Objective.
LC-MS analysis of polar 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	
• 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	
• -80°C Freezer			
• Vials	Eppendorf	022431081	LoBind tubes
• Vortexer			
• Temperature-controlled benchtop centrifuge			

Targeted analysis of polar metabolites
Polar metabolites, including amino acids, primary metabolites, components of the TCA cycle and many others are measured using targeted LC-MS methods. The metabolites are separated using hydrophilic interaction chromatography (HILIC) and the mass to charge ratio (m/z) is measured in both positive and negative ionization modes– typically with mass accuracies ≤2 ppm. Additionally, the retention times (RT) of a library of > 200 molecules has been determined for further metabolite confirmation.

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 can 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. Avoid using culture dishes with a surface area larger than 10 cm^2 to avoid salt contamination.

	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 **Extraction Solution**: This solution consists of 80% v/v LC-MS grade methanol, 20% v/v LC-MS grade H_2O and the heavy AA mix (final	*Salts are problematic for LC-MS analysis. It is important that wash buffer is completely aspirated.* *Example Preparation: 160 mL MeOH + 40 mL*

	concentration of 0.5-1 μM). It is recommended that this solution is pre-chilled for minimum 4h at -20°C.	*H_2O + 40-80 uL of 2.5 mM heavy AA mix (Refer to Table1). This solution can be stored long-term at -20°C.*
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 1 mL of cold **Extraction Solution** (see step 1) to each well and scrape the plate thoroughly. Transfer the mixture into a pre-chilled Eppendorf tube and vortex for 10 minutes at 4°C. Centrifuge at 16,000 RCF (or max. speed) at 4°C for 10 minutes to remove cell debris. Transfer the supernatant into a new Eppendorf tube. **Extraction from Suspension cells :** Re-suspend cell pellet with 1 mL of cold **Extraction Solution (see step 1)** and vortex for 10 minutes at 4°C. Centrifuge at 16,000 RCF (or max speed) at 4°C for 10 minutes to remove cell debris. Transfer the supernatant into a new Eppendorf tube. **Adherent and Suspension cells (optional):** The supernatant can be divided equally amongst two Eppendorf tubes. One vial can be stored at -80°C, to serve as a back-up, post evaporation.	*Note. You can save the cell pellet to measure protein concentration for sample normalization.*
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 (2-4 h).*
5	Fill out the metabolomics 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. You can substitute this with other isotopically labelled standard(s) so long as the extraction buffer does not contain any endogenous metabolites. Refer to **Table 1** for the composition of the MSK-A2-1.2 product.

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)