**Demannosylated LAM**

**SOP: PP059.2**
**Modified: 10/12/2022 MS**

**Materials and Reagents:**

1. QC verified purified Lipoarabinomannan (for demannosylation)
2. *M. smegmatis* purified Lipoarabinomannan
3. H37Rv purified Lipoarabinomannan
4. Sodium Acetate
5. Zinc sulfate
6. Digest Buffer: 0.02M Sodium Acetate, 5mM Zinc, pH 4.5
7. α-Mannosidase from Canavalia ensiformis (Jack bean) M7257-Sigma
8. 37°C water bath
9. 13 X 100 mm glass tubes w/ lids
10. Pronase (molecular biology grade)
11. Phenol:choloroform:iso-amyl alcohol (25:24:1)
12. Chloroform:iso-amyl alcohol (24:1)
13. 3,500 MWCO Slide-A-Lyzer cassette or dialysis tubing
14. Micro BCA kit
15. Water, endotoxin-free
16. Stir plate
17. SDS-PAGE gel supplies
18. Alditol Acetate supplies
19. Concanavalin A lectin peroxidase
20. PBS
21. HRP substrate
22. Endozyme II recombinant Factor C Endotoxin Assay Kit – 890030 bioMerieux

**Protocol:**

1. _____ Take 1mg of alpha-mannosidase (Jack Bean) per every 3mg of LAM and centrifuge the Jack bean α-mannosidase solution at 16,000xg for 20 min (α-mannosidase concentration will vary per lot purchased) (note 1).

2. _____ Discard supernatant from the centrifuged α-mannosidase (Jack Bean), then resuspend the pellet at a concentration of 1 mg/1 ml in Digest Buffer: 0.02M Sodium Acetate + 5mM Zinc pH:4.5 Buffer.

3. _____ Resuspend dried LAM at a concentration of 5 mg/ml in Digest Buffer.

4. _____ Add 0.5 mg α-mannosidase per 3 mg of LAM and incubate for 8 hours in 37°C water bath.

5. _____ After 8 hours, add the remaining 0.5 mg of α-mannosidase per 3 mg of LAM into the tube and incubate for an additional 16 hours in 37°C water bath.

6. _____ Add 10 µl of pronase (note 2) stock solution (at 10 mg/mL) for every 1 mL of sample. Incubate at 37°C for 1 hour.

7. _____ Double the sample volume with phenol:choloroform:iso-amyl alcohol (25:24:1) and rock in a fume hood for 30 min.

8. _____ Centrifuge at 12,000xg at 15°C for 30 min.

9. _____ Transfer aqueous later to new tube (discard bottom organic layer into a container for hazardous waste disposal) (note 3).

10. _____ Double the volume of the aqueous layer using chloroform:iso-amyl alcohol (24:1) and rock in a fume hood for 10 min.

11. _____ Centrifuge at 12,000xg at 15°C for 30 min.

12. _____ Transfer aqueous layer to new tube (discard bottom organic layer as stated above).

13. _____ Dialyze the digest in Milli Q water for 24 hours with 3 water exchanges (4 hour minimum between changes). Use 3,500 MWCO dialysis tubing or a Slide-A-Lyzer cassette (depending on sample volume) (note 4).
14. _____ Remove from dialysis and transfer to a new 13 X 100 mm glass tube.

15. _____ Run a Micro BCA to estimate protein concentration. Total protein amount must be less than 10% of the total LAM product (i.e. < 1 mg protein/10 mg LAM) (note 5).
16. _____ Run an Endozyme Assay per manufacturers protocol and calculate endotoxin amount relative to the sample concentration. Endotoxin amount must be less than 10 ng/1 mg of LAM.

17. _____ Run a gel for quantitation: Using a smegLAM standard with a known concentration, create a standard curve on the gel ranging from 1mg - 5µg, and run the demannosylated sample in various amounts (usually 0.5ml – 2 µl; alter this if demannosylated sample falls outside the standard curve). Silver stain the gel (note 6) and use the Image J software (note 7) to create a linear regression. This can be used to calculate the concentration of demannosylated LAM based on densitometry.
18. _____ Run 2 western blots using: 2 µg of sample, 2µg H37Rv LAM, and 5µg HSPX (negative control) per blot (note 8). Develop one using Con A (note 9) and the other using CS-35 (anti-LAM antibody).
19. _____ Run a gel and silver stain using: 5µg of sample, 5µg H37Rv LAM, and 5µg HSPX.

20. _____ Freeze dry by lyophilization.

**Notes:**

1. Starting material should be 10mg LAM or less (a greater amount of LAM hinders mannose cap removal)
2. Ensure pronase is not contaminated with endotoxin using an Endozyme Assay.
3. Interface layer containing white precipitate should be disposed of with phenol layer.
4. Soak cassette or tubing in Milli Q water for 20 minutes prior to sample injection.
5. If protein concentration is too high, repeat steps 6-15.
6. See SOP SP007 for running SDS-PAGE gels, and SOP SP012 for Silver Staining (use periodic acid step)
7. See SOP SP079 for Quantitation of LAM by Image J
8. See SOP SP011 for Western Blot
9. Follow manufacturer protocol for Con A western blot, but reduce the concentration of ConA lectin peroxidase to 5µg/10ml
10. If additional confirmation of demannosylation is needed, the sample can be run on GCMS for linkage analysis. To prepare the sample for analysis, a NaOH permethylation (Ciucanu, et.al.) needs to be done on 100 µg of the demannosylated LAM as well as Smeg and regular H37Rv LAM, followed by preparation of alditol acetate derivatives (SOP SP022).
    Materials:

    1. NaOH pellet (must be kept in decanter)
    2. Dry anhydrous DMSO
    3. Pasteur pipette
    4. Autosampler vials
    5. Mortar and pestle
    6. ACS grade Chloroform
    7. Methyl Iodide

GCMS: Linkages, Retention Times, and Ions

| Linkage | Retention Time | Ions |
| --- | --- | --- |
| t-Ara | 8.53 | 118,129,161 |
| 2-Ara | 10.12 | 129, 130, 161, 190 |
| 5-Ara | 10.77 | 118, 129, 189 |
| t-Man | 11.54 | 102, 118, 129, 145, 161, 162, 205 |
| 3,5-Ara | 12.16 | 118 |
| 2-Man | 13.09 | 129, 161, 190 |
| 6-Man | 13.69 | 102, 118, 129, 162, 189 |
| 2,6-Man | 15.38 | 129, 190 |

- Demannosylation can be confirmed if no 2-Man peak can be found in the sample

**References:**


Ciucanu, I and Kerek, F et al. (1984) A simple and rapid method for the permethylation of carbohydrates study

Snaith, S. M., & Levvy, G. A. (1968). Purification and properties of alpha-D-mannosidase from jack-bean meal. *The Biochemical journal*, *110*(4), 663–670.