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 chloroform ($CHCl_3$)	Sigma	650471	Do NOT use amylene stabilized $CHCl_3$
• 0.9% NaCl (prepare this using MilliQ water)			
• 2.5 mM pre-mixed Heavy Amino Acid (AA) mix (U-^{13}C, ^{15}N)	CIL	MSK-A2-1.2	Add heavy ISTDs to methanol if you are submitting the polar layer for metabolite profiling
• -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.

I. The extraction process will result in a methanol phase-which contains polar metabolites, and a chloroform 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 chloroform phase.

	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.	*Salts are problematic for LC-MS analysis. It is important that wash buffer is completely aspirated.* *Example Preparation: 200 mL MeOH + 80 µL of 2.5 mM heavy AA mix (Refer to Table1). This solution can*

	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 if the methanol layer will be retained for metabolite profiling. It is recommended that this solution and the $CHCl_3$ are pre-chilled at -20˚C. The H_2O can be pre-chilled at 4°C.	*be stored long-term at -20°C.* *Deuterated lipid standards like Avanti Splash ® (product #330707 or #330709 or d5-DG ISTD Mix I) can be added to the chloroform, prior to extraction, to serve as internal standards.*
2	**<u>Washing of Adherent cells</u>**: 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. **<u>Washing of Suspension cells</u>**: 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	**<u>Extraction from Adherent cells</u>** : • Add 0.6 mL of cold **MeOH extraction solution** (see step 1) to each well followed by 0.3 mL of **cold H_2O** and scrape the plate thoroughly. • Transfer the mixture into a pre-chilled Eppendorf tube and vortex for 5 seconds at 4°C. • Add 0.4 mL of **cold $CHCl_3$** to each sample. • Vortex the samples 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 3 phases ($CHCl_3$– Precipitate– MeOH). • Carefully collect the two layers separately and transfer each layer into a new Eppendorf tube. Avoid the protein interface between the chloroform (bottom) and methanol (top) layers. **<u>Extraction from Suspension cells</u> :** • Resuspend the cell pellet with 0.6 mL of cold **MeOH extraction solution** (see step 1) followed by addition of 0.3 mL of **cold H_2O**. Vortex for 5 seconds at 4°C. • Add 0.4 mL of **cold $CHCl_3$** to each sample.	*Note. You can save the cell pellet to measure protein concentration for sample normalization.* *Pipette tips and Eppendorf tubes do not have high chemical resistance to chloroform. Avoid polycarbonate-based consumables. Propylene/ polyethylene-based tubes can be used short term* (≤4°C).

	• 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 3 phases ($CHCl_3$– Precipitate– MeOH). • Carefully collect the two layers separately and transfer each layer into a new Eppendorf tube. Avoid the protein interface between the chloroform (bottom) and methanol (top) layers. **<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**.	
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 the chloroform layer will take ~1 hour. The methanol layer will take ~2-3 hours.*
5	Fill out the metabolomics/lipidomics submission form and submit the dried extracts to the PRC. **[https://formspolicies.rockefeller.edu/getfile.php?type=Form&file=Proteomics_Metabolomics_submission_form_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 2-4** and here; https://avantilipids.com/product/330707 , https://avantilipids.com/product/330709 and https://avantilipids.com/product/lm6001 .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. Deuterated fatty acids can be purchased from Cambridge Isotope Laboratories on a gram scale (<$300/1 G, e.g. Item #DLM-215-PK, DLM-208-PK, DLM-379-PK).

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**

Table 4 . Composition of the Avanti d5-DG Internal Standard Mix I product # LM6001.

Component Name	LipidMAPS ID	Exact Mass	Concentration by LC/MS
1,3-14:0 DG-d5	LMGL02010309	517.84	4.01 µM (2.07 µg/mL)
1,3-15:0 DG-d5	LMGL02010310	545.51	4.00 µM (2.18 µg/mL)
1,3-16:0 DG-d5	LMGL02010311	573.54	4.09 µM (2.34 µg/mL)
1,3-17:0 DG-d5	LMGL02010312	601.57	4.06 µM (2.45 µg/mL)
1,3-19:0 DG-d5	LMGL02010313	657.63	4.06 µM (2.67 µg/mL)
1,3-20:0 DG-d5	LMGL02010314	685.66	4.06 µM (2.78 µg/mL)
1,3-20:2 DG-d5	LMGL02010315	677.60	4.19 µM (2.84 µg/mL)
1,3-20:4 DG-d5	LMGL02010316	669.54	4.48 µM (3.00 µg/mL)
1,3-20:5 DG-d5	LMGL02010308	665.51	4.47 µM (2.97 µg/mL)