Standard Operating Procedure-Sample Preparation for LC-MS

1. Purpose

To prepare and analyze samples for LC-MS and/or LC-MS-MS lipidomics.

2. Scope

This SOP applies to all LC-MS samples submitted for lipidomic analysis. Samples may come from academic laboratories or outside companies.

3. Prerequisites

Agreement between the client and our lab.

4. Responsibilities

Dr. Alan Maschek is the primary researcher responsible for this SOP and the procedures involved herein. Dr. James Cox, Leon Catrow, Sandra Osburn and Tyler Van Ry are also covered in this SOP.

5. Procedures

Sample extraction from serum or cell pellets

a. Lipids are extracted from serum (10-100 µL) or cell pellets in a combined solution of 225 µL MeOH containing internal standards (Avanti Splash Lipid Mix, 10 µL each / sample; d4-succinate, 1 µg/sample; d9-carnitine, 1 µg/sample) and 750 µL MTBE (methyl tert-butyl ether). The samples are sonicated for 1 min, rested on ice for 1 hour, briefly vortexed every 15 min then an addition of 200 µL dd-H_2O is made to induce phase separation. All solutions are pre-chilled to 0 °C. The sample is then vortexed for 20 s, rested at room temperature for 10 min, and centrifuged at 14,000 g for 10 min at 4 °C. The upper (organic) and lower (aqueous) phases are collected separately and evaporated to dryness under vacuum. The remaining protein pellet is also kept separately. Lipid samples are reconstituted in at least 100 µL ACN:H_2O:IPA (1:1:2) + 0.1% formic acid and transferred to an LC/MS vial with insert for analysis.
b. Concurrently a process blank sample is brought forward as well as a quality control sample (5-10 µL per sample) was prepared by taking equal volumes from each sample after final resuspension.

Sample extraction from tissue

a. Chill MeOH, MTBE and dd H2O on ice for 15 min. Prepare organic + ISTD extraction solution to cover all samples + 2-5 extra. Calculate total volumes need for each component. In a clean glass vial add MeOH (225 uL/sample), MTBE (750 uL/sample) and ISTDs (Splash LIPID mix 10 uL/sample, d4-succinate 1uL/sample (1 mg/mL stock) and d9-carnitine 1uL/sample (1 mg/mL stock). Prepare a blank process sample concurrently. Transfer tissues to labeled (top and bottom labeled) bead mill tubes (1.4 mm, MoBio Cat# 13113-50). Depending on state of tissue, you may need a small scapula. To labeled bead-mill tubes add 987 uL of the extraction solution and

homogenize on bead-mill in one 30-sec cycle, rest on ice for 15 min with occasional vortexing. Add 300 – mass of tissue (if known assuming 1 mg/uL density) uL dd-H2O for phase separation and centrifuge at 14,000 G for 10 min at 4 C. Label new Eppendorf tubes coded with the project code and "O-#" and "Aq-#", including "QC" and "blank". Carefully remove top organic layer with pipette-man and transfer to corresponding "O-#". Carefully remove bottom layer and transfer to corresponding "Aq-#" tube leaving pellet/debris behind. Depending on sample this layer may be decanted. Some particulate is OK, re-centrifuge if necessary. Place tubes into speedvac at room temperature setting. Organic layer should be dried in 1-2 hours. Aqueous samples should take overnight. Store dried in fridge until ready. Resuspend dried organic samples in 100 uL of lipid buffer (IPA:ACN:H_2O, 2:1:1(v/v) + 10 mM ammonium formate + 0.1% formic acid). Vortex for 20 sec and then centrifuge at 14,000 G for 3 min at 4 C. Take 80 uL from supernatant and transfer into LC/MS vials. Take ~5-10 uL from each remaining sample into an Eppendorf, re-centrifuge and then transfer at-least 60 uL of this solution into LC/MS vial to build the pooled "QC" sample.

Sample extraction from yeast

a. All extraction procedures are performed on ice with solvents cooled to ~4C. Yeast pellets are resuspended in 1 mL of 150 mM NH_4HCO_3 (ammonium bicarbonate; pH 8) and transferred into bead tubes (0.5 mm). The yeast pellet is homogenized with the Omni Bead Ruptor 24 in two, 30 s cycles. Samples are centrifuged for at 14,000 g for 2 min at 4 °C. An aliquot (150 µL) of the yeast cell lysate is then diluted to 0.2 OD units per 200 µL with dd-H_2O (600 µL). Samples are next extracted with 750 µL $MTBE_3$/MeOH (17:1, v/v) containing an internal lipid standard mixture (Avanti Lipids, LM-1602, -1101 and -1202; 5 µL each / sample) for 2 hours, then the top layer is collected and transferred to an Eppendorf tube. The remaining aqueous layer was re-extracted with 750 µL MTBE/MeOH (2:1, v/v) for 2 hours, the bottom layer was removed and transferred to an Eppendorf tube. Lipid extracts were combined and dried under reduced pressure then resuspended in 65 µL MTBE/MeOH (1:2, v/v) and transferred to LC/MS vials for analysis.
b. Concurrently a process blank sample is brought forward as well as a quality control sample was prepared by taking equal volumes from each sample.

Sphingolipid extraction from tissue

a. Weigh and transfer samples to bead mill tubes, then prepare the internal standards, sonicating if necessary. Assign samples a numerical identity. Spike in either 50 or 500 pmol of each of the internal standards (2 or 20 µL of 25 uM stock). Standards are:
    a. Avanti Polar Lipids, INC. C17 Ceramide (d18:1/17:0) N-heptadecanoyl-D-*erythro*-sphingosine (860517)
    b. Avanti Polar Lipids, INC. C17 Sphingomyelin (d18:1/17:0) N-heptadecanoyl-D-*erythro*-sphingosylphosphorylcholine (860585)
    c. Avanti Polar Lipids, INC. C12 Dihydroceramide (d18:0/12:0) N-Lauroyl-D-*erythro*-sphinganine (860625)
    d. Avanti Polar Lipids, INC. Lactosyl (ß) C24 Ceramide N-(tetracosanoyl)-1- ß-lactosyl-sphing-4-ene (110762)
        i. LM-6002 can be used to replace all lipids but may not be cost effective depending on targets

b. Gently vortex and rest the samples for 30 seconds, then add 1.1 mL HPLC grade (if not MS grade) methanol. Incubate on ice for 15 minutes with brief vortexing every five minutes. Label a new set of non-lipid loss microcentrifuge tubes with numerical identity, then centrifuge the samples for 5 minutes at max speed (~15,000xg). Without disturbing the pellet, transfer the supernatant into the new eppendorfs.
c. Next, add 30 µL of 1 M KOH in methanol and mix. Incubate at 50̊C for at least four hours – this step can be done overnight. After, dry down the volume by about 1 mL in speedvac (~1 hour at room temperature) leaving 200-300 µL to make room for subsequent steps. Label a new set of eppendorfs. Once the samples are dried down sufficiently, add in 25 µL of glacial acetic acid to neutralize KOH basicity. Next, add 500 µL LC-grade MTBE and 400 µL ddH_2O. Vortex the tubes, then centrifuge them for 2 minutes at max speed (~15,000xg). Transfer the upper phase to the new eppendorfs. The lower phase is extracted again with another 25 µL of glacial acetic acid and 500 µL LC-grade MTBE. No water is added this time. Vortex and centrifuge again, then transfer the upper phase again into the new eppendorfs. Create a QC by transferring 5-10 µL from each remaining sample into a new Eppendorf, vortex and centrifuge. Dry the upper phase tubes under N_2 gas or speedvac (~2 hours at room temperature) – the tubes should be completely dry. Store in -20̊C until analysis.
d. Resuspend the samples in 200 µL of HPLC grade methanol or lipid buffer, then vortex or sonicate them. Centrifuge for 5 minutes at max speed (~15,000xg), then transfer the samples to glass vials and flush with nitrogen. Leave about 50 µL to avoid transferring garbage. Transfer samples to LCMS vials.

Maintenance of LC-MS instrument

a. Each week the instrument is calibrated (Tune -> Manual Mass Calibration-> Calibrate) in both positive and negative mode (passing with Max Residual < 1 ppm), each day the source is cleaned with a wipe-down (IPA & cloth) on the source.
b. Prime and purge binary pump each day, then condition with column on.
c. Ensure flex cube buffers are at a minimum of 500 mL.

LC-MS lipidomics analysis

a. Untargeted lipidomics (method names 'Lipidomics_POS_MS_vx' and 'Lipidomics_NEG_MS_vx') on Qtof: Lipid extracts are separated on an Acquity UPLC CSH C18 1.7 µm 2.1 x 100 mm column maintained at 60 °C connected to an Agilent HiP 1290 Sampler, Agilent 1290 Infinity pump, equipped with an Agilent 1290 Flex Cube and Agilent 6530 Accurate Mass Q-TOF dual ESI mass spectrometer. For positive more, the source gas temperature is set to 350 °C, with a gas flow of 11.1 (L/min) and a nebulizer pressure of 24 psig. VCap voltage is set at 5000 V, fragmentor at 250 V, skimmer at 74.4 V and Octopole RF peak at 750 V. For negative mode, the source gas temperature is set to 325 °C, with a drying gas flow of 12 L/min and a nebulizer pressure of 30 psig. VCap voltage is set at 4000 V, fragmentor at 225 V, skimmer at 75 V and Octopole RF peak at 750 V. Reference masses in positive mode (m/z 121.0509 and 922.0098) are infused with nebulizer pressure at 2 psig, in negative mode (1033.988, 966.0007, 112.9856 and 68.9958) are infused with a nebulizer pressure at 5 psig. Samples are analyzed in a randomized order in both positive and negative ionization mode in separate experiments acquiring with the scan range

between m/z 100 – 1700. Mobile phase A consists of ACN:H2O (60:40 v/v) in 10 mM ammonium formate and 0.1% formic acid, and mobile phase B consists of IPA:ACN:H_2O (90:9:1 v/v) in 10 mM ammonium formate and 0.1% formic acid. The chromatography gradient for both positive and negative modes starts at 15% mobile phase B then increases to 30% B over 4 min, it then increases to 52% B from 4-5 min, then increases to 82% B from 5-22 min, then increases to 95% B from 22-23 min, then increases to 99% B from 23-27 min. From 27-38 min it's held at 99%B, then decreases to 15% B from 38-38.2 min and is held there from 38.2-44 min. Flow is 0.35 mL/min throughout, injection volume is 1 µL for positive mode and 5 µL for negative mode. Tandem mass spectrometry is conducted using the same LC gradient at collision energies of 10 V, 20 V and 40 V.

b. Pressure for untargeted lipidomics on Agilent 6530 QTOF should range from ~350 bar (initial gradient) to ~775 bar (wash).

c. Targeted Sphingolipidomics on QqQ: Lipid extracts are separated on an Acquity UPLC CSH C18 1.7 µm 2.1 x 50 mm column maintained at 60 °C connected to an Agilent HiP 1290 Sampler, Agilent 1290 Infinity pump, equipped with an Agilent 1290 Flex Cube and Agilent 6490 triple quadrupole (QqQ) mass spectrometer. Sphingolipids are detected using dynamic multiple reaction monitoring (dMRM) in positive ion mode. Source gas temperature is set to 210°C, with a gas (N_2) flow of 11 L/min and a nebulizer pressure of 30 psi. Sheath gas temperature is 400°C, sheath gas (N_2) flow of 12 L/min, capillary voltage is 4000 V, nozzle voltage 500 V, high pressure RF 190 V and low pressure RF is 120 V. Injection volume is 2 µL and the samples are analyzed in a randomized order with the pooled QC sample injection eight times throughout the sample queue. Mobile phase A consists of ACN:H2O (60:40 v/v) in 10 mM ammonium formate and 0.1% formic acid, and mobile phase B consists of IPA:ACN:H_2O (90:9:1 v/v) in 10 mM ammonium formate and 0.1% formic acid. The chromatography gradient starts at 15% mobile phase B, increases to 30% B over 1 min, increases to 60% B from 1-2 min, increases to 80% B from 2-10 min, and increases to 99% B from 10-10.2 min where it's held until 14 min. Post-time is 5 min and the flowrate is 0.35 mL/min throughout. Collision energies and cell accelerator voltages were optimized using sphingolipid standards with dMRM transitions as $[M+H]^+ \rightarrow [m/z = 284.3]$ for dihydroceramides, $[M+H]^+ \rightarrow [m/z = 287.3]$ for isotope labeled dihydroceramides, $[M-H_2O+H]^+ \rightarrow [m/z = 264.2]$ for ceramides, $[M-H_2O+H]^+ \rightarrow [m/z = 267.2]$ for isotope labeled ceramides and $[M+H]^+ \rightarrow [M-H_2O+H]+$ for all targets. Sphingolipids without available standards are identified based on HR-LC/MS, quasi-molecular ion and characteristic product ions. Their retention times are either taken from HR-LC/MS data or inferred from the available sphingolipid standards.

d. All LC/MS sample queues begin with a minimum of three consecutive double blanks (e.g., MeOH in Vial 1), then proceed to three consecutive pooled QC injections. Adjust injection volume if needed (minimum 1 uL, max 5 uL). This is followed by a tandem MS run using 'Lipidomics_POS(or NEG)_MSMS'. Sample lists are randomized and contain a minimum of eight total injections of the pooled QC, three injections of the process blank and at least one double blank injection for every 10 injections.

Preparation of solutions

a. Preparation of Tuning Solution

Combine 88.5 mL ACN, 1.5 mL H_2O, 10 mL Agilent Low Concentration ESI Tuning Mix (G1969-85000), and 5 µL 322 Reference Ion (sonicate before use) then degas by sonication for 5 min. (100 mL will typically last months)

b. Preparation of Reference Mass Solution

Combine 95 mL ACN, 5 mL H_2O, 200 µL 5 mM 921 Reference Ion (Agilent G1969-85001; sonicate before use) and 250 µL 10 mM Purine Reference Ion (sonicate before use). Degas by sonication for 5 min.

c. Preparation of mobile phase A (60:40 ACN:water + 10 mM ammonium formate + 0.1% formic acid)

1. Pre-rinse three times 1 L glass bottle with pure ACN (~ 50 mL)
2. Measure exactly 600 mL of ACN in a pre-rinsed graduated cylinder and add them to the 1 L glass bottle.
3. Measure exactly 400 mL of MilliQ water in a pre-rinsed graduated cylinder and add them to the 1 L glass bottle
4. Add 1 mL formic acid
5. Weight 0.630 g of ammonium formate and add to the glass bottle
6. Sonicate for 10 min at room temperature until all the ammonium formate is dissolved

d. Mobile phase B (90:9:1 IPA:ACN:H2O + 10 mM ammonium formate + 0.1% formic acid)

1. Pre-rinse three times 1 L glass bottle with pure isopropanol (IPA; ~ 50 mL)
2. Add 10 mL H2O to a 1L glass bottle
2. Add 1mL formic acid to the same 1L glass bottle
3. Add 0.630g Ammonium Formate to the same 1L glass bottle
4. Gently shake 1L glass bottle to dissolve as much ammonium formate as possible
5. Add exactly 900 mL LC/MS grade IPA
6. Add exactly 90 mL LC/MS grade ACN
7. Sonicate for 10 min at room temperature.

e. Flex cube and needle wash buffers

1. A1 consists of 70% ACN + 0.1% formic acid
2. A2 and needle wash consist of 100% IPA + 1% cyclohexane
3. B2 consists of 90% MeOH + 0.1% formic acid

Data analysis

a. Results from LC-MS experiments are collected using Agilent Mass Hunter Workstation and analyzed using the software packages Mass Hunter Qual B.05.00 (Agilent Technologies, Inc.) and MZmine 2 (version 2.10).

b. Using Mass Hunter Qual, raw data files are exported as mzData files using the following parameters:peak filters (MS) absolute height >= 1000 counts and/or limit (by height) to the largest 100.

c. Using mzMine 2 chromatograms are processed in a chromatogram dependent manner as follows: mass detection, chromatogram builder, chromatogram deconvolution, deisotoper, join / RANSAC aligner, peak list row filter, duplicate removal and gap-filled thereby generating peak lists.
d. Data (m/z, RT, intensity) is subjected to different statistical approaches (e.g., PCA analysis) and peak lists are exported to Excel and sorted. Based on identified m/z & RT pairs, these values are then used to build preferred lists for subsequent tandem mass spectrometry (MS-MS) experiments on appropriate samples.
e. Results from LC-MS experiments are collected using Agilent Mass Hunter Workstation and analyzed using the software package Agilent Mass Hunter Quant B.07.00. Sphingolipids are quantitated based on peak area ratios to the standards added to the extracts.

Waste disposal

a. Chemical waste generated from sample extraction and LC-MS analysis are collected and pooled for collection from University of Utah Environmental Health and Safety.

6. Definitions

LC: Liquid Chromatography
MS: Mass Spectrometry
IPA: isopropyl alcohol
MeOH: methanol
MTBE: methyl *tert*-butyl ether
ACN: acetonitrile