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 ^oC 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 ^oC 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 ^oC for 1 hr min with occasional vortexing. Centrifuge samples at 20,000 rcf for 10 min at 4 ^oC. Transfer supernatant to new Eppendorf tube. Store at -80 ^oC 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 ^oC for 1 h. Centrifuge samples at 20,000 rcf for 10 min at 4 ^oC. Transfer supernatant to new Eppendorf tube. Store at -80 ^oC 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 ^oC for 1 h. Transfer sample to new Eppendorf tube. Centrifuge samples at 20,000 rcf for 10 min at 4 ^oC. Transfer supernatant to new Eppendorf tube. Store at -80 ^oC 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

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