SOP: SP050

Far Eastern Blot for M. leprae PGL-1

Materials and Reagents:

1. Four Thin layer chromatography plates (EMD Chemicals Silica Gel 60 F_{254} #5554-7)
2. Methanol (Alfa Aesar Ultrapure HPLC grade #22909)
3. Chloroform (Alfa Aesar HPLC grade #22920)
4. Ethanol (Pharmco Products 200 proof #111ACS200)
5. Acetone (for cleaning capillary glass pipettors)
6. Kimwipes
7. TLC (Kontes 416180-1020) with aluminum rack.
8. Scissors or razor blade
9. Weighing spatula
10. Foil
11. Ruler
12. Pencil
13. Capillary pipettor, 10 μL
14. Capillary pipets, glass, 10 μL
15. Certified chemical fume hood
16. TLC developer sprayer, 250 mL (Kontes 422530-0250)
17. TLC developer (α-napthol) SOP R012 Heat gun, 200-300°F
18. Flatbed Scanner
19. 10X Blocking Buffer (10% BSA in PBS)
20. Rabbit anti - M. leprae PGL-1 polyclonal antibody (BEI #NR-19355)
21. Goat anti-rabbit – AP IgG whole molecule (Sigma A3687)
22. Milli-Q H_2O
23. Shaker Table
24. Pyrex dish
25. Paper towels
26. BCIP/NBT Phosphatase Substrate (KPL #50-81-10)
27. PBS
28. 10% BSA in PBS
29. M. leprae total lipid diluted to 10mg/mL in 2:1 (v/v) in $CHCl_3$:MeOH
30. M. leprae PGL-1 native lipid diluted to 10mg/mL in 100% EtOH
31. Rabbit anti-PGL-1 polyclonal antibody (BEI # NR-19355)

Protocol:

1.___ Wear gloves during all steps of this protocol.
2.___ Using scissors or razor blade, cut TLC plate to appropriate size (For basic TLC analysis, cut aluminum-backed plates to 10 x 10 cm.
3.___ Using a ruler and pencil, draw a line 1 cm above and parallel to TLC plate bottom.
4.___ Mark and label spots for application of lipid at the origin to the TLC plate. It is best to make all markings no closer than one centimeter to plate edges (to prevent the solvent front from migrating unevenly). Mark and score the top of the TLC plate using a ruler and a weighing spatula to stop the solvent's migration. Turn the plate and repeat this on the right side at the 1cm marks as shown in Figure 1. Four TLC plates will be used, so the markings need to be consistent on all four plates.

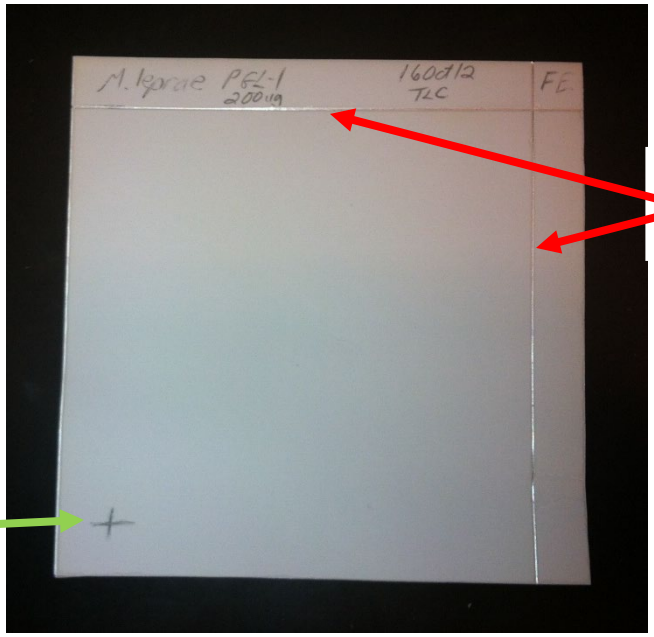

Figure 1 – Labeling and scoring of TLC plate

5.___ Label plates as follows:
i) M. leprae PGL-1 Far Eastern
ii) M. leprae PGL-1 α-napthol charred
iii) M. leprae total lipid Far Eastern
iv) M. leprae total lipid α-napthol charred
6.___ Using a 10 µl glass capillary tube in a 10µl glass capillary pipettor, pipette 100µg (10µl of 10mg/mL) of M. leprae total lipid in 2:1 $CHCl_3$:MeOH onto the M. leprae total lipid Far Eastern pre-marked TLC plate at the point of origin (figure 1). Repeat this for the M. leprae total lipid α-napthol charred plate (note 2).
7.___ Clean the glass capillary pipettors after each compound with 100% Acetone and a Kimwipe.
8.___ Using a new 10 µL glass capillary tube in a 10µL glass capillary pipettor, pipette 20µg (2µl of 10mg/mL) of M. leprae native PGL-1 in 100% EtOH onto the M. leprae PGL-1 Far Eastern pre-

marked TLC plate at the point of origin. Repeat this for the M. leprae PGL-1 α-napthol charred plate.

9.___ Let all compounds applied to TLC plate dry completely.

10.___ Make fresh TLC solvent (95:5 CH_3OH:MeOH v/v)and add to tank, Make 100 mL for TLC tank.

11.___ Allow tank to equilibrate for 5-10 minutes. Tank should be sealed as best as possible to minimize leakage of solvent fumes. Organic solvents need to be HPLC-grade chemicals, which will make the best TLC solvents systems. Fresh solvents are also important for solubilization of lipids for TLC analysis.

12.___ Place plates in equilibrated TLC tank with rack for first direction of TLC run until the solvent migrates to the top of the plate where the scoring mark is.

13.___ Remove plate and let dry completely in chemical fume hood. Keep TLC tank covered so that it can equilibrate for the second direction.

14.___ Turn TLC plate 90 degrees and let solvent run up to scoring mark in the second direction (note 3).

15.___ Once the plate has dried, F_{254} plates may be viewed under low and high wave ultraviolet light to ensure lipid migration.

16.___ Place the two TLC plates marked PGL-1 α-napthol and total lipid α-napthol into the TLC spraying area (Place a partially open box in a chemical fume hood and cover with foil. This will provide a place to set the TLC plate while spraying, and protect the inside of the hood from oxidizing chemicals.

17.___ Thoroughly spray TLC plate with α-napthol developer in TLC sprayer attached to compressed air line in chemical fume hood. Let dry completely.

18.___ Heat TLC plate with heat gun to complete plate development. The plate will look like Figure 2.

Figure 2 – PGL-1 TLC plate after α-napthol development

19.___ Immediately scan developed TLC plate to preserve results. All TLC plate developers will fade with time so it is important to document TLC results as soon as possible.

20.___ Wrap plates in plastic wrap and tape in notebook.

21.___ Prepare 3% blocking buffer in a small Pyrex dish. A minimum of 50 mL of blocking buffer is required for each TLC plate in a Pyrex dish.

22.___ For the Far Eastern PGL-1 and the Far Eastern total lipid TLC plates. Place Pyrex dishes with TLC plates on shaker table and let incubate for at least 1 hour at room temperature. Each plate will need to be in separate Pyrex dishes so that the silica does not come off the plate from friction with the other plate during the shaking incubation step.

23.___ Be very careful when handle wet TLC plates as the silica will come off the plate easily with handling.
24.___ Pour off the blocking buffer and rinse the TLC plate three times briefly with ~100mL PBS. Pour off remaining PBS.
25.___ For the Far Eastern PGL-1 and the Far Eastern total lipid TLC plates, dilute the primary antibody (rabbit anti-PGL-1 BEI# NR-19355) 1:200 in 50mL PBS for each TLC plate in separate Pyrex dishes. Incubate with primary antibody 1 hour.
26.___ Pour off primary antibody and rinse the membrane three times briefly with PBS. Pour off remaining PBS.
27.___ Dilute the secondary antibody (goat anti-rabbit IgG whole molecule) diluted 1:2500 in 50mL PBS. Again the TLC plates need to be in their own Pyrex dishes.
28.___ Incubate with secondary antibody for 35 minutes, too long of an incubation may increase the non-specific binding.
29.___ Rinse the TLC plates three times briefly with PBS.
30.___ Prepare substrate by bringing 50mL/plate of BCIP/NBT to room temperature a few minutes before developing. Apply the substrate to the plates and place on shaker until developed. This can take >30 minutes to develop on the silica matrix.
31.___ Pour off the substrate and rinse with Milli-Q H_20 several times to stop the reaction.
32.___ Place the blot on top of a paper towel to dry. The blot will look like Figure 3.

100μg M. leprae total lipid 20μg M. leprae PGL-1

Figure 3 – Developed Far Eastern Blot of M. leprae total lipid and PGL-1

33.___ Once dry, the Far Eastern Blot can be scanned and placed in a notebook. The blot will fade quickly, so scanning immediately is imperative.

Notes:

1. Taki et. al. recommend transferring Silica TLC plate to PVDF utilizing an iron. We were unable to optimize the protocol with that method at this time.
2. Only load 10μL at a time and letting sample dry in between loading additional volume. This keeps the sample size focused at the point of origin.
3. Thoroughly wash TLC tank. Wash tank using HPLC-grade CH_3OH and dry under compressed air. Then, rinse each surface with HPLC-grade acetone and dry under compressed air.

References:

http://www.hoeferinc.com/downloads/TE22-IME0.pdf

Antibodies: A Laboratory Manual. Ed Harlow and David Lane. Cold Spring Harbor Laboratory, New York. 1988. pp. 471-510.

Besra, GS (1998) Preparation of Cell-Wall Fractions from Mycobacteria. Methods in Molecular Biology, Vol 101: Mycobacterial Protocols (Parish T and Stoker, NG ed), Humana Press Inc, Towata NJ, pp 91-107.

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.

Belisle, JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, and Besra GS. Role of the Major Antigen of Mycobacterium tuberculosis in Cell Wall Biogenesis. Science (276): pp 1420-1422.

Dittmer, JC and Lester, RL. A Simple, Specific Spray for the Detection of Phospholipids on Thin-Layer Chromatography. Journal of Lipid Research (15): pp 126-127.

Kates, Morris. Methods in Lipidology.

Anal Biochem. 1994 Dec;223(2):232-8. *A simple and quantitative purification of glycosphingolipids and phospholipids by thin-layer chromatography blotting.* Taki T, Kasama T, Handa S, Ishikawa D. Department of Biochemistry Faculty of Medicine, Tokyo Medical and Dental University, Japan.

Methods Mol Biol. 2009;536:545-56. *TLC blot (far-eastern blot) and its applications*. Taki T, Gonzalez TV, Goto-Inoue N, Hayasaka T, Setou M.

Analytical Biochemistry. Volume 251, Issue 2, 5 September 1997, Pages 135–143. *TLC Blotting: Application to Microscale Analysis of Lipids and as a New Approach to Lipid–Protein Interaction.* Takao Taki, Dai Ishikawa.http://www.sciencedirect.com/science/article/pii/S0003269797922478 - AB972247FN1 Department of Biochemistry.