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

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	
• 0.9% NaCl (prepare this using MilliQ water)			
• 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			
• Benchtop centrifuge			

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.

The guidelines presented here have been used with a variety of cell lines including but not limited to; HEK293T, Jurkat, HeLa, A375, Hep G2. Cells should be cultured in a 6-well plate to yield $5e^5$-$2e^6$ cells at confluency, per replicate. We recommend that 3 biological replicates (n≥3) are prepared per condition - (1 well = 1 replicate). If internal standards will not be used, n≥4 replicates are preferred. Additional wells can be included for cell counting.

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	Prepare 0.9% NaCl in LC-MS grade H_2O and filter through a 0.2-0.45 micron filter. It is recommended that the 0.9% NaCl solution is chilled for minimum 2h. This solution can be stored long-term at 4°C. The PRC **cannot accept** samples washed with PBS or >0.9% NaCl. Prepare the **MeOH extraction solution**: This solution consists of 100% LC-MS grade methanol. Add the heavy AA mix to a final concentration of 2 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.	*Salts are problematic for LC-MS analysis. It is important that wash buffer is completely aspirated.* *Example Preparation: 200 mL MeOH + 160 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.*
2	**Washing of Adherent cells**: Place plates on wet ice and aspirate the cell culture media. Wash each well with 1-2 mL of ice-cold 0.9% NaCl. Aspirate the wash buffer carefully. Repeat the wash cycle once and store the plates on dry ice. **Washing of Suspension Cells**: Transfer cells into pre-chilled tubes and centrifuge (~200 x g) to pellet the cells. Remove the cell culture media and wash the cells with ~1.5 mL of ice-cold 0.9% NaCl. Centrifuge (~200 x g) the mixture and carefully remove the supernatant. Repeat the wash cycle once and store the tubes on dry ice.	*All steps must be performed on ice or at 4°C.* *Wet ice with NaCl can be used as an alternative to dry ice (temperature of approximately -10°C).*
3	**Extraction from Adherent Cells**: • Add 0.24 mL of cold **MeOH extraction solution** (see step 1) to each well followed by 0.2 mL of **cold H_2O** and scrape the plate thoroughly. • Transfer the mixture into a pre-chilled Eppendorf tube and vortex for 10 seconds at 4°C. • Add 0.8 mL of **cold MTBE** to each sample. [IV]	*Note. You can save the cell pellet to measure protein concentration for sample normalization or proteomic analysis.* *Pipette tips and Eppendorf tubes do not*

	• 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 (MTBE – MeOH/H_2O– protein). • 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. *IV. The desired ratio between MTBE and MeOH+H_2O is 1: 0.55. If there is sample loss during the transfer step, adjust the MTBE volume accordingly. Matyash Method → MTBE: MeOH: H2O = 4: 1.2: 1* **<u>Extraction from Suspension Cells</u>:** • Resuspend the cell pellet with 0.24 mL of cold **MeOH extraction solution** (see step 1) followed by addition of 0.8 mL of **cold MTBE**. Vortex the samples vigorously for 10 minutes at 4°C. • Add 0.2 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. **<u>Adherent and Suspension Cells (optional)</u>:** 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**. **<u>Re-extraction of Adherent/Suspension Cells (optional):</u>** 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.	*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 a nitrogen air evaporator 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 cell count, protein concentration or DNA concentration. Note that the biological samples (dry extracts) will be treated identically upon submission to the PRC.

The cell culture media from **STEP 2** can be retained and extracted to estimate cellular consumption and secretion of metabolites.

If you are treating the metabolites with reducing/oxidizing agents 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 metabolomics 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**