Objective.
LC-MS analysis of non-polar (and polar)[I] metabolites from biofluids (serum, plasma).

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	
• 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, but some 'less polar' metabolites may partition into the MTBE phase.
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	Collect blood into lithium heparin tube and vortex the sample. Centrifuge the tube for 5-10 minutes at max speed at room temperature. Transfer the supernatant into an Eppendorf tube, snap-freeze on dry ice or liquid nitrogen and store at -80°C till extraction or extract immediately. **NOTE:** It is crucial that the same type of vacutainer tube is used for the entire experiment. The salts/anti-coagulants will impact extraction efficiency.	*Prepare the plasma/serum sample as you see fit. Step 1 is a general guideline.*

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 3 µM **if the methanol layer will be retained for metabolite profiling**. 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: 30 mL MeOH + 36 µL of 2.5 mM heavy AA mix (Refer to Table1). 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	• Add 0.12 mL of cold **MeOH extraction solution** (see step 2) to a 10 µL aliquot of serum followed by addition of 0.4 mL of **cold MTBE**. Vortex the samples vigorously for 10 minutes at 4°C. • Add 0.1 mL of **cold H_2O** to each sample to induce phase separation. Vortex for 5 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. One vial can be stored at -80°C, to serve as a back-up, **post evaporation**. **Re-extraction of Serum (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.	*Note. You can save the protein 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 ice or at 4°C.*
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 (1-5 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**