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We use this protocol and it's working

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Untargeted Metabolomics & Targeted Lipidomics

Joanna Bi[1]

[1]Stanford University

NCIHTAN

Joanna Bi

ABSTRACT

Protocol for untargeted metabolomics & targeted lipidomics.

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Steps

1 Sample Preparation: Roughly 30 mg of flash frozen tissue were homogenized in 500 µl ice-cold methanol by bead beating (MP bioscience cat# 6913-100, Solon, OH) at 4°C (2 x 45 s). Metabolites and complex lipids were extracted using a biphasic separation with cold methyl tert-butyl ether (MTBE), methanol and water. Briefly, 1 ml of ice-cold MTBE was added to 300 µl of the homogenate spiked-in with 40 µl deuterated lipid internal standards (Sciex, cat# 5040156, lot# LPISTDKIT-102). The samples were then sonicated (3 x 30 s) and agitated at 4°C for 30 min. After addition of 250 µl of ice-cold water, the samples were vortexed for 1 min and centrifuged at 14,000 g for 5 min at 20°C. The upper organic phase contains the lipids, the lower aqueous phase contains the metabolites and the proteins are precipitated at the bottom of the tube. For quality controls, three reference plasma samples (40 µl plasma), two normal colon tissue, and one colonic polyp as well as one control lacking any sample (i.e. blank) were processed in parallel

2 Further Sample prep - Metabolites: Proteins were further precipitated by adding 500 µl of 33/33/33 acetone/acetonitrile/methanol spiked-in with 15 labeled metabolite internal standards to 300 µl of the aqueous phase and 200 µl of the lipid phase and incubating the samples overnight at -20°C. After centrifugation at 17,000 g for 10 min at 4°C, the metabolic extracts were dried down to completion and resuspended in 100 µl 50/50 methanol/water.

3 Further Sample prep - Complex lipids: 700 µl of the organic phase was dried down under a stream of nitrogen and resolubilized in 200 µl of methanol for storage at -20°C until analysis. The day of the analysis, samples were dried down, resuspended in 300 µl of 10 mM ammonium acetate in 90/10 methanol/toluene and centrifuged at 16,000 g for 5 min at 24°C

4 Untargeted Metabolomics by Liquid Chromatography (LC)-MS. Metabolic extracts were analyzed four times using HILIC and RPLC separation in both positive and negative ionization modes. Data were acquired on a Thermo Q Exactive HF mass spectrometer for HILIC (Thermo Fisher Scientific, Bremen, Germany) and a Thermo Q Exactive mass spectrometer for RPLC (Thermo Fisher Scientific, Bremen, Germany). Both instruments were equipped with a HESI-II probe and operated in full MS scan mode. MS/MS data were acquired on quality control samples (QC) consisting of an equimolar mixture of all samples in the study. HILIC experiments were performed using a ZIC-HILIC column 2.1 x 100 mm, 3.5 µm, 200Å (Merck Millipore, Darmstadt, Germany) and mobile phase solvents consisting of 10 mM ammonium acetate in 50/50 acetonitrile/water (A) and 10 mM ammonium acetate in 95/5 acetonitrile/water (B). RPLC experiments were performed using a Zorbax SBaq column 2.1 x 50 mm, 1.7 µm, 100Å (Agilent Technologies, Palo Alto, CA) and mobile phase solvents consisting of 0.06% acetic acid in water (A) and 0.06% acetic acid in methanol (B). Data quality was ensured by (i) injecting 6 and 12 pool samples to equilibrate the LC-MS system prior to

running the sequence for RPLC and HILIC, respectively, (ii) injecting a pool sample every 10 injections to control for signal deviation with time, and (iii) checking mass accuracy, retention time and peak shape of internal standards in each sample

5 Targeted Lipidomics using the Lipidyzer Platform. Lipid extracts were analyzed using the Lipidyzer platform that comprises a 5500 QTRAP system equipped with a SelexION differential mobility spectrometry (DMS) interface (Sciex) and a high flow LC-30AD solvent delivery unit (Shimazdu, Columbia, MD). Briefly, lipid molecular species were identified and quantified using multiple reaction monitoring (MRM) and positive/negative ionization switching. Two acquisition methods were employed covering 12 lipid classes; method 1 had SelexION voltages turned on while method 2 had SelexION voltages turned off. Data quality was ensured by i) tuning the DMS compensation voltages using a set of lipid standards (cat# 5040141, Sciex) after each cleaning, more than 24 hours of idling or 3 days of consecutive use, ii) performing a quick system suitability test (QSST) (cat# 5040407, Sciex) before each batch to ensure acceptable limit of detection for each lipid class, and iii) triplicate injection of lipids extracted from a reference plasma sample (cat# 4386703, Sciex) at the beginning of the batch.

6 Data was acquired in two separate batches and batch effect was controlled by running three samples in common in both batches.

7 Metabolomics: Data from each mode were independently analyzed using Progenesis QI software (v2.3) (Nonlinear Dynamics, Durham, NC). Metabolic features from blanks and those that didn't show sufficient linearity upon dilution in QC samples ($r<0.6$) were discarded. Only metabolic features present in >2/3 of the samples were kept for further analysis. Median normalization was applied to correct for differential starting material quantity. Missing values were imputed by drawing from a random distribution of low values in the corresponding sample. Batch effect was corrected by the ComBat model using the dbnorm package. Data quality post-normalization was verified by ensuring clustering of three biological replicates analyzed in two batches on a principal component analysis plot. Data from each mode were merged and 10,520 metabolic features were annotated as follows. Peak annotation was first performed by matching experimental m/z, retention time and MS/MS spectra to an in-house library of analytical-grade standards. Remaining peaks were identified by matching experimental m/z and fragmentation spectra to publicly available databases including HMDB, MoNA and MassBank using the R package 'metID' (v0.2.0). Metabolites were reported if the similarity score was above 0.65. We used the Metabolomics Standards Initiative (MSI) level of confidence to grade metabolite annotation confidence (level 1 - level 3). Level 1 represents formal identifications where the biological signal matches accurate mass, retention time and fragmentation spectra of an authentic standard run on the same platform. For level 2 identification, the biological signal matches accurate mass and fragmentation spectra available in one of the public databases listed above. Level 3 represents putative identifications that are the most likely name based on previous knowledge.

8 Targeted Lipidomics: Lipidyzer data were reported by the Lipidomics Workflow Manager (LWM, v1.0.5.0) software which calculates concentrations for each detected lipid as average intensity of the analyte MRM/average intensity of the most structurally similar internal standard (IS) MRM multiplied by its concentration. Lipids detected in less than 2/3 of the samples were discarded and missing values were imputed by drawing from a random distribution of low values class-wise in the corresponding sample. Median normalization (excluding TAG and DAG) was applied to correct for differential starting material quantity. Batch normalization was performed using quality control reference plasma samples run in both batches. Data quality post-normalization was verified by ensuring clustering of three biological replicates analyzed in two batches on a principal component analysis plot. We detected 514 individual lipid species belonging to 12 classes (e.g. CE, CER, DAG, FFA, HCER, LCER, LPE, LPC, PC, PE, SM, TAG) and their abundance was reported as concentrations in nmol/g.