SOP: PP016.6
Modified 03/22/2017 by SSP

Separation of LAM, LM, and PIM

Materials and Reagents:

1. LLP Preparation (see SOP PP015)
2. LPS Running Buffer (note 1)
3. Resuspension Butter (note 2)
4. 4x Deoxycholic Acid in LPS Running Buffer (note 3)
5. NaN_3 buffer (note 4)
6. LPS Dialysis Buffer (note 5)
7. 1M NaCl
8. 20% Ethanol (filtered)
9. 10% Methanol (filtered)
10. 0.2μm Steriflip or acrodisc filter
11. 10 or 3 ml syringe
12. 13x100 disposable glass culture tubes
13. SDS-PAGE supplies
14. 12-14,000 MWCO Spectra/Por Dialysis Membrane
15. 6-8,000 MWCO Spectra/Por Dialysis Membrane
16. 3,500 MWCO Spectra/Por Dialysis Membrane
17. 225 ml and 50 ml falcon tubes
18. HiPrep 26/60 Sephacryl-200 HR Column, 320 ml (GE Healthcare LS #17-1195-01)
19. HiPrep 26/60 Sephacryl-100 HR Column, 320 ml (GE Healthcare LS #17-1194-01)
20. HiPrep 16/60 Sepharcyl-200 HR Column, 120 ml (GE Healthcare LS #17-1166-01) For LepLAM only
21. Waters 2535 HPLC
22. Fraction Collector
23. Vortex
24. Sonicator

Protocol:

1._____ The day before you intend to run your sample, set up HPLC (note 6) with the S-200 and S-100 columns connected in tandem (note 7). Ensure that a 10 ml sample injection loop is installed on the HPLC. For LepLAM, set up 120 ml S-200 column and use a 5 ml sample injection loop.

2._____ Rinse columns in 1 column volume (1 CV = 640 ml for tandem columns, or 120 ml for single LepLAM column) filtered endotoxin free water.

3._____ Equilibrate columns in 2 CV of LPS Running Buffer. This step can be done overnight at approximately 1.4 ml/min depending on your start time (16 hours @ 1.4 ml/min = 1344 ml = 2.1 CV). For the LepLAM column, overnight equilibration can be done at 0.25 ml/min (16 hours @ 0.25 ml/min = 240 ml = 2.0 CV).

4._____ Set up fraction collector with glass culture tubes and add 0.5 ml NaN_3 buffer to each tube. The program for the fraction collector is:

- TBLAM or SmegLAM: 80 min wait, 120 fxns @1 min/fxn (Total run time = 200 min)
- LepLAM: 60 min wait, 80 fxns @ 2 min/fxn (Total run time = 240 min)

5._____ Resuspend dried LLP preparation in 4.8 ml LPS Resuspension Buffer per HPLC run (0.9 ml per run for LepLAM, note 8). Vortex and sonicate sample as necessary until it goes into solution. Filter sample through 0.2 μm acrodisc (note 9).

6._____ Collect 4.8 ml sample into a 10 ml syringe. Add 1.6ml of 4X Deoxycholic Acid in LPS Running Buffer just prior to injection and mix in syringe (for LepLAM, use 0.9 ml sample: 0.3 ml 4X Deoxycholic Acid). Then attach the HPLC injection needle. Be sure to expel any bubbles from the syringe and the needle before injection.

7._____ Set HPLC flow rate to 2.5 ml/min (0.5 ml/min for LepLAM).

8._____ Set up a 225 ml Falcon tube to collect the initial void volume off of the column (to be stored until after you verify your sample was properly separated).

9._____ Move the injection lever to LOAD.

10._____ Insert needle completely and inject sample.

11._____ Remove needle and switch the injection lever to INJECT. Hit START on the fraction collector.

12._____ Rinse the injection needle with Milli-Q water, followed by 20% ethanol.

13._____ When the fraction collector is done, run 1 CV buffer through the columns to wash them (note 10). Store fractions at room temperature, as the deoxycholic acid in the running buffer thickens at cold temperatures.

14._____ Run 4 CV of endotoxin-free water through the columns to clean them, followed by 4 CV 20% ethanol to store them. During the washes, continue to watch the pressure to make sure that it does not go over max psi.

15._____ After removing the columns, wash the injector thoroughly with water (in both the LOAD and INJECT positions) to prevent residual buffer from crystalizing. Prime all HPLC lines into 10% methanol for storage.

16._____ Run 10 μl of **every other** fraction on SDS-PAGE gels and develop by silver stain (note 11).

17._____ After staining the gel, run an α-LAM western blot on LM fractions and pool only those without reactivity to LAM for the LM pool (note 12).

18._____ Based on the gels, make pools of pure LAM, LM, and PIM6 and pools of mixed LAM+LM, and LM+PIM6 for later purification.

19._____ Prepare LPS Dialysis Buffer.

20._____ Hydrate dialysis membranes in endotoxin-free water for 30 minutes. The membranes needed for each pool are as follows:

LAM	12-14,000 MWCO
LAM + LM	6-8,000 MWCO
LM	6-8,000 MWCO
LM + PIM	3,500 MWCO
PIM	3,500 MWCO

21._____ Put pools in dialysis membrane and secure both ends. Be sure to leave room for the sample to expand during dialysis.

22._____ Put pools in LPS Dialysis Buffer and place in 37°C incubator for a minimum of 24 hours.

23._____ Change dialysis buffer to 1M NaCl (409.08 g NaCl in 7 L endotoxin free water) and leave stirring at room temperature for a minimum of 24 hours. Discard the LPS Dialysis Buffer as hazardous waste.

24._____ Change dialysis to running DI water for a minimum of 24 hours. Discard the NaCl Dialysis Buffer as hazardous waste.

25._____ Change dialysis to endotoxin free water and leave stirring at room temperature for a minimum of 24 hours, changing water two times during that 24 hours (minimum of 4 hr between changes).

26._____ Remove pools from dialysis and put in preweighed 225ml falcon tubes.

27._____ Freeze dry by lyophilization (note 13).

28._____ Weigh material and perform QC analysis on finished LAM, LM, and PIM6 (note 14). Save mixed pools for further separation (note 15).

Notes:

1. LPS Running Buffer
   1.21g Tris-Base
   11.68g NaCl
   5 g Deoxycholic acid
   2.0 ml 0.5M EDTA
   pH 8.0
   QS to 1L in endotoxin free water
   Filter through 0.2 µm filter
   NOTE: Deoxycholic acid can be irritating to the lungs if inhaled. A mask is recommended. Deoxycholic acid must be added in small increments (~1 g at a time). Allow each addition to go completely in solution before adding more. If necessary, the pH can be raised to encourage the deoxycholic acid to go into solution (be sure to readjust the pH to 8 before use). You will need approximately 4L buffer for 2 HPLC runs.

2. Resuspension Buffer
   0.121g Tris-Base
   1.168g NaCl
   0.2 ml 0.5 M EDTA
   pH 8.0
   QS to 100 ml in endotoxin free water
   A portion of the completed Resuspension Buffer will be used to make 4X Deoxycholic Acid (see below)

3. 4X Deoxycholic Acid
   1 g Deoxycholic acid
   QC to 50 ml Resuspension Buffer
   Filter through 0.2 µm filter

4. NaN_3 Buffer
   120 mg NaN_3
   QS to 100 ml in LPS Running Buffer
   NOTE: Sodium azide (NaN3) is hazardous. Always wear proper PPE (at minimum mask, gloves, and lab coat) while handling powered sodium azide. All buffers containing sodium azide must be disposed of as hazardous waste.

5. LPS Dialysis Buffer
   8.48g Tris-Base
   81.8g NaCl
   1.4g NaN3
   14ml 0.5M EDTA
   pH 8.0
   QS to 7L in endotoxin free water
   This buffer and the subsequent NaCl dialysis buffer must be disposed of as hazardous waste due to NaN_3 content.

6. See SOP SP026 for running Waters 2535 HPLC. All HPLC buffers must be filtered before use.
7. Due to the constraints of tubing length, the first columns (S200) will be mounted to the rings stand upside-down, followed by the second column (S100) mounted right-side-up. Columns are stored in 20% ethanol. Maximum flow rate is 2.6 ml/min. See column handbook for information of maximum pressure and how to determine pressure over the column bed. Also, for more rigorous column cleaning.
8. If the sample is prepared at too great a concentration, it can interfere with filtration and separation of sample on the columns. For this reason, no more than 250 mg sample should be loaded per HPLC run. If there is a large amount of material, more than one HPLC run will be necessary and the sample should be resuspended in no more than 6.4 ml per run (total volume includes 4X Deoxycholic Acid). For LepLAM, resuspend sample in no more than 1.2 ml buffer.

9. If sample will not go through a 0.2 μm filter, it can first be filtered through a 0.8 μm filter, then a 0.45 μm filter if necessary.
10. If doing more than one injection, run a minimum of 150 ml (1 hr @ 2.5 ml/min) Running Buffer through the columns between runs.
11. See SOP SP007 for running of SDS-PAGE gels and SOP SP012 for Silver Staining. Be sure to stop the gel before the dye front reaches the bottom in order to prevent PIM6 from running off the gel. Use the periodic acid step for silver staining.
12. See SOP SO011 for western blotting. Use CS-53 or other anti-LAM antibody.
13. See SOP SP004 for use of the Lyophilizer
14. See SOP PP017 for LAM and LM QC
15. It is best to repeat separation using several mixed pools in order to reduce loss. If small amounts of material are to be separated, use only the S100 column and CVs and run times accordingly.