SOP: PP016

Separation of LAM, LM, and PIM

Materials and Reagents:

1. LLP Preparation
2. LPS Running Buffer
3. LPS Dialysis Buffer
4. 1M NaCl
5. 0.2μm Acrodisc filter
6. 10ml syringe
7. 13x100 disposable glass culture tubes
8. SDS-PAGE supplies
9. 12-14,000 MWCO Spectra/Por Dialysis Membrane
10. 6-8,000 MWCO Spectra/Por Dialysis Membrane
11. 3,500 MWCO Spectra/Por Dialysis Membrane
12. 225ml falcon tubes
13. S-200 Column
14. S-100 Column
15. Waters 600 HPLC
16. Fraction Collector
17. Vortexer
18. Sonicator

Protocol:

1. _____ Set up HPLC (note 1) with the S-200 and S-100 columns connected in tandem (note 2).

2. _____ Rinse columns in 1L filtered endotoxin free water.

3. _____ Equilibrate columns in 600ml (one full column volume) of LPS Running Buffer (note 3).

4. _____ Set up fraction collector with the glass culture tubes. The program for the fraction collector is an 80 minute wait, followed by 120 fractions of 1 minute each.

5. _____ Resuspend dried LLP preparation (note 4) in 10ml LPS Running Buffer (note 5). Vortex and sonicate sample as necessary until it goes into solution.

6. _____ Filter sample through 0.2μm acrodisc (note 6).

7. _____ Collect 10ml sample into a syringe and attach the HPLC injection needle. Be sure to expel any bubbles from the syringe and the needle before injection.

8. _____ Set HPLC flow rate to 2.5ml/min (note 7).

9. _____ Move the injection lever to LOAD.

10. _____ Insert needle and inject sample.

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

12. _____ When the fraction collector is done, run another 500ml buffer through the columns to wash them.

13. _____ Run 1L of water through the columns to clean them, then 700ml 20% ethanol to store them. During the washes, continue to watch the pressure to make sure that it does not go over 100 psi.

14. _____ Run 10μl of every other fraction on SDS-PAGE gels and develop by silver stain (note 8).

15. _____ Based on the gels, make pools of pure LAM, LAM + LM, pure LM, and PIM.

16. _____ Prepare LPS Dialysis Buffer (note 9).

17. _____ Boil dialysis membranes. 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
PIM	3,500 MWCO

18. _____ Put pools in dialysis and place in 37°C warm room for 24 hours.

19. _____ Change dialysis buffer to 1M NaCl and leave stirring at room temperature for 24 hours.

20. _____ Change dialysis to running DI water for 24 hours.

21. _____ Change dialysis to endotoxin free water and leave stirring at room temperature for 24 hours, changing water two times during that 24 hours.

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

23. _____ Freeze dry by lyophilization (note 10).

24. _____ Weigh material and perform QC analysis on finished LAM and LM (note 11). Save the LAM + LM pool and when there are several, repeat protocol to obtain more pure LAM and LM.

Notes:

1. See SOP SP025 for running Waters 600 HPLC
2. Each column is packed in an XK26/70 column casing (Amersham) and has a volume of approximately 300ml. They are stored in 20% ethanol.
3. LPS Running Buffer
   - 1.21g Tris-Base
   - 11.68g NaCl
   - 0.2g NaN3
   - 2.5g Deoxycholic acid
   - 2.0 ml 0.5M EDTA
   - pH 8.0
   - QS to 1L in endotoxin free water

   You will need 1L per HPLC run plus 1L for the initial equilibration of the columns. The buffer must be filtered through a 0.2μm filter before it is put on the HPLC.
4. See SOP PP015 for Preparation of LAM, LM, and PIM
5. 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, it is a good idea to only run about 250mg sample per HPLC run. So, if there is a large amount of material, more than one HPLC run will be necessary and the sample should be resuspended in 10ml per run.
6. If sample will not go through a 0.2μm filter, it can first be filtered through a 0.8μm filter, then a 0.45μm filter if necessary.
7. The columns have a maximum pressure of 120psi, so the pressure should not be over 100psi before injecting your sample. The pressure at a given flow rate can vary depending on the age of the columns, so it is always best to wait and see how the columns behave. They can be run at a lower or higher flow rate depending on the pressure, but the fraction collection must be adjusted accordingly.
8. See SOP SP007 for running of SDS-PAGE gels and SOP SP012 for Silver Staining. Use the periodic acid step for silver staining
9. 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

10. See SOP SP004 for use of the Lyophilizer
11. See SOP PP017 for LAM and LM QC