Objective.
LC-MS analysis of non-polar (and polar)[I] metabolites from tissue.

Chemicals and Tools	**Vendor**	**Part#**	**Hazards/Notes**
• LC-MS grade methanol (MeOH)	Fisher	A456-4	
• LC-MS grade water (H_2O)	Fisher	W6-4	
• HPLC grade methyl tert-butyl ether (MTBE)	Sigma	34875-1L	
• Pre-filled Bead Mill Tubes	Fisher	15-340-154	Can be substituted with steel beads (e.g Qiagen 69989)
• Qiagen tissue lyser II			
• 2.5 mM pre-mixed Heavy Amino Acid (AA) mix (U-^{13}C, ^{15}N) [II, III]	CIL	MSK-A2-1.2	
• Avanti Splash LipidoMix [III]	Avanti	330707	
• -80°C Freezer			
• Vials	Eppendorf	022431081	LoBind tubes
• Vortexer			

Untargeted analysis of lipids
Lipids from various classes including ceramides, phospholipids, gangliosides, sphingosines, acylcarnitines, fatty acids and many others are measured using untargeted LC-MS/MS methods. The lipids are separated using reverse-phase chromatography (C18) and the mass to charge ratio (m/z) is measured in both positive and negative ionization modes (separate injections)– typically with mass accuracies ≤5 ppm. Raw data is searched against the Thermo Scientific™ LipidSearch™ Software which contains > 1.5 million lipid ions and their predicted fragment ions.

We recommend that 4 biological replicates (n≥4) are prepared per condition. If internal standards will not be used, n≥5 replicates are preferred.

I. The extraction process will result in a methanol phase-which contains polar metabolites, and a MTBE phase-which contains non-polar metabolites. We can carry out targeted metabolomics on the methanol extract.
II. Heavy AA mix is not required for lipidomics (non-polar) analysis only.
III. Other heavy internal standards can be substituted. Please speak to a PRC scientist prior to sample submission.

	Procedure.	*Examples, Tricks & Comments*
1	**<u>Preparing Tissue:</u>** Crush tissue to a powder in a liquid nitrogen-chilled mortar and pestle and weigh out 10-20 mg of powder. Alternatively, weigh the tissue in a pre-weighed tube (containing steel or ceramic beads).	*Prepare the tissue sample as you see fit. Step 1 is a general guideline.*

		Depending on the accuracy of your analytical balance, it may be more precise to use a larger tissue size.
2	Prepare the **MeOH extraction solution**: This solution consists of 100% LC-MS grade methanol. Add the heavy AA mix to a final concentration of 1 μM **<u>if the methanol layer will be retained for metabolite profiling</u>**. It is recommended that this solution and the MTBE are pre-chilled at -20°C. The H_2O can be pre-chilled at 4°C. Prepare the **MTBE extraction solution**: This solution consists of 100% HPLC grade MTBE. If heavy lipid standards will be used, add at this stage. Contact PRC regarding final concentration of lipid internal standards. This will vary based on lipid type.	*Example Preparation: 200 mL MeOH + 80 µL of 2.5 mM heavy AA mix (Refer to Table 1). This solution can be stored long-term at -20°C.* *Deuterated lipid standards like Avanti Splash ® (product #330707 or #330709) can be added to the MTBE, prior to extraction, to serve as internal standards.*
3	• While the samples are on dry ice, add 0.8 mL of cold **MTBE extraction solution** followed by 0.24 mL of cold **MeOH extraction solution** to each sample. • Homogenize the sample (if the tissue was not crushed to a powder). If using beads, ensure they are free-floating and not frozen at the bottom of the tube. If tissue was crushed to a powder, vortex vigorously and continue with the next step. **Suggested homogenization parameters:** cycle frequency 20-25/sec, 2 to 5-minute cycle. Repeat as needed. Keep samples very cold during this process. • Transfer the supernatant into a pre-chilled Eppendorf tube. • Add 0.2 mL of **cold H_2O** and vortex the samples vigorously for 10 minutes at 4°C followed by centrifugation at 16,000 RCF (or max. speed) at 4°C for 10 minutes to separate the phases. • Carefully collect the two layers separately and transfer each layer into a new Eppendorf tube (upper layer is the lipid-containing phase and bottom layer is the polar phase). Avoid the protein pellet at the bottom of the Eppendorf. **OPTIONAL:** Each layer can be divided equally amongst two Eppendorf tubes (use 20 mg tissue). One vial can be stored at -80°C, to serve as a back-up, **post evaporation**.	*Note. You can save the tissue pellet to measure protein concentration for sample normalization or proteomic analysis.* *Pipette tips and Eppendorf tubes do not have high chemical resistance to MTBE. Avoid polycarbonate-based consumables. Propylene/ polyethylene-based tubes can be used short term* (≤4°C). *All steps must be performed on dry ice or an ice bath containing salt.*

	Re-extraction of Tissue (optional): The polar phase can be re-extracted with cold MTBE (~1.8x volume of the remaining polar phase). Repeat as needed. The MTBE used for the 2nd/3rd extraction should not contain ISTDs.	
4	Dry the samples using nitrogen air or a temperature controlled centrifugal evaporator. Store the dried extracts at -80°C until LC-MS analysis.	*Drying time varies based on evaporation method, solvent volatility, and vacuum pump strength (1-3 h).*
5	Fill out the metabolomics/lipidomics submission form and submit the dried extracts to the PRC. https://www.rockefeller.edu/proteomics/uploads/www.rockefeller.edu/sites/216/2020/07/Metabolomics_submission_form_FY21.xlsx	*Required information:* • *Cell line/ Cell count* • *List of specific metabolites (or full profiling)* • *ISTD composition/ concentration* • *Cell treatment (e.g. labels, inhibitors, etc.)*

Comments.

The heavy amino acid mix (MSK-A2-1.2) is used as an internal standard for the polar phase. Refer to **Table 1** for the composition of the MSK-A2-1.2 product.

Information regarding the mixed heavy lipid standards can be found in **Tables 2-3** and here; https://avantilipids.com/product/330707 , https://avantilipids.com/product/330709 .You can substitute these with other isotopically labelled standard(s) so long as the extraction buffer does not contain any endogenous metabolites. Note: For lipidomics, it is best to use deuterated internal standards.
Samples can be normalized via sample volume, protein concentration or DNA concentration. Note that the biological samples (dry extracts) will be treated identically upon submission to the PRC.

If you are treating the samples with reducing/oxidizing agents, drugs or any other compounds that can be extracted during the extraction step, the reagent name and the final concentration (in the dry pellet) needs to be listed in the submission form.

Table 1. Composition of the Cambridge Isotope Laboratories MSK-A2-1.2 mixture.

Name	Product identifier
WATER UNLABELED	(CAS-No.) 7732-18-5 (EC-No.) 231-791-2
HYDROCHLORIC ACID	(CAS-No.) 7647-01-0 (EC-No.) 231-595-7 (EC Index-No.) 017-002-00-2
L-ALANINE (13C3, 99%; 15N, 99%)	(CAS-No.) 312623-85-1 (EC-No.) 200-273-8 (Unlabeled) (EC Index-No.)
L-LYSINE:2HCL (13C6, 99%; 15N2, 99%)	(CAS-No.) 657-26-1 (Unlabeled) (EC-No.) 211-518-3 (Unlabeled)
L-HISTIDINE:HCL:H2O (<5% D) (13C6, 97-99%; 15N3, 97-99%)	(CAS-No.) 5934-29-2 (Unlabeled)
L-ARGININE:HCL (13C6, 99%; 15N4, 99%)	(CAS-No.) 202468-25-5 (EC-No.) 214-275-1 (Unlabeled)
L-TYROSINE (13C9, 99%; 15N, 99%)	(CAS-No.) 202407-26-9 (EC-No.) 200-460-4 (Unlabeled)
L-PHENYLALANINE (13C9, 99%; 15N, 99%)	(CAS-No.) 63-91-2 (Unlabeled) (EC-No.) 200-568-1 (Unlabeled)
L-METHIONINE (13C5, 99%; 15N, 99%)	(CAS-No.) 63-68-3 (Unlabeled) (EC-No.) 200-562-9 (Unlabeled)
L-GLUTAMIC ACID (13C5, 99%; 15N, 99%)	(CAS-No.) 56-86-0 (Unlabeled) (EC-No.) 200-293-7 (Unlabeled)
L-ASPARTIC ACID (13C4, 99%; 15N, 99%)	(CAS-No.) 202468-27-7 (EC-No.) 200-291-6 (Unlabeled)
L-LEUCINE (13C6, 99%; 15N, 99%)	(CAS-No.) 202406-52-8 (EC-No.) 200-522-0 (Unlabeled)
L-ISOLEUCINE (13C6, 99%; 15N, 99%)	(CAS-No.) 73-32-5 (Unlabeled) (EC-No.) 200-798-2 (Unlabeled)
L-VALINE (13C5, 99%; 15N, 99%)	(CAS-No.) 72-18-4 (Unlabeled) (EC-No.) 200-773-6 (Unlabeled)
L-THREONINE (13C4, 97-99%; 15N, 97-99%)	(CAS-No.) 72-19-5 (Unlabeled) (EC-No.) 200-774-1 (Unlabeled)
L-CYSTINE (13C6, 99%; 15N2, 99%)	(CAS-No.) 1252803-65-8 (EC-No.) 200-296-3 (Unlabeled) (EC Index-No.)
L-PROLINE (13C5, 99%; 15N, 99%)	(CAS-No.) 147-85-3 (Unlabeled) (EC-No.) 205-702-2 (Unlabeled)
L-SERINE (13C3, 99%; 15N, 99%)	(CAS-No.) 202407-34-9 (EC-No.) 200-274-3 (Unlabeled)
GLYCINE (13C2, 99%; 15N, 99%)	(CAS-No.) 211057-02-2 (EC-No.) 200-272-2 (Unlabeled)

Table 2. Composition of the Avanti SPLASH LipidoMIX™ product # 330707.

Compound Name	Molecular Weight	Exact Mass	Chemical Formula	Concentration (µg/mL)*
15:0-18:1(d7) PC	753.11	752.61	$C_{41}H_{73}D_7NO_8P$	**150.6**
15:0-18:1(d7) PE	711.03	710.56	$C_{38}H_{67}D_7NO_8P$	**5.3**
15:0-18:1(d7) PS (Na Salt)	777.02	776.53	$C_{39}H_{66}D_7NNaO_{10}P$	**3.9**
15:0-18:1(d7) PG (Na Salt)	764.02	763.54	$C_{39}H_{67}D_7NaO_{10}P$	**26.7**
15:0-18:1(d7) PI (NH_4 Salt)	847.13	846.60	$C_{42}H_{75}D_7NO_{13}P$	**8.5**
15:0-18:1(d7) PA (Na Salt)	689.94	689.50	$C_{36}H_{61}D_7NaO_8P$	**6.9**
18:1(d7) Lyso PC	528.72	528.39	$C_{26}H_{45}D_7NO_7P$	**23.8**
18:1(d7) Lyso PE	486.64	486.35	$C_{23}H_{39}D_7NO_7P$	**4.9**
18:1(d7) Chol Ester	658.16	657.64	$C_{45}H_{71}D_7O_2$	**329.1**
18:1(d7) MAG	363.59	363.34	$C_{21}H_{33}D_7O_4$	**1.8**
15:0-18:1(d7) DAG	587.98	587.55	$C_{36}H_{61}D_7O_5$	**8.8**
15:0-18:1(d7)-15:0 TAG	812.37	811.77	$C_{51}H_{89}D_7O_6$	**52.8**
d18:1-18:1(d9) SM	738.12	737.64	$C_{41}H_{72}D_9N_2O_6P$	**29.6**
Cholesterol (d7)	393.71	393.40	$C_{27}H_{39}D_7O$	**98.4**

*Concentrations are based on the isotopic purity of each individual compound

Table 3 . Composition of the Avanti SPLASH II LipidoMIX™ product # 330709.

Compound Name	Molecular Weight	Exact Mass	Chemical Formula	Conc. (µg/mL)*	Conc. µM*
15:0-18:1(d7) PC	753.11	752.61	$C_{41}H_{73}D_7NO_8P$	**158.2**	**210**
15:0-18:1(d7) PE	711.03	710.56	$C_{38}H_{67}D_7NO_8P$	**5.0**	**7**
15:0-18:1(d7) PS (Na Salt)	777.02	776.53	$C_{39}H_{66}D_7NNaO_{10}P$	**7.8**	**10**
15:0-18:1(d7) PI (NH_4 Salt)	847.13	846.60	$C_{42}H_{75}D_7NO_{13}P$	**8.5**	**10**
18:1(d7) Lyso PC	528.72	528.39	$C_{26}H_{45}D_7NO_7P$	**23.8**	**45**
18:1(d7) Lyso PE	486.64	486.35	$C_{23}H_{39}D_7NO_7P$	**0.5**	**1**
18:1(d7) Chol Ester	658.16	657.64	$C_{45}H_{71}D_7O_2$	**348.8**	**530**
C18(Plasm)-18:1(d9) PC	781.19	780.67	$C_{44}H_{77}D_9NO_7P$	**7.8**	**10**
15:0-18:1(d7) DAG	587.98	587.55	$C_{36}H_{61}D_7O_5$	**11.8**	**20**
15:0-18:1(d7)-15:0 TAG	812.37	811.77	$C_{51}H_{89}D_7O_6$	**56.9**	**70**
d18:1-18:1(d9) SM	738.12	737.64	$C_{41}H_{72}D_9N_2O_6P$	**29.5**	**40**
C18(Plasm)-18:1(d9) PE	739.11	738.62	$C_{41}H_{71}D_9NO_7P$	**0.07**	**0.1**