Lipoarabinomannan (LAM) and Lipomannan (LM) Production Manual

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SOP: PP015

Preparation of LAM, LM, and PIM

Materials and Reagents:

1. 100g γ-irradiated H37Rv Cells
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3. 32% Triton X-114 (note 1)
4. Breaking Buffer (note 4)
5. Phosphate Buffered Saline (Gibco)
6. Cold 95% Ethanol
7. Water, endotoxin free
8. Alditol Acetate Reagents
9. Pronase Stock Solution
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12. 50ml Teflon Centrifuge Tubes
13. Aluminum Foil
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16. 13x100mm Glass Culture Tubes with Screw Caps
17. Slide-A-Lyzer Cassette 3,500 MWCO
18. 15ml Falcon Tubes
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21. Sorvall Centrifuge
22. Air Bath
23. Probe Sonicator
24. French Press
25. 37ºC Incubator or water bath
26. Savant
27. Mettler-Toledo balance

Protocol:

1. _____ Obtain approximately 100g γ-irradiated cells and freeze dry by lyophilization (note 2).

2. _____ Weigh out dry cells into teflon centrifuge tubes, approximately 3g in each tube.

3. _____ Delipidate cells by adding 30ml 10:10:3 and rocking for 2 hours at room temperature, vortexing the cells every 30 minutes.

4. _____ Centrifuge at 27,000xg at 15°C for 20 minutes.

5. _____ Decant organic supernatant (note 3).

6. _____ Repeat delipidation (steps 3-5) two more times.

7. _____ Cover tubes with foil and place on the air bath to dry (see SOP SP031 for use of the air bath). It will be necessary to stab the needle of the air bath through the foil. Allow cells to dry completely (will probably need to be left overnight to dry).*

8. _____ Use a glass rod to break up clumps of cells and create a fine powder.

9. _____ Add a minimum amount of breaking buffer to get cells into solution (note 4).

10. _____ Freeze/Thaw cells three times to ensure complete suspension.

11. _____ Break cells by passing over the French Press 8 times (notes 5 and 6).

12. _____ Perform an acid fast stain on a smear of the broken cells to check for at least 90% breakage.

13. _____ Spin cells at 2000 x g for 10 minutes to precipitate unbroken cells.

14. _____ Distribute the broken cells equally into 2 teflon tubes and fill the tubes the rest of the way with breaking buffer. Ratio of breaking buffer to cells should be 1:1.

15. _____ Rock at 4°C overnight.*

16. _____ Centrifuge at 27,000xg, 4°C for 1 hour.

17. _____ Decant supernatant into new tubes (note 7) and place pellet at 4°C.

18. _____ Place supernatant in 37°C incubator.

19. _____ When supernatant is partitioned, centrifuge at 27,000xg, 25°C for 15 minutes.

20. _____ Remove top aqueous layer and transfer it to the pellet from step 17. Pool detergent layers and store at 4°C.

21. _____ After adding aqueous layer to cell pellets, split between 2 tubes. Add 8.75ml 32% Triton and fill to 50ml with PBS. This will give an 8% solution.

22. _____ Rock at 4°C for 2 hours.

23. _____ Repeat Triton partition (step 16 to current) two more times (note 8).

24. _____ Add cold 95% ethanol to the pooled detergent layers at a 1:10 concentration and leave at –20°C overnight.*

25. _____ Collect precipitate in teflon tubes and centrifuge at 27,000xg, 4°C for 20 min.

26. _____ Decant supernatant (note 9).

27. _____ Once the precipitate is collected in one tube, transfer it to a preweighed 15ml falcon tube.

28. _____ Dry on the savant (note 10).

29. _____ Weigh material and resuspend in endotoxin free water at a concentration of 50mg/ml.

30. _____ Take a 100µg aliquot to perform alditol acetate derivation (note 11).*

31. _____ Run sample on GC to check for excessive glucan contamination (note 12).

32. _____ Add pronase at a ratio of 1:10 v/v (note 13) and incubate at 37°C overnight.*

33. _____ Dialyze the digest for 24 hours in running DI-water using the 3,500 MWCO Slide-A-Lyzer Cassette.*

34. _____ Remove from dialysis and transfer to preweighed 15ml falcon tube. Take a 50µl aliquot to run on gel (note 14) and check that all the protein has been removed (note 15).

35. _____ Dry remaining volume on savant and weigh material (note 16).

* These are good places to stop at the end of the day

Notes:

1. See SOP R001 for protocol on how to make 32 % Triton X-114
2. See SOP SP004 for use of Lyophilizer
3. Save organic phase for preparation of PIM
4. Breaking Buffer

    50ml 32% Triton X-114
    140µl 1mg/mL Pepstatin A
    100µl 1mg/mL Leupeptin
    400µl 1mg/mL PMSF
    400µl 0.5M EDTA
    150ml PBS

Immediately before using the French Press, add:

    300µg DNase
    330µg RNase

5. If you do not have access to a French Press, or have a cell mass lower than 3 g, you can break your cells with a probe sonicator (12 cycles of 60 seconds on and 90 seconds off).
6. See SOP SP027 for use of French Press. Use 225ml falcon tubes to collect cells while running through French press. When transferring cells to French press cell, rinse the tube with 2ml breaking buffer, but make sure that the cells remain in a small volume of buffer to obtain maximum breakage. If too much buffer is added and the cells are too thin, causing them to pass through the French Press with ease, freeze cells for a few minutes at –80°C after each pass.
7. If the supernatant is not clear at this point, transfer supernatant to new tubes and repeat centrifugation until clear supernatant is obtained. This may require several centrifugation, be sure to transfer to clean tubes each time.
8. After the third partition, the aqueous layer can be discarded. The pellet should be saved for preparation of MAGP (see SOP PP011)
9. There will be a large volume of ethanol, so it will be necessary to perform several centrifugations, each time adding to the tubes already containing precipitate, until all of the precipitate is collected and reduced into one or two tubes.
10. See SOP SP005 for use of the Savant
11. See SOP SP022 for preparation of Alditol Acetate Derivatives
12. If glucose concentration is reasonable, proceed with the rest of the protocol. If it is too high, repeat triton extraction, ethanol precipitation, and GC (steps 18-31)
13. A new stock of pronase should be made for each digestion. Solution made at 10mg/ml.
14. See SOP for Running of SDS-PAGE Gels. Because the concentration is unknown, it is best to run various amounts of sample in each lane of the gel, ranging from 0.5-20µl
15. If protein is seen on the gel, perform a pronase digestion as follows:
    - Add 10µl of pronase stock solution (at 10mg/ml) for every 1ml of sample
    - Incubate at 37°C for 1 hour

    Then extract residual pronase with phenol: chlorofrom: iso-amyl alcohol as follows:
    - Add an equal amount of 25:24:1 (phenol:chloroform:iso-amyl alcohol)
    - Rock in the fume hood for 30 minutes
    - Centrifuge at 12,000xg at 15°C for 30 min
    - Transfer aqueous layer to new tube (discard bottom organic layer into a container for hazardous waste disposal)
    - Add an equal amount of 24:1 (chloroform:iso-amyl alcohol)
    - Rock in the fume hood for 10 min
    - Repeat spin
    - Transfer aqueous layer to new tube
    - Freeze at –80°C and lyophilize
16. To continue purification further, see SOP PP016 for separation of LAM, LM, and PIM

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

SOP: PP017

SOP for QC Analysis of LAM and LM

Materials and Reagents:

1. Pure LAM or LM
2. Endotoxin Free Water
3. D_2O, 99%
4. D_2O, 100%, 0.75ml vials
5. Alditol Acetate Reagents
6. SDS-PAGE Supplies
7. Western Blotting Supplies
8. CS-35, α-LAM Antibody
9. LAL Assay Reagents
10. 16x100mm Glass Culture Tubes with Screw Caps
11. 13x100mm Glass Culture Tubes with Screw Caps
12. NMR Tube
13. 1.2ml Cryovials
14. Savant
15. GC

Protocol:

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

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

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

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 transfer to a clean NMR tube.

6. _____ Run NMR to check for contaminants from buffers (note 3).

7. _____ If NMR is clean, 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.

9. _____ From the original sample (the half not being used for NMR), transfer 50μg aliquots to each of three 13x100 glass tubes.

10. _____ Perform alditol acetate derivation on sample (note 4).

11. _____ Run GC on sample and calculate the concentration of LAM or LM (note 5).

12. _____ Based on the calculated concentration from step 11, run 3μg sample on a gel and 10μg of sample on a western blot (note 6).

13. _____ Place a 100μl aliquot of sample in a cryovial for LAL analysis.

14. _____ Run LAL assay in triplicate and calculate endotoxin amount relative to your sample concentration (note 7).

15. _____ Aliquot samples into 0.25mg, 0.5mg, and 1mg aliquots in cryovials. Dry on the savant, label and store at -80˚C.

Notes:

1. Be sure to record the exact volume removed because 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.
2. See SOP SP005 for operation of the Savant
3. If there are contaminants, repeat the dialysis described in steps 16-24 in the SOP PP016 for separation of LAM, LM, and PIM
4. See SOP SP022 for preparation of Alditol Acetate Derivatives
5. To calculate the concentration of LAM or LM in the sample, add the μg quantities for Mannose and Arabinose given by the GC analysis. Divide this number by the volume of the aliquot used to prepare the alditol acetate. Then multiply by 1.25 (this is a conversion factor). This gives the concentration. Use the average of all three GC runs as your final concentration. For determination of M. smeg LAM use the correction factor of 1.11 instead of 1.25

   ((μg Arabinose + μg Mannose) / μl Sample) x 1.25 = concentration
6. See SOP SP007 for running SDS-PAGE gels, SOP SP012 for Silver Staining (use periodic acid step), and 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
7. See SOP SP020 for LAL Assay. To calculate endotoxin amount, take the concentration given by the analysis (endotoxin units per ml) and divide by 10 (conversion factor to give you ng of endotoxin), then divide by the concentration of sample. This will give you ng endotoxin/mg sample. The endotoxin amount should be less then 10ng/mg
8. Make more of the 0.5mg aliquot because this is the default quantity given to investigators

SOP: R001

Preparation of 32% Triton X-114

Materials and Reagents:

1. 1 Liter bottle
2. PBS (Phosphate Buffered Saline) 1X, pH 7.4 (Gibco cat# 10010-023)
3. Triton® X-114 (Sigma cat# X114-1L)
4. Stir Bar
5. Stir Plate
6. 4°C refrigerator
7. 37°C Water Bath
8. Serological Pipets
9. Pipetaid

Protocol:

1. _____ In a 1 liter bottle combine 150 ml of Triton® X-114 and 150 ml of PBS.

2. _____ Place on stir plate and mix thoroughly. Several hours is recommended.

3. _____ Transfer mixture to 4°C and let stand until the mixture is clear (note 1).

4. _____ Transfer the mixture to a 37°C water bath and incubate until a biphase occurs (note 2).

5. _____ Carefully remove the top layer of the biphase and discard (note 3).

6. _____ Add an equal volume of PBS to the Triton® layer that was not removed (note 4).

7. _____ Return to the stir plate at room temperature and mix until the solution is clear.

8. _____ Return mixture to the 37°C water bath and incubate until the second biphase occurs (note5).

9. _____ Repeat steps 5-8 once more for a total of 3 biphases.

10. _____ On the third time, remove as much of the top layer as possible, then transfer the remaining Triton® to a stir plate to recombine any liquid not removed (note 6).

11. _____ Store at 4°C until use.

Notes:

1. Overnight incubation is recommended. If the mixture is not mixed well enough it will never go completely clear. If this happens remix and incubate again.
2. If the biphase does not occur after several hours of incubation, continue to step 6 (which would be to double the volume with PBS). The biphase may be too little to see; therefore, the liquid would not be able to be pulled off regardless.
3. Be carefully to not disrupt the Triton® layer.
4. For example: from 300 ml of starting material, after the 1st biphase 50 ml is removed from the top layer, then 250 ml of PBS will be added back to the Triton® layer.
5. A biphase will definitely occur from this step forward so do not proceed until a biphase occurs. The volume of the biphase layer will greatly increase on the 2nd and 3rd pass.
6. This will yield about 400-450 ml of 32% Triton® X-114.

SOP: SP020

LAL Endotoxin Assay

Materials and Reagents:

1. QCL-1000 LAL Endotoxin Kit (BioWhitaker Cat # 50-648U)
2. 200 µl Pipettor
3. 200 µl sterile pipet tips
4. 1000 µl Pipettor
5. 1000 µl sterile pipet tips
6. Pyrogen free cryovials (Cat# VWR 66021-944)
7. Pyrogen free 15 ml Falcon tubes (Cat# VWR 21008-918)
8. Endotoxin free water
9. Acetic acid
10. Aluminum foil
11. Lab timer
12. 37^{o}C Incubator
13. 37^{o}C Heat block
14. ELISA plate heat block
15. Plate reader with a 405 nm filter
16. Vortexer
17. Sterile pyrogen free 96-well assay plate (Cat# VWR 21100-006)

Protocol

1. _____ Remove the vial of purified endotoxin in the LAL Kit from the refrigerator and allow to warm to room temperature (note 1).

2. _____ Suspend the endotoxin in 1 ml of sterile endotoxin free water.

3. _____ Vortex the endotoxin for at least 30 minutes.

4. _____ Record the endotoxin level given in the manual for the LAL Kit, this is the EU level.

5. _____ Prepare 0.1, 0.25, 0.5, 0.75 and 1.0 EU/ml dilutions of the endotoxin for the standards with endotoxin free water in the pyrogen free cryovials (note 2).

6. _____ Vortex standards for at least 1 minute.

7. _____ Prepare the stop solution with 25% (v/v) acetic acid in endotoxin free water (note 3).

8. _____ Remove a vial of the chromogenic substrate from the LAL Kit.

9. _____ Prepare a small piece of aluminum foil to wrap around the chromogenic substrate vial.

10. _____ Suspend the chromogenic substrate in 6.5 ml of endotoxin free water and wrap the vial in the aluminum foil.

11. _____ Place the chromogenic substrate in the 37^{o}C incubator.

12. _____ Pipet 50 µl of each endotoxin standard into two wells in a sterile 96-well plate (note 4).

13. _____ Prepare 150 µl dilutions of the sample to be tested in pyrogen free cryovials using endotoxin free water. These dilutions can be 10, 50, 100, 250, 500 or any combination that will give a broad range so that the value will be a good fit on the EU curve (notes 5 and 6).

14. _____ Pipet 50 µl of each sample dilution into three wells on the 96-well plate.

15. _____ Place the 96-well plate on the 37°C ELISA plate block.

16. _____ Once the chromogenic substrate and the samples in the 96-well plate have come up to temperature, you can proceed with the rest of the assay (usually 1-2 hours to be safe).

17. _____ Set the lab timer for ten minutes, but do not hit start yet.

18. _____ Suspend the Limulus Amebocyte Lysate (LAL) in 3 ml of endotoxin free water, this should be done immediately prior to beginning the actual assay for maximum efficiency (note 7).

19. _____ Beginning with the first dilution of the standard curve, pipet 50 µl of the LAL into the well. Pipet up and down 3 times to ensure proper mixing. It is very important to pipet each well in the same manner to achieve maximum consistency among all wells.

20. _____ Immediately after pipetting the first well, start the lab timer.

21. _____ Continue pipetting until all wells have been mixed with the LAL substrate.

22. _____ Change the setting on the pipettor to 100 µl.

23. _____ When the timer comes close to the end, remove the chromogenic substrate from the incubator.

24. _____ When the timer sounds, reset it for six minutes.

25. _____ Using the same technique as before, pipet 100µl of the chromogenic substrate into each of the wells.

26. _____ When the timer sounds, pipet 100 µl of the stop solution (25% acetic acid) into each of the wells as before.

27. _____ Read the plate on the plate reader using the 405 nm filter.

28. _____ The readings from the plate reader are given in EU/ml. To calculate the level of endotoxin in the sample, perform the following calculation.

EU/ml x 1 ng/ 10 EU x 1 ml/? mg

This calculation begins with the value from the microplate manager printout
The 1 ng/ 10 EU is a conversion factor to change from EU to ng units
The 1 ml/? mg is the protein concentration from a BCA assay in inverse form.
This calculation will give nanograms endotoxin/milligrams protein as the measurement

Notes:
1. The LAL Endotoxin kit should be stored at 4°C.
2. The endotoxin standards should also be kept at 4°C, and are viable for up to two weeks. After that new standards should be prepared.
3. The stop solution is stable at room temperature for many months.
4. Extreme care should be taken when pipetting into the wells of the 96-well plate as to not touch anything but the inside walls of the wells to avoid contamination of the sample within the well.
5. Consistent pipetting is the key to achieving good results with this assay. Make sure to carefully and accurately pipet when making standards, and dilutions of the samples.
6. Generally for recombinant proteins dilutions of 1:10, 1:50, and 1:100 are sufficient.
7. If lysate is frozen at -20ºC immediately after use it can be thawed and used one more time.

SOP: SP022

Preparation of Alditol Acetate Derivatives

Materials and Reagents:

1. Sample, 20 to 50 μgs
2. Rhamose standard, 10 mg/ml in B & J water
3. Fucose standard, 10 mg/ml in B & J water
4. Ribose standard, 10 mg/ml in B & J water
5. Arabinose standard, 10 mg/ml in B & J water
6. Xylose standard, 10 mg/ml in B & J water
7. Mannose standard, 10 mg/ml in B & J water
8. Galactose standard, 10 mg/ml in B & J water
9. Glucose standard, 10 mg/ml in B & J water
10. Myo-inositol standard, 10 mg/ml in B & J water
11. Scyllo-inositol standard, 1 mg/ml in B & J water
12. Trifluoroacetic acid, concentrated, 1 ml ampule
13. Water, Burdick & Jackson HPLC-grade
14. Methanol, Burdick & Jackson HPLC-grade
15. Sodium borodeuteride ($NaBD_4$), solid
16. Ammonium hydroxide, concentrated
17. Ethanol, absolute
18. Acetic acid, glacial
19. Acetic anhydride, 2 ml ampule
20. Chloroform, Burdick & Jackson HPLC-grade
21. Savant speed-vac
22. 13 x 100 mm glass tubes (twice as many as number of samples plus standard)
23. 13 mm PTFE-lined lids (as many as number of samples plus standard)
24. Capillary pipettor, 0-100 μl
25. Glass capillary pipets, 100 μl
26. Heat block, 120°C
27. Air bath
28. Glass Pasteur pipets
29. Rubber Pasteur pipet bulb
30. Dessicator
31. Vortex
32. Benchtop centrifuge
33. Capillary pipettor, 0-10 μl
34. Glass capillary pipets, 10 μl

Protocol:

1. _____ Transfer each sample into a cleaned 13 x 100 mm glass tube.

2. _____ Combine 25 μg of each sugar standard, except scyllo-inositol, in a new 13 x 100 mm glass tube.

3. _____ Completely dry all samples and the neutral sugar standard on the savant (note 1).

4. _____ Add 250 μl of 2M TFA to each sample and the standard (notes 2 and 3).

5. _____ Cap each tube tightly and place in 120°C heat block for two hours.

6. _____ Remove samples from the heat block and let cool to room temperature.

7. _____ Add 10 μg of scyllo-inositol to each sample and the standard (note 4).

8. _____ Completely dry the contents of each tube on the air bath (note 5).

9. _____ Add approximately 100 µl of methanol to each sample (note 6).

10. _____ Completely dry on air bath.

11. _____ Repeat steps 9 and 10.

12. _____ Make $NaBD_4$ solution and add 250 µl to each sample (note 7).

13. _____ Cap each tube and let sit on benchtop overnight (note 8).

14. _____ Add two drops of glacial acetic acid to each sample (note 9).

15. _____ Add 200 µl of 10% acetic acid in methanol solution to each sample (note 10).

16. _____ Dry completely on air bath.

17. _____ Repeat steps 15 and 16.

18. _____ Add approximately 100 µl of methanol to each sample (note 6).

19. _____ Completely dry on air bath.

20. _____ Repeat steps 18 and 19.

21. _____ Add 100 µl of acetic anhydride from ampules to each sample.

22. _____ Cap each tube and heat at 120^{o}C for two hours in a heating block.

23. _____ Remove samples from the heat block and let cool to room temperature.

24. _____ Completely dry on air bath.

25. _____ Add 1 ml of water to each sample.

26. _____ Add 2 ml of chloroform to each sample.

27. _____ Cap each tube and mix by vortexing vigorously.

28. _____ Centrifuge at 2,500 x g, 4^{o}C for five minutes.

29. _____ Transfer the lower, organic layer from each sample into new 13 x 100 mm glass tubes and discard water layer (note 11).

30. _____ Completely dry on air bath.

31. _____ Sample is now ready for GC analysis.

Notes:
1. See SOP SP005 for use of savant
2. Use a fresh ampule of concentrated trifluoroacetic acid (TFA) to make 2M TFA for each alditol acetate preparation. Concentrated TFA is 12.98 M; use 153 µl of acid to every 847 µl of water to make each 1 ml of 2M TFA. Make in a glass container.
3. Use only glass capillary pipets to transfer liquids from this step on, as the GC will detect plastic components from Pipetman tips.

4. Scyllo-inositol is used as an internal standard for the GC to calculate the amount of neutral sugar in the sample.
5. See Air Bath SOP SP031.
6. This approximation is five drops from a glass Pasteur pipet with a rubber bulb. It is not necessary to add a specified amount of methanol, only enough to saturate the sample and remove any residual water from the sample.
7. The $NaBD_4$ solution is 10 mg of $NaBD_4$ in 1 ml of 1 M NH_4OH in ethanol, and must be freshly made prior to use. $NaBD_4$ is extremely hygroscopic and must be kept in a dessicator. 1 M NH_4OH in ethanol must be made fresh every two months. To make, add 6.6 ml of concentrated NH_4OH to 93.4 ml of absolute ethanol; as NH_4OH is caustic, make in a chemical fume hood.
8. The reduction reaction is complete in one hour, but overnight reduction provides the best results.
9. The addition of glacial acetic acid should cause the sample to bubble and fizz, indicating the required excess of reducing agent was present.
10. Make 10 % acetic acid in methanol as any other standard v/v solution; glacial acetic acid is caustic, so make in chemical fume hood.
11. It is important not to contaminate the organic layer with debris from the water layer. To do this, expel several drops of air while passing the Pasteur pipet through the water layer until the tip is in the organic layer. It is better to leave a small amount of organic layer in the tube than to risk water contamination.

Reference:

McNeil, M., D. Chatterjee, S. W. Hunter, and P. J. Brennan. 1989. Mycobacterial glycolipids: isolation, structures antigenicity and synthesis of neoantigens. Methods Enzymology. 179: 215-242.

SOP: SP027

Operation of French Press

Materials and Reagents:

1. Whole cells (note 1)
2. Breaking buffer (note 2)
3. Ice bucket
4. Ice
5. Glycerol
6. French Press cell (note 3)
7. French Press cell stand
8. Falcon centifuge bottle, 225 ml
9. Lysol I.C. solution, 10%
10. Ethanol solution, 70%
11. Paper towels

Protocol:

1. _____ Suspend cells in breaking buffer at a concentration of 0.5 ml/gram cells (note 4).

2. _____ Place suspended cells on ice in ice bucket.

3. _____ Use a small amount of glycerol to lubricate French Press cylinder and bottom.

4. _____ Attach bottom of French Press to French Press cell and place unit on French Press stand.

5. _____ Attach Pressure Release knob and spout to bottom, making sure knob is closed completely.

6. _____ Place French Press cylinder into unit until reaching “max fill” mark.

7. _____ Turn unit upside-down, place on French Press stand, and remove bottom from unit.

8. _____ Add cell suspension to French Press unit, leaving enough room to attach bottom to the unit.

9. _____ Attach bottom to unit.

10. _____ Turn complete unit right-side up and place in French Press (note 5).

11. _____ Turn French Press on, and move lever to Up/High setting (Med setting if using the mini-cell).

12. _____ Using Pressure Release knob, keep pressure on cell between 1000 and 1500 while collecting eluent into the 225ml Falcon centrifuge bottle (note 6).

13. _____ When French Press cell is empty, turn machine lever to down.

14. _____ Place eluent collection bottle on ice in ice bucket, and carefully remove cell from the French Press.

15. _____ Repeat steps 7 to 14 until cell suspension has been passed through the French Press cell a total of six times.

16. _____ Check breakage by acid fast staining (note 7).

17. _____ When finished, thoroughly clean French Press using Lysol I.C. solution followed by 70% ethanol.

18. _____ Thoroughly clean French Press cell by completely disassembling unit, and washing each part with Lysol I.C. solution, distilled water, then 70% ethanol.

19. _____ Let all parts completely air dry prior to storage.

Notes:

1. *M. tuberculosis* cells must have been γ-irradiated according to SOP PP004 prior to breaking. If *M. tuberculosis* cells are live, protocol MUST be completed under BioSafety Level III conditions in room 101D at BHRB
2. Breaking buffer is made according to SOP PP007, PP008 or PP015, depending on your needs.
3. French Press cell unit contains the following parts: French Press cell, cylinder, bottom, pressure release valve, and spout. The French Press Mini-Cell will also need a silicone bead on the pressure release valve.
4. Cell solution should be viscous in order for proper breaking to occur.
5. Care should be taken to hold onto the unit bottom, otherwise gravity may cause loss of sample due to bottom removal. When the cell is placed in the French Press, be sure that the unit is flush against the bottom pegs, and be sure to turn the cylinder handle perpendicular to the bracing bar.
6. Care should be taken to point mouth of bottle away from eyes and head, as small air pockets inside the French Press cell may cause violent eruption of eluent when expelled.
7. Cells should be more than 90% broken. See SOP SP035 for Acid-Fast staining.

Reference:

Thermo IEC Operation Manual OMFA 078A Revision 0