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Tissue Preparation for Spatial Metabolomics V.1

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KPMP Metabolomics Protocols ...

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We use this protocol and it's working

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Abstract

Mass spectrometry imaging (MSI) is a cutting-edge molecular technology that enables simultaneous analysis of multiple molecular components directly from single cells, tissues, and organs. For MSI, cryosections are prepared from flash-frozen tissue and mounted on a contuctive glass slide. We use matrix-assisted laser desorption ionization (MALDI)-MSI, where tissue sections are coated with a MALDI matrix in order to facilitate laser ionization and detection of metabolites with mass spectrometry. We do it using an automated robotic sprayer (TM-Sprayer) with 2,5- dihydroxybenzoic acid (DHB) MALDI matrix for positive ion mode analysis, N-(1-naphthyl) ethylenediamine hydrochloride (NEDC) for negative ion mode analyses, or 1,5-Diaminonaphthalene (DAN) for dual-polarity analysis, prior to being loaded into the MALDI-Q Exactive HF-X Orbitrap-MSI or MALDI-FTICR-MSI for untargeted metabolomics analysis. We have optimized a method for matrix application to maximize analyte extraction from tissue and increase MALDI sensitivity and to create the most homogenous matrix films possible for best lateral resolution of lipids. A key capacity that will be critical for scale-up in KPMP is the use of a TM-sprayer robotic sprayer for MALDI matrix application. This will make inter-lab studies viable, by increasing the reproducibility of sample preparation and is currently in use at UTHSA and PNNL. Our lipid extraction protocol for LC-MS/MS is a robust and universal protocol for single-sample integrative proteomics, metabolomics, and lipidomics analyses (see citation).

Guidelines

Key characteristics of TM-Sprayer robotic sprayer for MALDI-specific tissue preparation

1. Patented technology providing very small matrix droplets (<20 microns)
2. High flow rate and fast sample prep (10 to 20 minutes per plate)
3. Highly consistent matrix deposition across entire sample area (+/- 3% by weight)
4. Unique use of temperature and nitrogen flow to control evaporation rate and matrix crystal formation
5. Validated protocols for most matrices (e.g.: DHB, DAN, NEDC)
6. Continuous matrix coverage as needed for high-resolution imaging
7. Rugged operation and easy clean-up

Data types and file formats

Data generated from MS imaging is in the format of either a .d/.mis or .raw (Bruker- and Thermo-based instruments, respectively). These files are converted to the universal MSI file format (.imzML/.ibd) for further data processing and metabolite annotations in SCiLS and METASPACE.

Quality Control

As quality control, in all MALDI imaging optimization, the number of ions annotated by METASPACE (**https://metaspace2020.eu/**) at 20% false discovery rate (FDR) was used as the main benchmark. All experiments are performed at least in duplicates and results are presented accordingly.

Materials

MATERIALS

- Dry ice
- Parafilm™ M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #**1337410
- CryoStar NX50 Cryostat with height adjustment, Vacutome, Cold D, 100V **Thermo Fisher Catalog #**957090
- CM1950 Cryostat **Leica Biosystems**
- Tissue-Tek Accu-Edge Disposable Microtome Blades **Sakura Finetek Catalog #**4689
- Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker Catalog #**8237001
- Superfrost Plus Microscope Slides **Fischer Scientific Catalog #**12-550-15
- 4 × 6 Zipper Bag
- Pocket Scriber Carbide w/Magnet End **Catalog #**Westward #4KY32
- Flatbed Scanner
- MTP Slide-Adapter II **Bruker Catalog #**8235380
- Tissue MALDI TM-Sprayer **HTX Technologies, LLC**
- 25-Dihydroxybenzoic acid (DHB >98%) **Merck MilliporeSigma (Sigma-Aldrich)**
- 15-Diaminonaphthalene (DAN 99%) **Merck MilliporeSigma (Sigma-Aldrich)**
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Water (miliQ)
- Acetone (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Ethanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Hydrogen chloride solution (HCl 1M) **Merck MilliporeSigma (Sigma-Aldrich)**
- Vacuum Desiccator
- FastPrep-24 Homogenizer **MP Biomedicals Catalog #**116004500
- Balance (500 mg)
- Container to hold dry ice
- Weigh boat
- Spatula
- Falcon 50mL Conical Centrifuge Tubes **Fisher Scientific Catalog #**14-432-22
- Metal Bead Lysing Matrix **MP Biomedicals Catalog #**6925050

- Wild type adult mouse kidneys (avg 200mg/kidney 12 kidneys >2g total)
- Sphingomyelin (d18:1/16:0) **Cayman Chemical Company** **Catalog #**10007946
- 13C Sphingomyelin 1:(d18:1/16:0) **Cayman Chemical Company** **Catalog #**24452
- Centrifuge
- Pipette and tips (20 - 300 µL)
- 2 mL screw cap vials (1 per layer of the mimetic model)
- Water (deionized)
- 3 mL Syringe **Becton Dickinson (BD)** **Catalog #**309657
- Beaker
- Dry ice-cooled ethanol
- Ammonium Acetate (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Acetonitrile (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Isopropyl alcohol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- 2 mL Sorenson MµlTI™ SafeSeal™ Microcentrifuge Tubes **VWR International (Avantor)** **Catalog #**53550
- Waters autosampler vial
- NEDC (N-(1-naphthyl) ethylenediamine hydrochloride)

STEP MATERIALS

- 2 mL Sorenson MµlTI™ SafeSeal™ Microcentrifuge Tubes **VWR International (Avantor)** **Catalog #**53550
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Tissue MALDI TM-Sprayer **HTX Technologies, LLC**
- 4 × 6 Zipper Bag
- MTP Slide-Adapter II **Bruker** **Catalog #**8235380
- Flatbed Scanner
- Tissue-Tek Accu-Edge Disposable Microtome Blades **Sakura Finetek** **Catalog #**4689
- Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker** **Catalog #**8237001
- Superfrost Plus Microscope Slides **Fischer Scientific** **Catalog #**12-550-15

Pocket Scriber Carbide w/Magnet End **Catalog #**Westward #4KY32

Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker Catalog #**8237001

Protocol materials

- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- 4 × 6 Zipper Bag
- 25-Dihydroxybenzoic acid (DHB >98%) **Merck MilliporeSigma (Sigma-Aldrich)**
- Water (miliQ)
- Ethanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Waters autosampler vial
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Tissue MALDI TM-Sprayer **HTX Technologies, LLC**
- Falcon 50mL Conical Centrifuge Tubes **Fisher Scientific Catalog #**14-432-22
- Pipette and tips (20 - 300 µL)
- Ammonium Acetate (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Flatbed Scanner
- Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker Catalog #**8237001
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Balance (500 mg)
- Weigh boat
- Acetonitrile (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
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- MTP Slide-Adapter II **Bruker Catalog #**8235380
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- CryoStar NX50 Cryostat with height adjustment, Vacutome, Cold D, 100V **Thermo Fisher Catalog #**957090
- Container to hold dry ice
- 13C Sphingomyelin 1:(d18:1/16:0) **Cayman Chemical Company Catalog #**24452
- Centrifuge
- Superfrost Plus Microscope Slides **Fischer Scientific Catalog #**12-550-15
- Vacuum Desiccator
- Spatula

- Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker** **Catalog #**8237001
- 2 mL Sorenson MµlTI™ SafeSeal™ Microcentrifuge Tubes **VWR International (Avantor)** **Catalog #**53550
- 4 × 6 Zipper Bag
- Parafilm™ M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher** **Catalog #**1337410
- Acetone (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Hydrogen chloride solution (HCl 1M) **Merck MilliporeSigma (Sigma-Aldrich)**
- Water (deionized)
- Beaker
- 15-Diaminonaphthalene (DAN 99%) **Merck MilliporeSigma (Sigma-Aldrich)**
- 3 mL Syringe **Becton Dickinson (BD)** **Catalog #**309657
- MTP Slide-Adapter II **Bruker** **Catalog #**8235380
- Superfrost Plus Microscope Slides **Fischer Scientific** **Catalog #**12-550-15
- Pocket Scriber Carbide w/Magnet End **Catalog #**Westward #4KY32
- Sphingomyelin (d18:1/16:0) **Cayman Chemical Company** **Catalog #**10007946
- Isopropyl alcohol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- FastPrep-24 Homogenizer **MP Biomedicals** **Catalog #**116004500
- CM1950 Cryostat **Leica Biosystems**
- Dry ice-cooled ethanol
- Tissue-Tek Accu-Edge Disposable Microtome Blades **Sakura Finetek** **Catalog #**4689
- 2 mL Sorenson MµlTI™ SafeSeal™ Microcentrifuge Tubes **VWR International (Avantor)** **Catalog #**53550
- NEDC (N-(1-naphthyl) ethylenediamine hydrochloride)
- Flatbed Scanner
- Dry ice
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Wild type adult mouse kidneys (avg 200mg/kidney 12 kidneys >2g total)
- Metal Bead Lysing Matrix **MP Biomedicals** **Catalog #**6925050
- 2 mL screw cap vials (1 per layer of the mimetic model)
- Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**
- Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker** **Catalog #**8237001
- Pocket Scriber Carbide w/Magnet End **Catalog #**Westward #4KY32

Tissue-Tek Accu-Edge Disposable Microtome Blades **Sakura Finetek** **Catalog #**4689

Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker** **Catalog #**8237001

Superfrost Plus Microscope Slides **Fischer Scientific** **Catalog #**12-550-15

Pocket Scriber Carbide w/Magnet End **Catalog #**Westward #4KY32

4 × 6 Zipper Bag

MTP Slide-Adapter II **Bruker** **Catalog #**8235380

Flatbed Scanner

Tissue MALDI TM-Sprayer **HTX Technologies, LLC**

Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker** **Catalog #**8237001

2 mL Sorenson MµlTI™ SafeSeal™ Microcentrifuge Tubes **VWR International (Avantor)** **Catalog #**53550

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Troubleshooting

Snap frozen (liquid N_2) sample preparation and sectioning

1 Remove fresh frozen (liquid nitrogen) kidney sample stored at -80 °C and place in cryostat set at -15 °C

2 Mount the sample on chuck with minimal amount of water (one droplet) and make 10 µm sections, while keeping chuck temperature at -15 °C and blade temperature at -20 °C .

Tissue-Tek Accu-Edge Disposable Microtome Blades **Sakura Finetek Catalog #**4689

3 Thaw-mount in sequential order as outlined in Figure 6. Dry the sections immediately after sectioning in the cryostat chamber. Each slide will be marked with a number via a scribe.

Figure 6. Serial sectioning sandwich model protocol for MALDI-MS and histological imaging.

Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker Catalog #**8237001

Superfrost Plus Microscope Slides **Fischer Scientific Catalog #**12-550-15

Pocket Scriber Carbide w/Magnet End **Catalog #**Westward #4KY32

4 Tissues will be cut to generate adjacent slides that are alternatively subjected to histological staining or MALDI-MSI. This sandwich like assignment allows proper orientation and localization of each MALDI-MSI ion image to its histological counterpart.

5 ITO slides with mounted coss-sections are then transferred directly from the cryostat to either the vacuum desiccator OR zipper bag.

4 × 6 Zipper Bag

5.1 For imaging later: Place ITO slides in a zipper bag to keep vacuum inside of the bag for storing in the -80 °C freezer (sectioned samples can be stored in the freezer for months but should be analyzed as soon as possible). When removing the bag from the freezer, keep it close for ~5 minutes to reach RT before opening it.

5.2 For immediate imaging: Place ITO slides in the vacuum desiccator at Room temperature for drying 00:20:00

6 Regular glass slides are stored at -80 °C untiil staining

7 All slides (regular and ITO) stored at -80 °C are placed in the vacuum desiccator to defrost

8 For the 15T Fourier Transform Ion Cyclotron Resonance (FTICR) Mass Spectrometry, MALDI imaging slides are mounted in the Bruker MTP Slide Adapter II and scanned with a flatbed scanner with at least 3200 dpi resolution (output JPEG, TIFF, or PNG).

Note

This is performed so that the optical image can be co-registered with the imaging experiment run. Visible fiducials (e.g., "X" marks) are previously placed onto MTP Slide Adapter to use as teaching points to register the optical and MS images.

MTP Slide-Adapter II **Bruker Catalog #**8235380

Flatbed Scanner

8.1 Visible fiducials (e.g., "X" marks) are previously placed onto MTP Slide Adapter to use as teaching points to register the optical and MS images.

Matrix application for MALDI-MSI: Start-up

9 Turn on TM-Sprayer unit. Set valve to LOAD position.

Tissue MALDI TM-Sprayer **HTX Technologies, LLC**

10 Launch TM-Sprayer Software

> Note
>
> IMPORTANT: Check that exhaust fan is operational. Do not start solvent pump if proper active venting is not functioning.

11 Start solvent pump at 0.100 mL/min. Backpressure should be normal. ~ 500 psi (3.4 MPa)

12 Start compressed air flow to TM-Sprayer. Set at 10 psi (70 kPA).

13 Adjust operating temperature on the sprayer device per solvent mixture.

> Note
>
> Follow safety instructions. SOLVENT MIXTURE SHOULD CONTAIN 30% WATER MINIMUM.

14 Prepare matrix solution. Typical concentration is [M] 5 mg/mL

Note

Positive ion mode:

DHB is used at [M] 40 mg/mL in 1:1 MeOH:Water; 80 °C ; 3mm/track spacing; 8 cycles; 1200 mm/min spraying velocity; 0.05 mL/min matrix flow

25-Dihydroxybenzoic acid (DHB >98%) **Merck MilliporeSigma (Sigma-Aldrich)**

DAN is used at [M] 4.4 mg/mL in 9 mL of 1:1 Ethanol:Water (Add 500 µL of 1M HCl in 4 mL milli'Q' water and 4.5 mL of ethanol); 90 °C ; 3 mm/track spacing; 16 cycles; 1250 mm/min spraying velocity; 0.05 mL/min matrix flow

15-Diaminonaphthalene (DAN 99%) **Merck MilliporeSigma (Sigma-Aldrich)**

Note

Negative ion mode

NEDC is used at [M] 7 mg/mL in 7:3 Methanol:Water; 70 °C ; 3 mm/track spacing; 8 passes; 1200 mm/min spraying velocity; 0.12 mL/min matrix flow

NEDC (N-(1-naphthyl) ethylenediamine hydrochloride)

DAN is used at [M] 4.4 mg/mL in 9 mL of 1:1 Ethanol:Water (Add 500 µL of 1M HCl in 4 mL milli'Q' water and 4.5 mL of ethanol); 90 °C ; 3 mm/track spacing; 16 cycles; 1250 mm/min spraying velocity; 0.05 mL/min matrix flow.

15-Diaminonaphthalene (DAN 99%) **Merck MilliporeSigma (Sigma-Aldrich)**

NOTE: These are, for now, the optimal condition for matric spraying based on METASPACE output and spatial resolution achieved, and we will continue to optimize both matrix applications.

15 With valve in LOAD position, use a syringe to fill loop with matrix.

Note

20% overfill is recommended (e.g. Use 6 mL syringe to fill 5 mL loop)

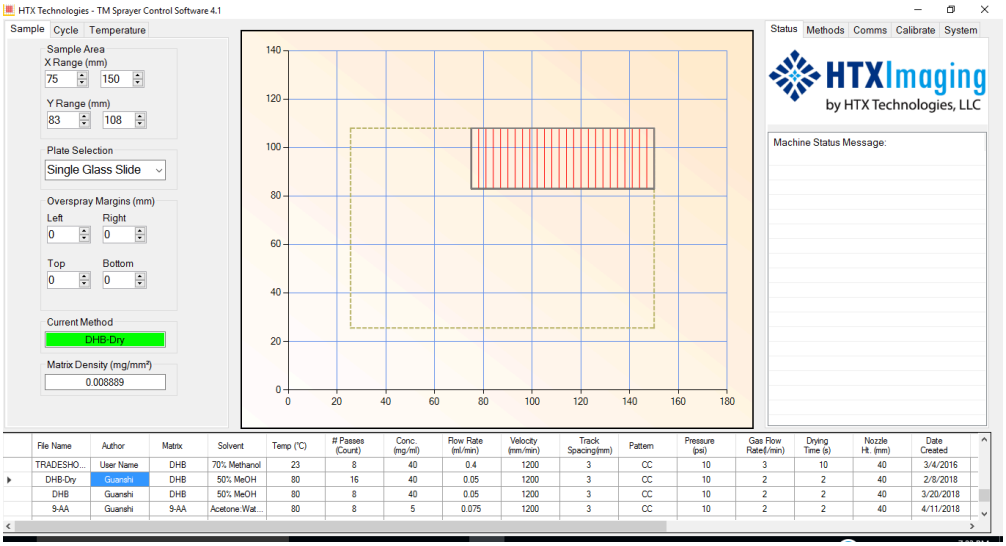
Matrix application for MALDI-MSI: Sample preparation

16 Bring ITO glass slide with affixed sample(s) from dessicator.

Conductive ITO Coating Glass Slides for MALDI Imaging **Bruker Catalog #**8237001

17 Check that the flow rate of the solvent pump and temperature of the spray nozzle are correct and stable.

18 Review sample position and define spraying area in TM-Sprayer software.



19 Select Method by clicking on left column. Current Method field will confirm selection.

20 Press START. Option changes to CONTINUE. Follow STATUS tab for prompts.

21 When prompted, switch valve to Spray and confirm by clicking Continue.

22 Software will automatically delay start to allow purging of liquid lines.

23 At end of the run the spray nozzle automatically goes to Waste position.

24 Follow end of run prompts. Software will keep track of usage and remaining matrix volume.

Matrix application for MALDI-MSI: Pause Mode

25 At the end of sample prep sequence, swith the valve back to LOAD.

Note

IMPORTANT: Keep solvent pump flow on so that clean solvent flows to nozzle and prevents matrix residues from crystallizing and clogging the capillary and spray nozzle.

26 Spray at 0.200 mL/min for 00:10:00 , then at 0.010 mL/min until ready to resume.

27 To resume, start at step 16.

Matrix application for MALDI-MSI: Shut down

28 Switch valve back to LOAD position. Set solvent pump flow rate at 0.500 mL/min.

29 Set Temperature to 30 °C to start cool down.

30 Fill syringe with 5 mL to 6 mL of clean solvent and flush loop completely. Repeat 3 times.

31 Toggle valve to wash matrix residue. Leave valve in LOAD position.

32 Keep airflow and solvent pump flow on until temperature is below 50 °C .

Note

This is important to prevent clogging.

33 Turn N_2 flow off (droplet will form at nozzle tip).

34 Stop solvent pump flow.

35 Exit TMSP Software.

36 Power OFF TM-Sprayer. Power OFF solvent pump.

Lipid extraction for LC-MS/MS

37 Add MilliQ water (200 µL to 300 µL) to the biopsy tube containing the remaining fresh frozen (liquid N_2) biopsy sample (i.e. not sectioned) and lyse the remaining biopsy sample using a tissue lyser. Quantify the amount of tissue remaining by weight.

38 Place the sample into a 2 mL microcentrifuge tube.

2 mL Sorenson MµlTI™ SafeSeal™ Microcentrifuge Tubes **VWR International (Avantor) Catalog #**53550

Note

It's been shown these tubes do **not** leach polymers into the lipid layer from the chloroform.

39 Add cold (-20 °C) chloroform:methanol mix (prepared 2:1 v/v) to sample in 4:1 ratio over sample volume and vortex.

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Note

i.e. add 400 µl of the 2:1 chloroform:methanol mixture to 80 µl of sample

39.1 Vortex for 00:00:05 to 00:00:10

39.2 Let stand on ice for 00:05:00

39.3 Vortex for 00:00:05 to 00:00:10

39.4 Centrifuge the sample 12000 x g, 4°C, 00:10:00 , 5-10 min

40 Carefully remove the upper aqueous metabolite layer until the interphase contracts without disturbing the protein disk, and discard.

41 Carefully puncture the protein interphase with a pipette tip, remove the organic lipid phase from the bottom of the tube into a conical bottom Waters autosampler vial.

Note

Be sure to gently push out any protein or upper methanol phase that might have entered the pipette tip.

42 The organic layer (containing lipids) is placed into the speed vac to dry.

43 500 µL of 2:1 Chl:MeOH and cap (no septa) is stored at -70 °C until ready for analysis.

Note

Requires extra solution volume for safe storage.

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

44 Prior to LC analysis, dry sample in speed vac and reconsititute in 100 µL of 95:5 MeOH:Chl.

Methanol (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**

Chloroform (HPLC grade) **Merck MilliporeSigma (Sigma-Aldrich)**