Standard Operating Procedures (SOP)

Protocols and SOPs used throughout the University of Utah Metabolomics Core

- Untargeted Metabolomics SOP
- GC/MS Fly Larvae
- GC/MS Serum or Plasma
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Untargeted Metabolomics SOP

Standard Operating Procedure - Sample Preparation for Untargeted Metabolomic Analysis

1. Purpose

To analyze samples for changes in polar metabolites by liquid-chromatography mass spectrometry. This procedure covers the preparation of extraction solvents, sample extraction, setup of the instrument for analysis, analytical methods, and data analysis.

2. Scope

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

3. Prerequisites

The following must be completed before any samples can be analyzed.

An agreement between the client and our lab.

A pre-submission consultation.

Electronic submission of experimental details, sample details, and billing information.

4. Responsibilities

Dr. Leon Catrow is the primary researcher responsible for this SOP and the assays involved herein. Dr. Alan Maschek. Dr. James Cox, Quentinn Pearse, and Trevor Lonergan are also covered in this SOP.

5. Procedures

Preparation of Extraction Solvent

A. *Plasma, serum, or other liquid matrix*: Eighty microliters of extraction solvent is required per 20 µL plasma sample. Prepare enough extraction solvent to cover all samples plus a minimum of two process blanks (Pb). Prepare extraction solvent by adding carnitine-d9 and tyrosine-d4 to distilled ACN for a final concentration of 0.1 µg/mL and 1 µg/mL, respectively. If analyzing redox metabolites or acyl-coenzyme As (acyl-CoAs), add ammonium hydroxide for a final concentration of 0.1%. Chill to -20 ^{o}C prior to extraction.

B. *Cell pellets*: One hundred microliters of extraction solvent is required per 1x10^{6} cells in the sample. Prepare enough extraction solvent to cover all samples plus a minimum of two Pbs. Prepare extraction solvent by adding carnitine-d9 and tyrosine-d4 to 4:1 distilled ACN:ddH_2O for a final concentration of 0.1 µg/mL and 1 µg/mL,

respectively. If analyzing redox metabolites or acyl-CoAs, add ammonium hydroxide for a final concentration of 0.1%. Chill to -20 ^{o}C prior to extraction.

C. *Tissue*: Prepare enough extraction solvent to cover all samples at a final concentration of 40 mg tissue per mL of solvent plus a minimum of two Pbs. Prepare extraction solvent by adding carnitine-d9 and tyrosine-d4 to 4:1 distilled ACN:ddH_2O for a final concentration of 0.1 µg/mL and 1 µg/mL, respectively. If analyzing redox metabolites or acyl-CoAs, add ammonium hydroxide for a final concentration of 0.1%. Chill to -20 ^{o}C prior to extraction.

Sample Extraction

A. *Plasma, serum, or other liquid matrix*. Extract plasma with the addition of chilled extraction solvent at a ratio of 4:1 extraction solvent:plasma. Create a Pb containing 20 µL ddH_2O and 80 µL of chilled extraction solvent. The Pb will be carried through the remainder of the extraction as if it were a sample. Vortex samples for 30 s. Chill samples at -20 ^{o}C for 1 hr min with occasional vortexing. Centrifuge samples at 20,000 rcf for 10 min at 4 ^{o}C. Transfer supernatant to new Eppendorf tube. Store at -80 ^{o}C until ready for analysis.

B. *Cell pellet*. Extract each cell pellet of 1x10^6 cells with 100 µL of extraction solvent. Create a Pb containing 100 µL of chilled extraction solvent. The Pb will be carried through the remainder of the extraction as if it were a sample. Then, vortex samples for 30 s, followed by sonication on ice for 5 min. Vortex the sample for another 30 s. Chill samples at -20 ^{o}C for 1 h. Centrifuge samples at 20,000 rcf for 10 min at 4 ^{o}C. Transfer supernatant to new Eppendorf tube. Store at -80 ^{o}C until ready for analysis.

C. *Tissue sample.* Weigh tissue samples and transfer to a ceramic bead mill tube. The maximum amount of tissue per tube is 100 mg. Add extraction solvent to a final concentration of 40 mg tissue/mL of solvent. Create a Pb at this time consisting of 500 µL extraction solvent in a bead mill tube. The Pb will be carried through the remainder of the extraction. Homogenize samples for 15 s. Chill samples at -20 ^{o}C for 1 h. Transfer sample to new Eppendorf tube. Centrifuge samples at 20,000 rcf for 10 min at 4 ^{o}C. Transfer supernatant to new Eppendorf tube. Store at -80 ^{o}C until ready for analysis.

Instrument Setup

A. Preparation of buffers: Untargeted LC-MS metabolomics is performed with an aqueous buffer (Buffer A) of 25 mM ammonium carbonate. Buffer A should be prepared fresh for every run. All flasks used for buffers should be rinsed at least 3 times with ddH_2O (Buffer A flask) or ACN (Buffer B flask) prior to use. Weigh out 1.2 $^{+}/_{-}$ 2% g ammonium carbonate. Add the ammonium carbonate to 1 L PTFE flask and add 500 mL ddH_2O. Buffer B is 95% ACN in ddH_2O. Prepare Buffer B by adding 950 mL of distilled ACN to a PTFE flask. After the addition of distilled ACN, add 50 mL ddH_2O. It is critical that the order of this addition be in the above manner.

B. Install a Waters Acquity BEH zhilic 2.1x100 mm column with Phenomenex KrudKatcher Ultra precolumn in the column compartment.

C. Perform check tune if none has previously been performed on that day.

D. Purge both pumps at 5 mL/min for 5 min if not already done the same day with the same solvents.

E. Condition the column for 20 min immediately prior to run. Column pressure should be no higher than 260 psi prior to running. If column pressure is over 260 psi, replace precolumn or column as necessary.

LC-MS HILIC Metabolomics Analysis

A. Prepare a worklist for all samples to be analyzed starting with three double blanks (no injection), followed by a Pb and a minimum of three and maximum of six pooled quality control samples, these QC samples should be used for iterative exclusion MS/MS analysis. The rest of the samples will be randomized with a

QC sample every 3-10 samples. At the end of the analysis, run another QC followed by a Pb and a double blank.

B. Untargeted LC-MS analysis is performed on an Agilent 6545B mass spectrometer coupled to an Agilent 1290 Infinity UPLC. Chromatographic separation of metabolites is achieved using buffers and column described under instrument set-up. Buffer B is held at a concentration of 99% at a flow rate of 0.4 mL/min for 2 min. Next, buffer B is decreased to 75% over 3 min, then further decreased to 60% over 3 min. Buffer B is then decreased again to 40% over 1 min and held for 1 min. Finally, buffer B is decreased to 1% over one minute and held for 1 minute. The system is returned to starting conditions and allowed to equilibrate for 5 min between runs. Column temperature was is set to 35 ^{O}C.

Mass Spectrometry is performed by an Agilent 6545B in MS acquisition mode in both positive and negative ion polarity. Source conditions for positive-mode are as follows: Gas Temp = 336 ^{O}C, Drying Gas = 12 L/min, Nebulizer = 35 psi, Sheath Gas Temp = 350 ^{O}C, Sheath Gas Flow = 12 L/min. VCap = 3009 V, Nozzle Voltage (Expt) = 500 V, Fragmentor = 107 V, Skimmer = 65 V, Oct 1 RF Vpp = 750 V. Source conditions for Negative-mode are as follows: Gas Temp = 227 ^{O}C, Drying Gas = 12 L/min, Nebulizer = 35 psi, Sheath Gas Temp = 350 ^{O}C, Sheath Gas Flow = 12 L/min. VCap = 3513 V, Nozzle Voltage (Expt) = 1490 V, Fragmentor = 90 V, Skimmer = 65 V, Oct 1 RF Vpp = 750 V. Scan times for both modes are 1.7 scans/second.

Data Analysis

A. LC-MS data will be analyzed by Agilent Mass Hunter Qual (Agilent Technologies, Inc.), Agilent Mass Hunter Quant, Agilent Profinder, and Microsoft Excel.

B. Molecular feature (MF) analysis is performed using Agilent Profinder to analyze MFs found in QC samples. These MFs are then exported as .CEF files for the creation of an Agilent Quant method. The MFs found are combined with an in-house library of common metabolites. Peaks are integrated using the Agile algorithm in Agilent Mass Hunter Quant and transferred to an Excel sheet for further analysis.

C. Prior to statistical analysis, the data will be reviewed for quality and scaled to carnitine-d9 (positive-mode) or tyrosine-d4 (negative-mode) response. Metabolites and MFs with a percentage coefficient of variation (%CV) > 30% in the QC samples will be removed. Metabolites and molecular features with an average QC value of < 1.5X the average value of the Pb will also be rejected for further analysis.

D. Molecular features of interest will be annotated by MS/MS fragmentation data and retention time.

E. A report containing extraction methods, LC-MS methods, and basic statistical analysis of samples will be prepared for the researcher. The report will also contain instructions for performing any further analysis. The data generated in Excel format will accompany the form.

Waste disposal

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

6. Definitions

LC: Liquid Chromatography

MS: Mass Spectrometry

QC: Quality Control

Pb: Process blank

ACN: Acetonitrile

MF: Molecular Feature

PTFE: Polytetrafluoroethylene

GC/MS Fly Larvae

Standard Operating Procedure - Extraction of Fly Larvae Samples for GC-MS

1. Purpose

To extract metabolites from fly larvae for general metabolomics analysis by GC-MS. This procedure covers processing the actual samples, preparation of process blanks (PB) samples to determine sources of possible contamination, and quality control (QC) samples.

2. Scope

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

3. Prerequisites

Agreement between the client and our lab.

4. Responsibilities

Quentinn Pearce is the primary researcher responsible for this SOP and the assays involved herein. Dr. Alan Maschek and Dr. James Cox are also covered in this SOP.

5. Procedure

Fly Larvae sample preparation-Researcher

a) Sample Preparation. Fly larvae should be collected so the volume of the larvae is estimated to be around 50 µL, snap frozen and kept at -80?C.

b) Submit samples to Core with a hard copy of sample submission form. This will be placed into sample box for identification.

Fly Larvae sample extraction-Core

c) Determine the quantity of 90% methanol (MeOH) solution needed for extraction. For each sample 9 parts of 90% HPLC grade methanol is added to each fly larvae sample. **Example:** If one has 12 samples of fly larvae then each sample will need 50X9 = 450 µL 90% MeOH; totally 12 X 450 µL 90% MeOH= 5,400 µL is needed. Include 4 extra samples for process blanks. For accurate measurement assume that you will need enough for 15 samples, 15 X 450 µL= 6,750 µL or 6.75 mL. Make this up in a 15 mL or 50 mL conical tube.

d) Determine quantity of d4-succinate internal standard to add from stock solution. Total amount of d4-succinate per sample is always 1 µg. The stock solution is made of 2 mg/mL of d4-succinate made up in nanopure water. Add to 90% methanol solution and chill to -20?C in freezer. ***Use positive displacement tip for this.*** **Example:** For methanol prepared in c) add 7.5 µL of stock solution of d4-succinate.

e) Determine quantity of d27-myristic acid internal standard to add from stock solution. Total amount of d27-myristic acid per sample is always 15 µg. Add to 90% methanol solution and chill to -20?C in freezer. ***Use positive displacement tip for this.***

f) Label a set of bead tube (bioExpress, Cat. #: G-3290-1, Ceramic Bead Tube Kit, 1.4mm) according the sample names. Chill the tubes in -80?C freezer. Label appropriate number of bead tubes as PB (Process Blank). These PB tubes will not receive larvae samples, but will be otherwise processed in the same way and at the same time as real samples.

g) While the fly larvae samples are still in frozen state, quickly transfer the samples to the corresponding labeled bead tube. Put the bead tube containing the larvae back to -80?C freezer.

h) Using **piston pipette** add the cold 90% methanol solution containing the internal standard into each tube including the PB. ***It is important to use the piston pipette for accurate addition. Make certain no bubbles are in the tip!***

i) Make sure the cap of the bead tube is securely screwed on. Put bead tubes onto OMNI Bead Ruptor 24. Use the setting #3 for homogenizing. This setting will homogenize samples at S=6.45 (MHz) for C=01 (1 cycle) for T=0:30 (30 seconds) with D=0:00 (0 waiting time between cycles). After homogenization place the tubes into -20?C freezer and incubate for 1 hour. ***Use timer!***

j) Chill Beckman centrifuge and rotor to 4?C during this time. Label a fresh set of 1.5 mL microcentrifuge tubes with the exact label as found on the sample tubes.

k) After incubation in step i) centrifuge at 10,000 x g for 10 minutes at 4?C.

l) Transfer supernatant from each sample tube into the labeled fresh microcentrifuge tubes.

m) Make up QC samples by removing 20% volume of each sample and adding to a single microcentrifuge tube. From this aliquot an amount equal to the final volume of the actual samples. **Example**: For the 12 fly larvae samples used in step c) remove 90 µL from the samples in j). This gives a final volume of each sample of 1080 µL. Mix the pooled QC by vortex then aliquot 360 µL into 3 tubes labeled QC. *THIS STEP IS CRITICAL-DO NOT SKIP!*

n) Dry samples in speed-vac using *–OH* setting overnight. It is critical that extracted samples are completely dry.

o) Store at room temperature in box containing several desiccant bags.

GC-MS sample preparation

p) Prepare GC-MS using operating SOP

q) Make up a 40 mg/mL solution of N-methoxy amine (MOX) in dry pyridine. The MOX is stored in room temperature desiccators. The dry pyridine is stored in the hood. Dry the needle and syringe prior to use.

Waste disposal

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

6. Definitions

GC: gas chromatography

MS: mass spectrometry

PB: process blank

QC: quality control

GC/MS Serum or Plasma

Standard Operating Procedure-Plasma/Serum for GC-MS

1. Purpose

To extract metabolites from serum/plasma for general metabolomics analysis by GC-MS. This procedure covers processing the actual samples, preparation of process blanks (PB) samples to determine sources of possible contamination, and quality control (QC) samples.

2. Scope

This SOP applies to all serum/plasma samples submitted for GC-MS analysis. Samples may come from academic laboratories or outside companies.

3. Prerequisites

Agreement between the client and our lab.

4. Responsibilities

Quentinn Pearce is the primary researcher responsible for this SOP and the assays involved herein. Dr. Alan Maschek and Dr. James Cox are also covered in this SOP.

5. Procedure

Serum/plasma sample preparation-Researcher

a) Sample Preparation. Samples (serum or plasma) should be prepared, aliquoted into 40 µL fractions using 1.5 mL microcentrifuge tubes, snap frozen and kept at -80? C.

b) Submit samples to Core with a hard copy of sample submission form. This will be placed into sample box for identification.

Serum/plasma sample extraction-Core

c) Determine quantity of 90% methanol (MeOH) solution needed for extraction. For each sample 9 parts of 90% HPLC grade methanol is added to each part serum/plasma. **Example:** If one has 12 samples of 40 µL of serum then 12 X 360 µL 90% MeOH= 4320 µL is needed. Include 4 extra samples for process blanks. For accurate measurement assume that you will need enough for 15 samples, 15 X 360 µL= 5400 µL or 5.4 mL. Make this up in a 15 mL or 50 mL conical tube.

d) Determine quantity of d4-succinate internal standard to add from stock solution. Total amount of d4-succinate per sample is always 1 µg. The stock solution is made of 1 mg/mL of d4-succinate made up in nanopure water. Add to 90% methanol solution and chill to -20?C in freezer. ***Use positive displacement tip for this.*** **Example:** For methanol prepared in c) add 1 µL of stock solution of d4-succinate.

e) Determine quantity of d27-myristic acid internal standard to add from stock solution. Total amount of d27-myristic acid per sample is always 15 µg. Add to 90% methanol solution and chill to -20?C in freezer. ***Use positive displacement tip for this.*** **Example:** For methanol prepared in c) add 15 µL of stock solution of d27-myristic acid.

f) Thaw samples on ice or in a 4?C refrigerator prior to extraction. Start this after solutions are prepared. Label 1.5 mL microfuge tubes as PB (Process Blank).

g) Using **piston pipette** add the cold 90% methanol solution containing the internal standard into each tube including the PB. ***It is important to use the piston pipette for accurate addition. Make certain no bubbles are in the tip!***

h) Vortex for 30 seconds each sample, place into -20?C freezer and incubate for 1 hour.

i) Chill Beckman centrifuge and rotor to 4?C during this time. Label a fresh set of 1.5 mL centrifuge tubes with the exact label as found on the sample tubes.

j) After incubation in step h) centrifuge at 20,000 x g for 10 minutes at 4? C.

k) Pour supernatant from each sample into the labeled tubes.

l) Make up QC samples by removing 20% volume of each sample and adding to a single micro centrifuge tube. From this aliquot an amount equal to the final volume of the actual samples. **Example**: For the 12 x 40 µL of serum used in step c) remove 80 µL from the samples in j). This gives a final volume of each sample of 320 µL. Mix the pooled QC by vortex then aliquot 320 µL into 3 tubes labeled QC. *THIS STEP IS CRITICAL-DO NOT SKIP!*

m) Dry samples in speed-vac using *–OH* setting overnight. It is critical that extracted samples are completely dry.

n) Store at room temperature in box containing several desiccant bags.

GC-MS sample preparation

o) Prepare GC-MS using operating SOP

p) Make up a 40 mg/mL solution of N-methoxy amine (MOX) in dry pyridine. The MOX is stored in room temperature desiccators. The dry pyridine is stored in the hood. Dry the needle and syringe prior to use

Waste disposal

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

6. References

7. Definitions

GC: gas chromatography

MS: mass spectrometry

PB: process blank

QC: quality control

GC/MS Tissue

Standard Operating Procedure - Extraction of Liver Tissue Samples for GC-MS

1. Purpose

To extract metabolites from liver tissue for general metabolomics analysis by GC-MS. This procedure covers processing the actual samples, preparation of process blanks (PB) samples to determine sources of possible contamination, and quality control (QC) samples.

2. Scope

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

3. Prerequisites

Agreement between the client and our lab.

4. Responsibilities

Quentinn Pearce is the primary researcher responsible for this SOP and the assays involved herein. Dr. Alan Maschek and Dr. James Cox is also covered in this SOP.

5. Procedure

Liver tissue sample preparation-Researcher

a) Sample Preparation. Liver tissue should be collected so the volume of the sample is estimated to be around 25 mg, snap frozen and kept at -80?C.

b) Submit samples to Core with a hard copy of sample submission form. This will be placed into sample box for identification. The Researcher will be responsible for submitting the proper paperwork to our online submission system.

Liver tissue sample extraction-Core

c) Determine the quantity of 90% methanol (MeOH) solution needed for extraction. 18 parts of 90% HPLC grade methanol is added to each liver tissue sample. **Example:** If one has 12 samples of liver tissue then each sample will need 25X18 = 450 µL 90% MeOH; totally 12 X 450 µL 90% MeOH= 5,400 µL is needed. Include 4 extra samples for process blanks. For accurate measurement assume that you will need enough for 15 samples, 15 X 450 µL= 6,750 µL or 6.75 mL. Make this up in a 15 mL or 50 mL conical tube.

d) Determine quantity of d4-succinate internal standard to add from stock solution. Total amount of d4-succinate per sample is always 1 µg. The stock solution is made of 2 mg/mL of d4-succinate made up in nanopure water. Add to 90% methanol solution and chill to -20?C in freezer. ***Use positive displacement tip for this.*** **Example:** For methanol prepared in c) add 7.5 µL of stock solution of d4-succinate.

e) Label a set of bead tube (bioExpress, Cat. #: G-3290-1, Ceramic Bead Tube Kit, 1.4mm) according to the sample names. Chill the tubes in -80?C freezer. Label appropriate number of bead tubes as PB (Process Blank). These PB tubes will not receive tissue samples, but will be otherwise processed in the same way and at the same time as real samples.

f) While the liver tissue samples are still in frozen state, quickly transfer the samples to the corresponding labeled bead tube. Put the bead tube containing the tissue back to -80?C freezer.

g) Using **piston pipette** add the cold 90% methanol solution containing the internal standard into each tube including the PB. ***It is important to use the piston pipette for accurate addition. Make certain no bubbles are in the tip!***

h) Make sure the cap of the bead tube is securely screwed on. Put bead tubes onto OMNI Bead Ruptor 24. Use the setting #3 for homogenizing. This setting will homogenize samples at S=6.45 (MHz) for C=01 (1 cycle) for T=0:30 (30 seconds) with D=0:00 (0 waiting time between cycles). After homogenization place the tubes into -20?C freezer and incubate for 1 hour. ***Use timer!***

i) Chill Beckman centrifuge and rotor to 4?C during this time. Label a fresh set of 1.5 mL microcentrifuge tubes with the exact label as found on the sample tubes.

j) After incubation in step i) centrifuge at 10,000 x g for 10 minutes at 4?C.

k) Transfer supernatant from each sample tube into the labeled fresh microcentrifuge tubes.

l) Make up QC samples by removing 20% volume of each sample and adding to a single microcentrifuge tube. From this aliquot an amount equal to the final volume of the actual samples. **Example**: For the 12 liver tissue samples used in step c) remove 90 µL from the samples in j). This gives a final volume of each sample of 1080 µL. Mix the pooled QC by vortex then aliquot 360 µL into 3 tubes labeled QC. *THIS STEP IS CRITICAL-DO NOT SKIP!*

m) Dry samples in speed-vac using *–OH* setting overnight. It is critical that extracted samples are completely dry.

n) Store at room temperature in box containing several desiccant bags.

GC-MS sample preparation

o) Prepare GC-MS using operating SOP

p) Make up a 40 mg/mL solution of N-methoxy amine (MOX) in dry pyridine. The MOX is stored in room temperature desiccators. The dry pyridine is stored in the hood. Dry the needle and syringe prior to use.

Waste disposal

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

6. Definitions

GC: gas chromatography

MS: mass spectrometry

PB: process blank

QC: quality control

GC/MS Sample Preparation

Standard Operating Procedure-Sample Preparation for GC-MS

1. Purpose

To prepare samples for GC-MS analysis. This procedure covers the preparation of FAMES (Fatty Acid Methyl Ester) GC reference standard, MOX (Methoxyamine) derivatization of GC samples and setup of the instrument for analysis.

2. Scope

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

3. Prerequisites

Agreement between the client and our lab.

4. Responsibilities

Quentinn Pearce is the primary researcher responsible for this SOP and the assays involved herein. Dr. Alan Maschek and Dr. James Cox are also covered in this SOP.

5. Procedures

MOX (Methoxyamine) derivatization

a) Determine the quantity of MOX solution needed for the sample derivatization. For each sample 40 µL will be added. **Example:** If one has 20 samples, 20 X 40 µL = 800 µL MOX is needed; make 1000 µL for extra samples. MOX solution consists of 40 mg/ml MOX in dry pyridine. **ATTENTION:** keep in mind that dryness is crucial for GC-MS analysis as water will consume the derivatization reagent. Pyridine used for making MOX (and FAMES solution) is DriSolv Pyridine from EMD. Before using the syringe to remove pyridine, briefly dry the syringe with a heat gun. Keep a positive flow of dry nitrogen through the solvent bottle (using another syringe) to keep out the moisture throughout the solvent-removing process. Weigh the appropriate amount of MOX (e.g. 40 mg for making 1000 µL MOX solution) in a microcentrifgue tube and add needed amount of pyridine. Briefly warm the solution with heat gun, dissolve MOX by inverting the tube several times.

b) To each sample tube, add 40 µL MOX solution prepared in a). Always use freshly-prepared MOX solution. Vortex the tube for 30 seconds and put into 40 ?C sand bath incubator for 1 hour. Use timer to keep track of the time.

c) After 1 hour incubation, take out the sample tubes and remove the sand stuck to the outside of the tubes. Spin at 14,000 rpm for 5 min with the Eppendorf 5418 microcentrifuge.

d) Prepare a set of GC glass vials and inserts. Glass vial used for GC-MS is Agilent Part # 5181-3376 (Vial, crimp, 2ml ambr, WrtOn, cert, 100PK), insert used for GC-MS is Agilent Part # 5183-2086 (400 µL Silanized Flat bottom Insert). Put an insert into each glass vial then label the vials appropriately.

e) Transfer 25 µL of cleared supernatant obtained after centrifugation in c) into corresponding labeled vial. Seal the vial using a crimper with the vial cap, using Agilent Part # 5188-5386 (Crimp cap, 11mm Magnetic CTC, 100pk; golden-colored). The sample vials are ready to be loaded onto the autosampler sample tray.

Instrument Setup

f) Change liner and tune/calibrate the GCT Premier if necessary. Normally the liner should be changed after running 20 or more samples; GCT Premier should be tuned/calibrated once a week. Please refer to SOP (Instrument Preparation _ GCT Premier) for detailed procedures.

g) Add sample information to the corresponding sample list in Mass Lynx software. Sample lists are named as: GCT_Month_YEAR. Add appropriate number of sample files to the sample list. For routine sample run, required number of sample files to be added will be 2XN+N/6 (N is the number of samples to be run, 2XN is because a defaultShort and a defaultLong will be run for each sample; N/6 is because a hexane blank needs to be run every 6 samples). **Example:** total samples are 17, sample files to be added will be 2X17 + 17/6 = 34 + 17/6 (here in the case of non-integer, round up to next integer, 17/6 = 2.83, round up to 3) = 34 + 3 = 37. Need to add 37 sample files to the sample list.

h) **Randomize the samples** and put them into the sample tray. In the sample list add the sample information into the sample file following the sample order. **Example:** first sample is D4-suc/AA-IS, a defaultShort and a defaultLong method will be run on this sample; then the first sample file will be named as: 100:1 D4 AA (100:1 is the split ratio for default short method, meaning this is a default short run), the second sample file will be named as: 10:1 D4 AA (10:1 is the split ratio for default long method, meaning this is a default long run). Remember to add the hexane blank every 6 samples. Return the sample tray to the autosampler tray holder.

i) Check the inlet methods for defaultShort and defaultLong. The split ratio for defaultShort should be 100:1 and the split ratio for defaultLong should be 10:1.

j) In MassLynx select the samples files that will be run, start the sample list run. (Note: at this time no sample injection will occur as it will be waiting for the autosampler.)

k) In the MassHunter software that controls the autosampler select the appropriate prepSequence file, for the first run, add 60 uL MSTFA, for subsequent runs, add 20 uL of MSTFA.

l) Make sure the MSTFA (Vials 2 and 3) vials contain sufficient reagents for the run. It is critical that MSTFA + 1% TMCS is used. Also make sure the washing solution bottles Acetonitrile and hexanes (vials A1 and B1) in both towers contain enough washing reagents.

m) Start the Sequence from the MassHunter software.

Waste disposal

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

6. Definitions

GC: Gas Chromatography

MS: Mass Spectrometry

MOX: Methoxyamine

FAMES: Fatty Acid Methyl Ester

Lipidomics SOP

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, Dr. Leon Catrow and Trevor Lonergan are also covered in this SOP.

. Procedures

Sample extraction from plasma, serum or cell pellets

All solutions are pre-chilled on ice. Lipids are extracted in randomized order from 5-50 µL plasma (*preferred*), serum or cell pellets in a combined solution of 225 µL MeOH (LC-MS grade Burdick & Jackson) containing internal standards (IS) (Avanti SPLASH LIPIDOMIX, 10 µL each / sample; d4-succinate, 1 µg/sample; d9-carnitine, 1 µg/sample) and 750 µL methyl tert-butyl ether (MTBE; HPLC grade Fisher Sci). The samples are sonicated for 1 min, rested on ice for 1 hour, briefly vortexed every 15 min then an addition of 188 µL PBS is made to induce phase separation. The sample is then vortexed for 20 s, rested at room temperature for 10 min, and centrifuged at 14,000 g (3,000 g for glass tubes) for 10 min at 4 °C. The upper (organic) and lower (aqueous) phases are collected separately. The bottom layer is re-extracted with 1 mL of the upper phase of MTBE:MeOH:H_2O (10:3:2), briefly vortexed, and centrifuged as above. The upper layer is combined with the previously collected upper layer and evaporated to dryness under vacuum. The remaining protein pellet is also kept separately. Lipid samples are reconstituted in at least 100 µL mobile phase B (IPA or MeOH is OK) and transferred to an LC/MS vial with insert (e.g., Agilent 5182-0554 and Agilent 5183-2086) for analysis.

Concurrently a process blank sample is brought forward as well as a pooled quality control (QC) sample (5-10 µL per sample) was prepared by taking equal volumes from each sample after final resuspension.

Sample extraction from tissue

All reagents are pre-chilled on ice. Transfer tissues to labeled (top and side 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 200 – mass of tissue µL PBS. For example, if tissue is 48 mg, then add 152 µL PBS, 225 µL MeOH containing IS (Avanti SPLASH LIPIDOMIX 10 µL/sample, d4-succinate 1 µL/sample (1 mg/mL stock) and d9-carnitine 1µL/sample (1 mg/mL stock) and homogenize on bead-mill in one 30-sec cycle, rest on ice for 15 min with occasional vortexing. Transfer to glass vial or tube and add MTBE (750 µL/sample). Prepare a blank process sample concurrently. Vortex samples for 30 sec and centrifuge at 14,000 G (3,000 G for glass tubes) for 10 min at 4 °C. If no phase separation has occurred add 50 uL H_2O and repeat. Carefully remove top organic layer with pipette-man and transfer to new vial. The bottom

layer is re-extracted with 1 mL of the upper phase of MTBE:MeOH:H_2O (10:3:2), briefly vortexed, and centrifuged as above. The upper layer is combined with the previously collected upper layer and evaporated to dryness under vacuum. Place tubes into speedvac at room temperature setting (max 40 °C). Organic layer should be dried in 1-2 hours. Resuspend organic samples in a minimum of 100 µL of mobile phase B (IPA ok). Vortex for 20 sec and then centrifuge at 14,000 G for 3 min at 4 C. Take 80 µL from supernatant and transfer into LC/MS vials. Take ~5-10 µL from each remaining sample into an Eppendorf, re-centrifuge and then transfer at-least 60 µL of this solution into LC/MS vial to build the pooled QC sample.

Sample extraction from yeast

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 (3,000 G for glass tubes) 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 Polar Lipids SPLASH LIPIDOMIX; 10 µL each / sample) for 2 hours, then the top layer is collected and transferred to an Eppendorf tube. The bottom layer is re-extracted with 1 mL of the upper phase of MTBE:MeOH:H_2O (10:3:2), briefly vortexed, and centrifuged as above. The upper layer is combined with the previously collected upper layer and evaporated to dryness under vacuum. 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 (e.g., Agilent 5182-0554 and Agilent 5183-2086) for analysis.

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

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

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.

Next, add 30 µL of 1 M KOH in methanol and mix. Incubate at 50?C 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 -20?C until analysis.

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 LC/MS vials (e.g., Agilent 5182-0554 and Agilent 5183-2086).

Maintenance of LC-MS instrument

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.

Prime and purge binary pump each day, then condition with column on.

Ensure flex cube buffers are at a minimum of 500 mL.

LC-MS lipidomics analysis

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.

LC-MS grade methanol, acetonitrile and isopropyl alcohol purchased from Honeywell - Burdick & Jackson. Formic acid purchased from Honeywell Fluka. Ammonium formate purchased from Millipore.

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

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 $[M+H]^+$?[*m/z* = 284.3] for dihydroceramides, $[M+H]^+$?[*m/z* = 28

7.3] for isotope labeled dihydroceramides, [M-H_2O+H]$^+$?[*m/z* = 264.2] for ceramides, [M-H_2O+H]$^+$?[*m/z* = 267.2] for isotope labeled ceramides and [M+H]$^+$?[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.

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. Add 10 mL of 1 M ammonium formate 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 isopropanol (IPA; ~ 50 mL) followed by one rinse of dd-H_2O
2. Add 10 mL of 1 M ammonium formate to the glass bottle
2. Add 1mL formic acid to the same 1L glass bottle
3. Gently shake 1L glass bottle to dissolve as much ammonium formate as possible
4. Add exactly 900 mL LC/MS grade IPA
5. Add exactly 90 mL LC/MS grade ACN
6. Sonicate for 10 min at room temperature.

e. Flex cube and needle wash buffers

1. A1 consists of 90% ACN (nebulizer flush)

2. A2 and needle wash consist of 100% IPA (left over Lipid buffer B also acceptable)

3. B2 consists of 90% MeOH + 0.1% formic acid

Data analysis

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).

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.

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.

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.

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

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

Definitions

LC: Liquid Chromatography

MS: Mass Spectrometry

IPA: isopropyl alcohol

MeOH: methanol

MTBE: methyl *tert*-butyl ether

ACN: acetonitrile