SOP: PP017.3
Modified 01/24/2013 by SOS

SOP for QC Analysis of LAM, LM, and PIM6

Materials and Reagents:

1. Pure LAM, LM, or PIM6 (see SOP PP016)
2. Endotoxin Free Water
3. D_2O, 99%
4. D_2O, 100%, 0.75 ml vials
5. Alditol Acetate Reagents
6. SDS-PAGE Supplies
7. Western Blotting Supplies
8. CS-35, α-LAM Antibody
9. LAL Assay Reagents
10. 13x100mm Glass Culture Tubes with Screw Caps
11. NMR Tube
12. 9" Borosilicate glass Pasteur pipets
13. 1.2ml Cryovials
14. Savant
15. GC

Protocol:

NMR

1._____ Resuspend dried sample in endotoxin free water at a concentration of about 10 mg/ml based on weight and transfer to 16x100 glass tube that has been acetone washed and air dried.

2._____ Remove approximately half of the sample and transfer to another tube, being sure to note the volume removed (note 1) and dry on savant (note 2).

3._____ To the dried material, add 1 ml 99% D_2O and dry on the savant.

4._____ Repeat D_2O exchange (step 3) once more.

5._____ Add the entire contents of one vial of 100% D_2O. Get sample into suspension and then use an extra-long (9") pasteur pipet to transfer to a clean NMR tube (note 3), cap the tube, and wipe the outside dry with a kimwipe.

6._____ Run NMR (note 4).

7._____ Using an extra-long pasteur pipet, transfer sample back into the 16x100 glass tube and place on the savant to dry.

8._____ Resuspend sample in the same volume of water as was removed in step 2. This will restore the sample to its original concentration.

Quantitation by GC

9._____ From the half of the material, transfer 10 µl aliquots to each of three 13x100 glass tubes that have been acetone washed and air dried. This will be 9 tubes total, 3 each of LAM, LM and PIM6 (note 5).

10._____ Perform alditol acetate derivation on sample (note 6).

11._____ Run GC on sample and calculate the concentration of LAM, LM, or PIM6 (note 7).

Gel & Blot

12._____ Based on the calculated concentration from step 11, run 3 µg of each sample on a SDS gel and silver stain the gel with the periodic acid step (note 8).

13._____ Run 3 μg of each sample on a western blot developed with CS-35 or other α-LAM antibody (note 9).

Endotoxin determination by LAL

14._____ Run a LAL assay in triplicate and calculate endotoxin amount relative to your sample concentration (note 10).

MALDI (for PIM6 Only)

15._____ **This Step For PIM6 Only:** Submit 50 μg PIM6 (at 2 mg/ml in 2:1, chloroform:methanol) for MALDI-TOF analysis.

Aliquot

16._____ Aliquot samples into cryovials in the following amounts:

LAM, SmegLAM – 500 μg
LM, LepLAM – 100 μg
PIM6 – 250 μg

17._____ Freeze-dry by lyophilization (note 11).

Notes:

1. This is the volume that will be added back to the sample after the NMR is complete. While D2O exchanges and NMR are being performed on this half of the sample, you can continue the QC with the rest of the sample, starting with step 9. If your sample is less than 6 ml, it will be necessary to use the whole sample for NMR. NMR is not necessary for PIM6.
2. See SOP SP005 for operation of the Savant
3. To clean NMR tubes do a three wash with each of the following solvents, in order: MilliQ endotoxin-free H_2O, methanol, acetone. After the washes, rinse each tube once with 99% D_2O and allow to air dry.
4. The NMR in the chemistry department. Training/orientation should be scheduled before first use. SOPs are available on-site. If NMR shows buffer contaminants, repeat the dialysis described in SOP PP016 for separation of LAM, LM, and PIM.
5. LepLAM is not quantitated by GC, as the quantity is generally too small to sacrifice large amounts to alditol acetate. Instead, LepLAM concentration is estimated by visualization on a gel as follows:
    a. Run various quantities of a known LAM standard on SDS-PAGE: 1 μg, 2 μg, and 3 μg
    b. On the same gel, run 3 μl of LepLAM to be quantified
    c. Develop the gel by periodate staining (SOP SP012 for Silver Staining using periodic acid step)
    d. Compare band intensity and estimate the number of μg loaded on the gel
    e. Divide the estimated number of μg by 3 (or number of μl loaded on the gel) to obtain mg/ml

    Once concentration is determined, continue SOP starting at step 13.
6. See SOP SP022 for preparation of Alditol Acetate Derivatives
7. To calculate the amount of LAM, LM, and PIM6, see below table:

	NS1	NS2	LAM1	LAM2	LAM3	LM1	LM2	LM3	PIM1	PIM2	PIM3	Areas
Arabinose												
Mannose												
Myo												
Scyllo												

	NS	LAM	LM	PIM	Ave. Areas
Ara					
Man					
Myo					
Scyllo					

	NS	LAM	LM	PIM	All / Scyllo
Ara					
Man					
Myo					

	LAM	LM	PIM	Sample/ External Std	LAM: Add up Ara, Man, and Myo to give you concentration of LAM per 10 µl (or sample volume used to prepare alditol acetate). LM: Add up Man and Myo to give you concentration of LM per 10 µl (or sample volume used to prepare alditol acetate). PIM: Add up Man and Myo to give you concentration of PIM per 10 µl (or sample volume used to prepare alditol acetate).
Ara					
Man					
Myo					
	LAM	LM	PIM	Sample x 25	
Ara					
Man					
Myo					

8. See SOP SP007 for running SDS-PAGE gels, SOP SP012 for Silver Staining (use periodic acid step).
9. See SOP SP011 for Western Blot. When developing the western blot, use CS-35 as the primary antibody and anti-mouse IgG as the secondary antibody. LM should be negative for LAM by western blot. PIM6 requires only a silver stain for QC.
10. See SOP SP020 for LAL Assay procedure and endotoxin calculation. Endotoxin amount must be less than 10 ng endotoxin /mg sample.
11. See SOP SP004 for Lyophilization.