Preparation and Fractionation of Cell Wall Proper

Version 1
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SOP: PP011

Preparation of mAGP

Materials and Reagents:

1. Cell wall from *M. tuberculosis*
2. Phosphate buffered saline (PBS), sterile (Gibco 10010-023)
2. Sodium dodecylsulfate (SDS)/Lauryl sulfate (Sigma L-3771)
3. Water, HPLC-grade (VWR BJ365-4)
4. Acetone, HPLC-grade (VWR BJ010-1)
5. Proteinase K (Roche 1000144), stock solution of 10 mg/ml
6. Magnetic stir bar, small
7. Magnetic stir/heat plate
8. Magnetic stir bar retriever
9. Sorvall centrifuge
10. Sorvall SS-34 centrifuge rotor
11. Oakridge centrifuge tube, 50 ml, teflon, sterile
12. Warm room or reach-in incubator, 37^{o}C
13. Beaker, 250 ml
14. Thermometer
15. Chemical fume hood
16. Glass tube with PTFE-lined lid, 16 x 100 mm
17. Mettler-Toledo balance

Protocol:

1. _____ Transfer *M. tuberculosis* cell wall to a sterile 50 ml Oakridge tube.

2. _____ Add 30 ml of 2% SDS solution and a small magnetic stir bar (note 1).

3. _____ Cap tube and place on magnetic stir plate at room temperature for 30 minutes.

4. _____ Remove the stir bar and centrifuge at 27,000 xg, 25^{o}C for 10 minutes.

5. _____ Decant the supernatant.

6. _____ Repeat steps 2 to 5 twice more.

7. _____ Add 26 ml of 2% SDS solution, 4 ml of Proteinase K stock solution and a small magnetic stir bar.

8. _____ Cap tube and place on magnetic stir plate at room temperature for 30 minutes.

9. _____ Remove the stir bar and centrifuge at 27,000 xg, 25^{o}C for 10 minutes.

10. _____ Decant the supernatant.

11. _____ Add 30 ml of 2% SDS solution and a small magnetic stir bar.

12. _____ Cap tube and place on magnetic heat/stir plate at 90^{o}C for one hour (note 2).

13. _____ Remove the stir bar and centrifuge at 27,000 xg, 25^{o}C for 10 minutes.

14. _____ Decant the supernatant.

15. _____ Repeat steps 11 to 14 twice more.

16. _____ Add 30 ml of water and a small magnetic stir bar.

17. _____ Cap tube and place on magnetic stir plate at room temperature for 30 minutes.

18. _____ Transfer sample, but not the stir bar, to a sterile 50 ml Teflon Oakridge tube.

19. _____ Centrifuge at 27,000 xg, 25^{o}C for 10 minutes.

20. _____ Decant the supernatant.

21. _____ Add 5 ml of acetone and a small magnetic stir bar.

22. _____ Cap tube and place on magnetic stir plate at room temperature for 10 minutes.

23. _____ Remove the stir bar and centrifuge at 27,000 xg, 25^{o}C for 10 minutes.

24. _____ Decant acetone and place in chemical fume hood and allow to dry completely (note 3).

25. _____ Transfer the purified mAGP to a new, pre-weighed 16 x 100 mm glass tube and weigh.

Notes:
1. The 2% SDS solution is made with sterile PBS. Use 2 g of SDS per 100 ml of PBS.
2. Fill a 250 ml beaker with water and heat to 90^{o}C on a heat/stir plate; monitor temperature of water with a thermometer, and add water as needed.
3. Do not dry sample on air bath, as the sample will be blown away with the acetone. Before placing in chemical fume hood, decant as much acetone as possible without decanting the pellet.

Reference:
Daffe, M., *et. al.* Journal of Biological Chemistry. 1990. 265:6734.

SOP: PP014

Preparation of Mycolic acid methyl esthers

Materials and Reagents:

1. Purified mAGP, 50 to 150 mg
2. Tetrabutyl ammonium hydroxide (TBAH) (Fluka 86854), 15% solution
3. Dichloromethane (Aldrich 270997-100ml)
4. Iodomethane (Aldrich 289566-100g)
5. Diethyl ether (VWR BJ107-1)
6. Glass tubes with PTFE-lined lids, 13 x 100 mm
7. Glass pipet, 5 ml
8. Pipet bulb, rubber
9. Heat block, 100^{o}C
10. Glass capillary pipet, 100 µl
11. Glass capillary pipettor, 100 µl
12. Platform rocker
13. Benchtop centrifuge
14. Pasteur pipets, glass
15. Pasteur pipet bulb, rubber
16. N_2 bath
17. Vortex
18. Pan sonicator
19. Mettler-Toledo balance
20. Materials and equipment for TLC
21. Petroleum ether (VWR BJ317-1)

Protocol:

1. _____ Transfer mAGP into a 13 x 100 mm glass tube.

2. _____ Add 2 ml of 15% TBAH solution (note 1).

3. _____ Cap tube and place in heat block at 100^{o}C for two hours (note 2).

4. _____ Remove from heat block and let cool to room temperature.

5. _____ Add 2 ml of dichloromethane.

6. _____ Add 100 µl of iodomethane (note 3).

7. _____ Cap tube and place on platform rocker at room temperature for one hour.

8. _____ Centrifuge at 2,500 x g, 4^{o}C for 10 minutes.

9. _____ Transfer organic, lower layer to a 13 x 100 mm glass tube (note 4). The insoluble pellet should be saved for further purification (note 5).

10. _____ Completely dry organic layer under a stream of nitrogen (note 6).

11. _____ Add 3 ml of diethyl ether, cap tube and re-suspend dried organic layer (note 7).

12. _____ Transfer to a new, pre-weighed 13 x 100 mm glass tube

13. _____ Completely dry under a stream of nitrogen and weigh.

14. _____ Re-suspend in a minimal amount of dichloromethane.

15. _____ Load 10 to 20 μg onto a 10 x 10 cm aluminum-backed TLC plate (note 8).

16. _____ Run the plate six times in solvent system petroleum ether-diethyl ether (95:5), allowing the plate to dry between runs.

17. _____ Visualize purified MAME with charring spray (note 9).

Notes:
1. All organic and acidic compounds need to be transferred using glass pipets or glass capillary pipets.
2. Two hours is the minimum time for hydrolysis, but the reaction may be run overnight.
3. Iodomethane acts as a catalyst for phase transfer to make methyl esthers.
4. It is important not to contaminate the organic layer with debris from the aqueous layer. To do this, expel several drops of air while passing the Pasteur pipet through the upper layer until the tip is in the lower layer.
5. The insoluble material is de-mycolated AGP, and should be saved and used for the purification of arabinogalactan and peptidoglycan (SOP PP012 and PP013). Using a glass Pasteur pipet, remove the aqueous layer and dry the pellet in a chemical fume hood.
6. See SOP SP031 for use of the N_2 Bath. A white, salty precipitate should be remaining in the tube.
7. The pellet is difficult to re-supsend, and will need to be pan sonicated and vortexed vigorously. This step removes contaminating salts from the MAME.
8. See SOP SP033 for running TLC.
9. See SOP R011.

Reference:
Slayden R. A., C. E. Barry III. Analysis of the Lipids of Mycobacterium tuberculosis. Methods in Molecular Medicine, Mycobacterium tubeculosis Protocols. T. Parish and N. G. Stoker, ed. 2001. 229-246.

Personal correspondance with Dr. Richard A. Slayden, Mycobacterial Research Laboratories, CSU, Fort Collins, CO.

SOP: PP012

Preparation of Arabinogalactan

Materials and Reagents:

1. AGP (mAGP without mycolates), 50 to 150 mg
2. Sulfuric acid, 0.05M
3. Barium carbonate
4. Water, HPLC-grade (VWR BJ365-4)
5. Reagents for alditol acetates (note 6)
6. Glass tubes with PTFE-lids, 16 x 100 mm
7. Magnetic stir bar, small
8. Magnetic stir plate
9. Warm room or reach-in incubator, 37^{o}C
10. Magnetic stir bar remover
11. Benchtop centrifuge
12. Pasteur pipet, glass
13. Pasteur pipet bulb, rubber
14. Savant speed-vac
15. Glass tubes with PTFE-lined lids, 13 x 100 mm
16. Glass capillary pipet, 10 µl
17. Glass capillary pipetor, 10 µl
18. Gas Chromatograph

Protocol:

1. _____ Transfer AGP into a new 16 x 100 mm glass tube (note 1).

2. _____ Add 5 ml of 0.05M sulfuric acid and a small magnetic stir bar.

3. _____ Cap tube and place on a magnetic stir plate at 37^{o}C for four days.

4. _____ Remove the small magnetic stir bar and centrifuge at 3,000 x g, 25^{o}C for 15 minutes.

5. _____ Transfer the supernatant to a new 16 x 100 mm tube (note 2).

6. _____ Add a few grains of barium carbonate (note 3).

7. _____ Cap tube and place at room temperature overnight.

8. _____ Centrifuge at 3,000 x g, 25^{o}C for 15 minutes.

9. _____ Transfer the supernatant to a new, pre-weighed, 16 x 100 mm tube (note 4).

10. _____ Completely dry on savant speed-vac (note 5).

11. _____ Re-suspend dried material in 5 ml of HPLC-grade water.

12. _____ Transfer two 2.5 µl aliquots to two 13 x 100 mm glass tubes.

13. _____ Completely dry on savant and prepare alditol-acetate derivatives (note 6).

14. _____ Analyze derivatives by GC to ensure the purified AG contains only arabinose and galactose.

15. _____ Make 0.25 mg aliquots based on the dry weight, dry on the savant, and store at -80˚C.

Notes:

1. AGP is obtained from SOP PP014.

2. At this point an insoluble pellet will be remaining; this is peptidoglycan, and should be further processed according SOP PP013.
3. Barium carbonate neutralizes the weak acid, and only a small amount is required to accomplish this.
4. It is important to leave behind the residual salt pellet in the bottom of the tube.
5. See SOP SP005 for use of the savant.
6. See SOP SP022 for derivative preparation and SP045 for GC operation.

Reference:

Personal correspondance with Dr. Michael R. McNeil, Mycobacterial Research Laboratories, Colorado State University, Fort Collins CO and Dr. Phillip Draper, National Institute for Medical Research, Mill Hill, London United Kingdom.

SOP: PP013

Preparation of Peptidoglycan

Materials and Reagents:
1. AGP (mAGP without mycolates), 50 to 150 mg
2. Sulfuric acid, 0.05M
3. Water, HPLC-grade (VWR BJ365-4)
4. Reagents for alditol acetates (note 4)
5. Glass tubes with PTFE-lids, 16 x 100 mm
6. Magnetic stir bar, small
7. Magnetic stir plate
8. Warm room or reach-in incubator, 37^{o}C
9. Magnetic stir bar remover
10. Benchtop centrifuge
11. Vortex
12. Pasteur pipet, glass
13. Pasteur pipet bulb, rubber
14. Savant speed-vac
15. Glass tubes with PTFE-lined lids, 13 x 100 mm
16. Glass capillary pipet, 10 µl
17. Glass capillary pipetor, 10 µl
18. Gas Chromatograph

Protocol:
1. _____ Transfer AGP into a new 16 x 100 mm glass tube (note 1).

2. _____ Add 5 ml of 0.05M sulfuric acid and a small magnetic stir bar.

3. _____ Cap tube and place on a magnetic stir plate at 37^{o}C for four days.

4. _____ Remove the small magnetic stir bar and centrifuge at 3,000 x g, 25^{o}C for 15 minutes.

5. _____ Transfer the supernatant to a new 16 x 100 mm tube (note 2).

6. _____ Add 5 ml of HPLC-grade water to the pellet.

7. _____ Cap tube and vortex vigorously.

8. _____ Centrifuge at 3,000 x g, 25^{o}C for 15 minutes.

9. _____ Decant the supernatant into a new, preweighed 16 x 100 mm tube and completely dry on savant speed-vac (note 3).

10. _____ Re-suspend the dried material in 5 ml of HPLC-grade water.

11. _____ Transfer two 25 µl aliquots to two new 13 x 100 mm glass tubes.

12. _____ Completely dry on savant and prepare alditol-acetate derivatives (note 4).

13. _____ Analyze derivatives by GC to ensure the purified PG does not contain arabinose and galactose (note 5).

14. _____ Make 0.25 mg aliquots based on the dry weight, dry and store at -80˚C.

Notes:
1. AGP is obtained from SOP PP014.

2. At this point the supernatant is soluble arabinogalactan, and should be further purified using SOP PP012.
3. See SOP SP005 for use of savant.
4. See SP022 for derivative preparation and GC operation.
5. If the GC analysis shows contaminating arabinose and/or galactose, then the protocol needs to be repeated, until the peptidoglycan is devoid of arabinogalactan.

Reference:

Personal correspondance with Dr. Michael R. McNeil, Mycobacterial Research Laboratories, Colorado State University, Fort Collins CO and Dr. Phillip Draper, National Institute for Medical Research, Mill Hill, London United Kingdom.

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: SP033

Thin-Layer Chromatography

Materials and Reagents:

1. Thin layer chromatography plates (note 1)
2. Organic solvents (note 2)
3. TLC tank (note 3)
4. Scissors or paper cutter
5. Foil
6. Ruler
7. Pencil
8. Capillary pipettor, 10 µl
9. Capillary pipets, glass, 10 µl
10. Plastic wrap
11. Lead weight
12. Chemical fume hood
13. TLC developer sprayer, 250 ml (Kontes 422530-0250)
14. TLC developer (note 4)
15. Heat gun, 200-300^{o}F
16. Scanner

Protocol:

1. _____ Using scissors or paper cutter, cut TLC plate to appropriate size (note 5).

2. _____ Using a ruler and pencil, draw a line 1 cm above and parallel to TLC plate bottom.

3. _____ Mark and label spots for application of compound to TLC plate (note 6).

4. _____ Using a 10 µl glass capillary tube in a 10 µl glass capillary pipettor, add compound(s) to TLC plate (note 7).

5. _____ Let all compounds applied to TLC plate dry completely.

6. _____ Thoroughly wash TLC tank (note 8).

7. _____ Make fresh TLC solvent and add to tank (note 9).

8. _____ Cover tank opening with plastic wrap, followed by tank lid, and place lead weigh on top. Allow tank to equilibrate for 5-10 minutes (note 10).

9. _____ Place plate(s) in equilibrated TLC tank.

10. _____ Let plate(s) sit in tank until solvent front reaches top of plate.

11. _____ Remove plate and let dry completely on foil in chemical fume hood.

12. _____ Place plate in TLC spraying area (note 11).

13. _____ Thoroughly spray TLC plate with desired developer in TLC sprayer attached to compressed air line in chemical fume hood.

14. _____ If necessary, heat TLC plate with heat gun to complete plate development.

15. _____ Immediately scan developed TLC plate to preserve results (note 12).

16. _____ Wrap plates in plastic wrap and tape in notebook.

Notes:
1. Depending on the type of TLC needed, there are three basic types of TLC plates to use. For TLC analysis, use aluminum-backed EM Science F254 plates (EM Science 5554/7). For small preparative TLC, use plastic-backed EM Science F254 plates (EM Science 5735/7). For large preparative TLC, use glass-backed EM Science F254 plates (EM Science 13792/7). For performing preparative TLC, please see SOP SP032 Preparative Thin Layer Chromatography.
2. Fresh HPLC-grade chemicals, preferably Burdick & Jackson, make the best TLC solvents systems. Commons systems are $CHCl_3$-CH_3OH-H_2O (60:30:6) for PIM preparation/analysis and $CHCl_3$:CH_3OH:NH_4OH (80:20:2) for TDM preparation/analysis. Fresh solvents are also important for solubilization of lipids for TLC analysis.
3. For small TLC's (5 x 10 cm or 10 x 10 cm) use a small Kontes tank (Kontes 416180-1020). For large TLC's (20 x 10 cm or 20 x 20 cm) use a large Kontes tank (Kontes 416180-0000).
4. There are numerous detection sprays, based on what molecule is to be detected. See SOP for charring spray, α-napthol and Dittmer-Lester for details. Additionally, F_{254} plates may be viewed under low and high wave ultraviolet light, and all plates may be placed in a tank with iodine crystals, which will temporarily stain some compounds.
5. For basic TLC analysis, cut aluminum-backed plates to 5 x 10 cm, 10 x 10 cm or 20 x 10 cm.
6. It is best to make all spots one centimeter apart, and no closer than one centimeter to plate edges (to prevent the solvent front from migrating unevenly).
7. Ideally, compound should be suspended in $CHCl_3$.CH_3OH (2:1) at a concentration around 5-10 μg per μl, and 10-30 μg should be applied to plate. Each spot should be applied as a small band. New glass capillaries should be used for each compound
8. Wash tank using mild detergent soap. Rinse each side three times with hot water, three times with deionized water, once with HPLC-grade CH_3OH and dry under compressed air. Then, rinse each surface with HPLC-grade acetone and dry under compressed air.
9. Make approximately 10 ml for small TLC tanks and 100 ml for large TLC tanks.
10. Tank should be sealed as best as possible to minimize leakage of solvent fumes. Preferably, place tank in area with low air flow.
11. Place a partially open box in a chemical fume hood and cover with foil. This will provide a place to set the TLC plate while spraying, and protect the inside of the hood from oxidizing chemicals.
12. All TLC plate developers will fade with time; Dittmer-Lester may disappear within minutes, so it is important to document TLC results as soon as possible.

References:
Besra, GS (1998) Preparation of Cell-Wall Fractions from Mycobacteria. Methods in Molecular Biology, Vol 101: Mycobacterial Protocols (Parish T and Stoker, NG ed), Humana Press Inc, Towata NJ, pp 91-107.

Slayden, RA and Barry 3rd, CE (2001). Analysis of the Lipids of Mycobacterium tuberculosis. Mycobacterium tuberculosis Protocols (Parish T and Stoker, NG ed), Humana Press Inc, Towata NJ, pp 229-246.

Belisle, JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, and Besra GS. Role of the Major Antigen of Mycobacterium tuberculosis in Cell Wall Biogenesis. Science (276): pp 1420-1422.

Dittmer, JC and Lester, RL. A Simple, Specific Spray for the Detection of Phospholipids on Thin-Layer Chromatography. Journal of Lipid Research (15): pp 126-127.

SOP: R011

Preparation of Charring Spray for TLC analysis

Materials and Reagents:

1. Graduated beaker, 150 ml
2. Magnetic stir bar
3. Magnetic stir plate
4. Graduated cylinder, 100 ml
5. Cupric sulfate pentahydrate (VWR MK475210)
6. Phosphoric acid (VWR MK279618)
7. Water, HPLC-grade (VWR BJ365-4)
8. Serological pipet, glass, 10 ml
9. Pipet bulb
10. Chemical fume hood
11. TLC sprayer, 250 ml (Kontes 422530-0250)

Protocol:

1. _____ Measure 70 ml of HPLC-grade water into the graduated beaker.

2. _____ Add magnetic stir bar and place on a magnetic stir plate located inside a chemical fume hood.

3. _____ Carefully and slowly add 9.4 ml of phosphoric acid to the water using a glass serological pipet with a rubber bulb (note 1).

4. _____ Add 10 g of cupric sulfate pentahydrate to the phosphoric acid/water solution (note 2).

5. _____ Allow components to mix thoroughly.

6. _____ Transfer contents to 100 ml graduated cylinder, and bring volume to 100 ml with HPLC-grade water (note 3).

7. _____ Transfer spray to TLC sprayer for use (note 4).

Notes:

1. The acid may react violently with the water and create an exothermic reaction, as it comes as 14.7M in an 85% solution. Use caution with this step, including personal protective equipment such as gloves and lab coat, and perform only in a certified chemical fume hood.
2. Use only cupric sulfate pentahydrate, as any other type (i.e. anhydrous) will not go into solution.
3. This provides a final concentration of 10% cupric sulfate (w/v) and 8% phosphoric acid (v/v).
4. This spray, once charred, will detect all organic compounds.