SOP: PP032

Purification of Mycobactin

Materials

$FeCl_3$ (5% w/v in 95% EtOH)
100% EtOH
13x100 mm glass tubes + caps
Oakridge tubes (teflon)
Centrifuge bottles (teflon)
Ultracentrifuge
Filter paper cones (Whatman)
Glass funnel
Sep-Pak C18 filters (Waters)
20 x 20 cm preparative TLC plates (1 mm)
Large TLC tank
Pasteur pipets
Capillary pipets (10 µl, 100 µl)

B&J Grade Reagents

chloroform
methanol
water
petroleum ether
n-butanol
ethyl acetate

Protocol

Growth, Harvest, and Initial Extraction

1._____Make media and plates for transport to BRB. [SOP M013]

2._____At BRB inoculate each large agar plate with one *M.tb* glycerol stock. Growth will proceed about 6 weeks before harvest. [SOP PP032]

3._____At BRB inactivate cells by harvesting into 100% ethanol. [SOP PP032]

4._____Begin extraction after cells have been suspended in ethanol 24 hours.

5._____Add $FeCl_3$ solution until solution appears amber and ceases to darken. Filter entire solution through filter cones and save the cells. (Note 1)

6._____Add an equal volume $CHCl_3$, then slowly add water until organic/aqueous layers form.

7._____Separate the chloroform layer and partition into Teflon tubes or bottles to commence Folch washes. Add an amount of $CHCl_3/CH_3OH/H_2O$ (1:47:48) such that the total volume fills at least 80% of the bottle or tube. (Note 2)

8._____Spin at *3000 x g* or less. Time and speed may need to be adjusted based on rotor and efficiency of separation. Siphon off the aqueous layer. (Note 3)

9._____Repeat steps (7) and (8) twice more for a total of three washes.

10._____Run a 10 x 10 cm TLC as a cursory check for two mycobactin bands, visible without staining. Use petroleum ether/*n*-butanol/ethyl acetate (2:3:3) as the developing system.

11._____If the crude chloroform/ethanol extract is positive for mycobactin, proceed with preparative TLC steps. Otherwise, reduce volume of extract with rotary evaporation, back-extract the concentrate with $CHCl_3$, and repeat TLC analysis on the lower organic layer. (Note 4)

Preparative Thin Layer Chromatography

12._____Load small volume of crude mycobactin extract onto a silica gel 60 preparative TLC plate (20 x 20 cm, 1 mm thickness, F_{254} fluorescent indicator). Run in petroleum ether/*n*-butanol/ethyl acetate (2:3:3) as before, about 1½ hour.

13._____Look for good separation of the two mycobactin bands. If the bands are not straight, decrease the quantity of crude extract loaded on subsequent plates. Save at least 200 µl of crude extract as a total mycobactin control.

14._____Scrape silica for the two bands separately, then extract with CH_3OH twice, and then with $CHCl_3/CH_3OH/H_2O$ (15:4:0.2) twice. It may be more efficient to use Teflon Oakridge tubes rather than glass here. Keep these extracts separate.

15._____Dry down CH_3OH and 15:4:0.2 extracts of upper and lower mycobactin bands separately by rotary evaporation. Resuspend in small volume $CHCl_3$ and evaluate on 10 x 10 cm TLC using previous conditions.

Cleanup and Quality Control

16._____Dilute small volumes of $CHCl_3$ extracts ten-fold in CH_3OH and passage through C18 Sep-Pak cartridges. (Note 5)

17._____Run 50-100 µg of separate extracts on duplicate TLC sheets, one of which should be charred to check for impurities. (Note 6)

18._____Submit upper and lower band mycobactin T for MALDI-TOF analysis. Include MB-J or contract MB-T.U/L (upper or lower band) as positive control.

19._____Perform LAL endotoxin analysis by drying 40-50 µg aliquot, resuspending in CH_3OH, and testing 10-fold through 200-fold dilutions. Include a CH_3OH control for the least-diluted sample.

20._____Complete the QC sheet and inventory the mycobactin.

Notes

(1) The reddish amber color is due to the formation of ferric mycobactin.

(2) This is to prevent collapse of the vessel. Adding more than ¼ volume as 1:47:48 may lead to irretrievable loss.

(3) The aqueous layer should pull the excess iron in the organic layer out, so this should be more amber-tinted. May also see oligosaccharides and lipids precipitate out.

(4) Rotary evaporation will pull out the $CHCl_3$ first, and then the water bath temperature may need to be increased to 45-50ºC to reduce the volume of EtOH. At this point the mycobactin can be retrieved with $CHCl_3$.

(5) Retain at least 200 µg of each extract prior to filtering through Sep-Pak to use as control.

(6) Develop with pet ether/*n*-butanol/ethyl acetate (2:3:3) and check for minimalization of any bands outside of the visible bands, especially near the solvent line. Sample may need to be repassaged through Sep-Pak C18 filter to clear or at least attenuate those impurities before moving on to MALDI analysis.

References

RM Hall and C Ratledge (1982) A simple method for the production of mycobactin, the lipid-soluble siderophore, from mycobacteria. FEMS Microbiology Letters 15:133-36.

JJ De Voss, K Rutter, BG Schroeder, H Su, YQ Zhu and CE Barry III (1 Feb 2000) The salicyclate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. PNAS 97(3):1252-57.

J Gobin, CH Moore, JR Reeve, Jr., DK Wong, BW Gibson and MA Horwitz (May 1995) Iron acquisition by *Mycobacterium tuberculosis*: Isolation and characterization of a family of iron-binding exochelins. PNAS 92:5189-193.