Rv cells and freeze dry by lyophilization (note 1).

2._____ Transfer cells to 500 mL bottle.

3._____ Delipidate cells by adding 10:10:3 at 10 mL/g of cells and adding a stir bar, stir overnight in a chemical fume hood. Make sure the bottle is tightly closed.

4._____ Transfer overnight extract into 35 mL Teflon Oak Ridge tube(s) and centrifuge at 27,000 x g at 15° C for 20 minutes.

5._____ Decant organic supernatant.

6._____ Repeat delipidation (steps 2 -5) two more times, reducing incubation time to 2 hours for each repeat by transferring cells from tubes back to 500 mL bottle. Be sure to thoroughly break up cells before repeating. (note 2)

7._____ Collect all extracts into one bottle and label appropriately.

8._____ Measure total volume and aliquot 0.5 mL into tared 2 mL vial, dry under N2 bath. Calculate mg/mL of total volume based on weight.

9._____ Take 50 mg of your 10:10:3 total lipid and completely dry down via N2 bath. Resuspend in MilliQ water at a concentration of 5 mg/mL (10 mL total). Use a water bath sonicator to thoroughly resuspend – may take several minutes. If necessary, can also add more water to further dilute and ensure complete resuspension. (note 7)

10._____ Prep your Amicon® Ultra-15 Centrifugal Filter Unit by adding 5 mL of water and centrifuging at 3500 x g at 20° C for 5 minutes. Dispose of flowthrough. (note 3)

11._____ Add your total lipid extract from step 9 to the Amicon® filter and centrifuge at 3500 x g at 20° for 30 minutes. Check volume level: if there is only a small amount of flowthrough, increase speed to 3750 – 4000 x g.

12._____ Repeat centrifugation if necessary until most of the volume is passed as flowthrough and the retentate volume is ~ 1 mL.

13._____ Rinse 10 kDa filter membrane using Pasteur pipette by adding 2 – 3 mL of water, pipette up and down several times. Centrifuge for 10 minutes. (note 4)

14._____ Collect flowthrough, including volume from rinse step in 13, and transfer to a Microsep™ Advance Centrifugal Device with Omega Membrane 3K in increments of 4 mL, which is the max volume.

15._____ Centrifuge at 3500 x g at 20° C for 10 minutes, dispose of flowthrough. Add more 10 kDa flowthrough until max volume (4 mL) is reached again. Continue this process until all the 10 kDa flowthrough has been passed through the 3 kDa filter and retentate volume is reduced to ~ 1 mL.

16._____ Transfer retentate to 13 x 100 tube and thoroughly rinse 3 kDa filter with 2x 1 mL of water, pipetting up and down to collect any residual product before transferring to 13 x 100 tube.

17._____ Evaluate total amount of crude material in 3 kDa retentate by aliquoting 0.5 mL into a pre-tared 2 mL vial. Calculate mg/mL of total volume based on weight.

18._____ Aliquot 0.5 mg of 3 kDa retentate into a 13 x 100 tube and completely dry down on N2 bath. Resuspend in 500 uL of water and dilute 1:10 by adding 4.5 mL of additional water. (note 5)

19._____ Prep your Sep-Pak C18 cartridge with the following using the 10 mL glass syringe:

a. 4 mL 95% acetonitrile

b. 5 mL 100% methanol

c. 2 mL 80% methanol in chloroform

20._____ Equilibrate your cartridge by adding 3 mL of water; leave enough water in the column so that it is saturated and a droplet is visible at the bottom. Let sit for 5 minutes.

21._____ Load your diluted crude sample into the column and slowly elute.

22._____ Wash with 4 mL of water.

23._____ Perform a series of elutions with the following:

a. 4 mL 30% methanol in water

b. 4 mL 60% methanol in water

c. 4 mL 90% methanol in water

Perform a wash step with 4 mL of water in between each elution; collect each elution and wash step in a labeled 13 x 100 tube.

24._____ Dry each fraction on N2 bath and resuspend in 500 mL of water.

25._____ Repeat Sep-Pak chromatography for as much material is needed. (note 8)

26._____ Perform analytical TLC and MALDI-TOF for QC. (note 6, 9)

Notes

1. See SP004.2 for instructions for lyophilization.
2. The overnight step can be done for either the 1st or 2nd round of extraction.
3. It is important to not let the filter over spin and dry out – can check halfway through.
4. Ensure total volume of flowthrough does not exceed 15 mL. If it does, transfer FT to new tube before adding more water.
5. Can go above 0.5 mg but most importantly a 1:10 dilution is needed. 0.5 – 2.0 mg is acceptable – generally, increasing the amount will lead to lower product yield.
6. See SP033.1 for instructions for TLC.
7. Do not let water resuspension sit out to avoid contamination build up. If necessary, can store overnight in fridge. Thorough resuspension is important to ensure there is not clogging during 10 kDa filtration.
8. Save FT wash tubes but do not need to dry down and run on TLC unless needed. Will only run TLC for 30%, 60%, and 90% fractions.
9. For MALDI, use HCCA matrix. Can start in positive mode, but switching to negative mode will give better spectra results.