Oct 01, 2024

JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Polar HILIC-Z

DOI
dx.doi.org/10.17504/protocols.io.kxygxydwkl8j/v1

Katherine B. Louie[1], Suzanne Kosina[2], Thomas Harwood[1], Meghana Faltane[1], Marie Lynde[1], Benjamin P. Bowen[1,2], Trent Northen[1,2]

[1]Lawrence Berkeley National Laboratory, Joint Genome Institute, Berkeley, CA, United States;
[2]Lawrence Berkeley National Laboratory, Environmental Genomics and Systems Biology Division, Berkeley, CA, United States

Katherine B. Louie
Lawrence Berkeley National Laboratory, Joint Genome Institut...

DOI: dx.doi.org/10.17504/protocols.io.kxygxydwkl8j/v1

Protocol Citation: Katherine B. Louie, Suzanne Kosina, Thomas Harwood, Meghana Faltane, Marie Lynde, Benjamin P. Bowen, Trent Northen 2024. JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Polar HILIC-Z. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygxydwkl8j/v1

Protocol status: Working
We use this protocol and it's working

Created: September 26, 2024

Last Modified: October 01, 2024

Protocol Integer ID: 108480

Keywords: metabolomics, JGI, HILIC, LBNL, Joint Genome Institute, LC-MS, Thermo Orbitrap, Berkeley Lab, Environmental Metabolite Atlas

Funders Acknowledgement:
The work conducted by the U.S. Department of Energy Joint Genome Institute (https://ror.org/04xm1d337), a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy
Grant ID: Contract No. DE-AC02-05CH11231
The work conducted by ENIGMA – Ecosystems and Networks Integrated with Genes and Molecular Assemblies (http://enigma.lbl.gov), a Science Focus Area Program at Lawrence Berkeley National Laboratory is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research and used resources of the National Energy Research Scientific Computing Center, a Department of Energy Office of Science User Facility
Grant ID: Contract No. DE-AC02-05CH11231

Abstract

This protocol describes the standard LC-MS/MS ESI method developed at Lawrence Berkeley National Laboratory (LBNL) by JGI Metabolomics and EGSB's Northen Lab to analyze polar metabolites (e.g. amino acids, nucleic acids, sugars, organic acids, primary metabolites, etc.) using normal phase chromatography (HILIC-Z) coupled to a Thermo Orbitrap Mass Spectrometer with ESI source. This robust method of detection is easily reproduced and adapted onto similar LC-MS/MS systems to achieve consistent outcomes across mass spectrometry datasets, foster inter-lab and inter-experiment comparability, and enable effective data integration and analysis. Since its inception in 2018, this method has been used to analyze tens of thousands of experimental samples, with many of these datasets publicly available in the MassIVE data repository[1,2]. This method has also been used to run thousands of compound standards to collect characteristic retention time, *m/z* and fragmentation spectra. This forms the basis of Berkeley Lab's Environmental Metabolite Atlas (EMA), a database used for confident annotation of metabolites and publicly available through GNPS/GNPS2[3].

Overview

1 This protocol describes the standard LC-MS/MS ESI method developed at Lawrence Berkeley National Laboratory (LBNL) by JGI Metabolomics and EGSB's Northen Lab to analyze polar metabolites (e.g. amino acids, nucleic acids, sugars, organic acids, primary metabolites, etc.) using normal phase chromatography (HILIC-Z) coupled to a Thermo Orbitrap Mass Spectrometer with ESI source.

Instrumentation / Equipment

2 **Mass Spectrometry Instrumentation**

	A	B	C	D
	Mass Spectrometer	**Source / Probe**	**ESI needle (calibration)**	**ESI needle (running samples)**
	Thermo Q Exactive HF Orbitrap (QE-HF)	Thermo Ion Max API Source w/ H-ESI II probe	32G Metal Needle High Flow (OPTON-53010, Thermo)	32G Metal Needle High Flow (OPTON-53010, Thermo)
	Thermo Q Exactive Orbitrap (QE)	Thermo Ion Max API Source w/ H-ESI II probe	32G Metal Needle High Flow (OPTON-53010, Thermo)	32G Metal Needle High Flow (OPTON-53010, Thermo)
	Thermo Orbitrap Exploris 120 (Exp120)	Thermo OptaMax NG™ API source w/ H-ESI probe	35G Metal Needle Low Flow, 50 um ID (OPTON-30139)	32G Metal Needle High Flow, 100 um ID (OPTON-30694)
	Thermo Orbitrap IQ-X Tribrid (IQX)	Thermo OptaMax NG™ API source w/ H-ESI probe	35G Metal Needle Low Flow, 50 um ID (OPTON-30139)	32G Metal Needle High Flow, 100 um ID (OPTON-30694)

Table 1. Mass spectrometer and source.

Note: Other Thermo Orbitrap mass spectrometers may also be used (e.g. IDX, Exploris 240, Astral, etc) with appropriate source, ESI needle and modified acquisition parameters.

3 **UHPLC System**

	A	B	C	D
	Module Name	**Module Type**	**Part Numbers**	**Alternative Part Numbers**
	1290 Infinity DAD*	Diode Array Detector	G4212A	G7115A, G7117BR
	1200 Infinity Series TCC	Column Compartment	G1316A	G7116B
	1290 Infinity Sampler	Autosampler	G4226A	G7167B - Multisampler w/ thermostat

	A	B	C	D
	1290 Infinity Thermostat	Autosampler Thermostat	G1330B	G7167B - Multisampler w/ thermostat
	1290 Infinity Bin Pump	Binary Pump	G4220A	G7120A

Table 2. Agilent 1290 Infinity UHPLC Modules and type.
* Optional

Note: To use Agilent LC systems in line with Thermo Orbitrap mass spectrometers, communication / compatibility requires either installation of Chromeleon software, or a contact closure board (for QE-HF or QE) or Universal Interface Box (UIB) (for IQX, IDX, and Exploris models), as well as specialized software packages and cables. Information, manuals and installation procedures are available from Agilent and Thermo.

4 UHPLC Column information

	A	B
	Column name	InfinityLab Poroshell 120 HILIC-Z
	Part #	#683775-924
	Manufacturer	Agilent
	Column chemistry	HILIC-Z, zwitterionic
	Inner Diameter (ID)	2.1 mm
	Length	150 mm
	Particle size	2.7 µm
	Pore size	100 Å
	Max pressure	600 bar
	pH range	2-12
	Max temperature	80 °C @ pH 7; 35 °C @ pH 2-12

Table 3. UHPLC column information.

5 Chemicals / solvents

	A	B
	Chemicals / solvents	**Product Number**
	acetonitrile (LC-MS grade)	AX0156, Sigma
	water (LC-MS grade)	9831-03, VWR
	ammonium acetate (LC-MS grade)	73594, Sigma
	acetic acid (glacial, ≥99.7%)	JT9515-3, VWR
	methylene-di-phosphonic acid (medro	64255, Sigma

	A	B
	nic acid)	
	methanol (LC-MS grade)	MX0486, Sigma

Table 4. Chemicals and solvents. These are used to prepare mobile phase and resuspend extracts. For solvents, other LC-MS grade products can also be used. For chemicals, high purity compounds of analytical grade or listed as suitable for mass spectrometry can be used.

LC-MS/MS Method Parameters

6 **LIQUID CHROMATOGRAPHY**

	A	B
	Mobile Phase A	99.8% H2O and 0.2% acetic acid, w/ 5 mM ammonium acetate and 5 µM methylene-di-phosphonic acid
	Mobile Phase B	99.8% 95:5 v/v ACN:H2O and 0.2% acetic acid, w/ 5 mM ammonium acetate

Table 5. Mobile phase composition for HILIC. Sufficient mobile phase for all injections of a sample set are prepared prior to starting a run. To prepare Mobile Phase B, ammonium acetate is first dissolved in water prior to adding acetonitrile and other components (for solubility).

Column

InfinityLab Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 µm, 100 Å (Agilent, #683775-924)

Column Temperature 40 °C

Autosampler Temperature 4 °C

	A	B	C	D	E
	Time (min)	**Flow (mL/min)**	**%A**	**%B**	**minutes for segment**
	0	0.45	0	100	
	1	0.45	0	100	1
	11	0.45	11	89	10
	15.75	0.45	30	70	4.75
	16.25	0.45	80	20	0.5
	18.5	0.45	80	20	2.25

	A	B	C	D	E
	18.6	0.45	0	100	0.1
	21	0.45	0	100	2.4

Table 6. Mobile phase gradients for HILIC. Each segment is a linear gradient to the new mobile phase composition.

Typically, depending on the length of the lines connecting the ESI needle through to the autosampler, the first 0.3-0.8 minutes of the run does not contain signal from the injected sample (void volume). This is then followed by a large peak (solvent front) comprised of metabolites that do not retain on the column. Signals acquired during this time window are typically not used in analysis. Also, signals acquired in the time window following final isocratic elution (here, between 18.5 to 21 minutes during column re-equilibration), are also not used in analysis.

Salty samples: When analyzing samples that still have a lot of salt in the extract, diverting flow to waste for the first 1 minute (void volume containing the most salt) may help keep the ESI needle and source cleaner throughout the run and preserve data quality.

7 MASS SPECTROMETRY

Source settings

	A	B	C	D	E
	Parameter	**QE-HF**	**QE**	**Exp120**	**IQX**
	Sheath Gas Flow Rate (au)	55	55	50	50
	Auxillary Gas Flow Rate (au)	20	20	10	10
	Sweep Gas Flow Rate (au)	2	2	1	1
	Spray Voltage (V) - POS	3000	3000	3500	3500
	Spray Voltage (V) - NEG	3000	3000	2500	2500
	Capillary Temperature (°C)	400	400	325	325
	Vaporizer Temperature (°C)	N/A	N/A	300	300
	S-Lens RF Level (%)	50	50	70	50

Table 7. ESI source settings. These settings are used with these specific Orbitrap models. Other mass spectrometers will need these source settings adjusted to achieve similar results.

- au = arbitrary units

MS1 Settings

	A	B	C	D	E
	Parameter	**QE-HF**	**QE**	**Exp120**	**IQX**

	A	B	C	D	E
	Microscans	1	1	1	1
	Resolution	60,000	70,000	60,000	60,000
	AGC Target	3e6	3e6	Standard (100%)	1e5
	Maximum IT (ms)	100	100	Auto	118
	Scan range (m/z)	70 - 1050	70 - 1050	70 - 1050	70 - 1050
	Spectrum data type	Centroid	Centroid	Centroid	Centroid

Table 8. MS1 scan settings. Full MS spectra are collected in both positive and negative ionization modes. These settings are used with these specific Orbitrap models. Other mass spectrometers will need these source settings adjusted to achieve similar results.

Collection time: 21 minutes

MS2 Settings

	A	B	C	D	E
	Parameter	**QE-HF**	**QE**	**Exp120**	**IQX**
	Microscans	**1**	**1**	1	1
	Resolution	15,000	17,500	15,000	15,000
	AGC Target	1e5	1e5	Standard (100%)	5e4
	Maximum IT (ms)	50	50	Auto	22
	Loop count	2 (or 4)	2 (or 4)	4	N/A
	Cycle time	N/A	N/A	N/A	0.8 sec
	MSX count	1	1	1	1
	TopN	2 (or 4)	2 (or 4)	4	10-15
	Exclusion duration (sec)	7 - 10	7 - 10	4	5
	Stepped Collision Energies (eV)	10, 20, 40 (or 20, 50, 60)	10, 20, 40 (or 20, 50, 60)	10, 20, 40 (or 20, 50, 60)	10, 20, 40 (or 20, 50, 60)

Table 9. MS2 scan settings for collecting fragmentation data. Stepped and then averaged collision energies of 10, 20, 40 eV and/or 20, 50, 60 eV. A full MS1 scan is followed by "N" MS2 scans of the most intense precursor ions (TopN), excluding those precursors already fragmented in the previous time period (exclusion duration).

Source and acquisition settings listed here are the settings used for standard LC-MS/MS runs. Depending on experimental details and goals, these can be adjusted (e.g. instrument resolution, customization of data-dependent MS2, scan range, etc) as needed.

Sample Vial Preparation

8 **Quality Control (QC) Mix**

This is a custom formulation of compounds with annotated *m/z*, retention time (RT), and MS2 spectra dissolved in 100% MeOH. Compounds are representative of the metabolite classes detected using this LC-MS/MS method, as well as *m/z* and retention time ranges.

Usage: QC injections are interspersed throughout the LC-MS run to monitor instrument performance (calibration, intensity, retention time, etc.) as well as adjust compound retention times between runs.

	A	B	C	D	E	F	G	H
	Compound	**Formula**	**Monoisotopic mass**	**Adduct (POS)**	**m/z (POS)**	**Adduct (NEG)**	**m/z (NEG)**	**Expected RT**
	1-methyladenosine	C11H15N5O4	281.1124	[M+H]+	282.1197	[M-H]-	280.1051	10.78
	2,4-dihydroxypteridine	C6H4N4O2	164.0334	[M+H]+	165.0407	[M-H]-	163.0261	1.27
	2'-deoxyadenosine	C10H13N5O3	251.1018	[M+H]+	252.1091	[M-H]-	250.0946	2.23
	2'-deoxyguanosine	C10H13N5O4	267.0968	[M+H]+	268.1040	[M-H]-	266.0895	6.87
	2-hydroxyphenyl acetic acid	C8H8O3	152.0473	[M+H]+	153.0546	[M-H]-	151.0401	1.62
	2-oxovaleric acid	C5H8O3	116.0473	[M+H]+	117.0546	[M-H]-	115.0401	4.45
	4-coumaric acid	C9H8O3	164.0473	[M+H]+	165.0546	[M-H]-	163.0401	1.51
	4-guanidinobutanoic acid	C5H11N3O2	145.0851	[M+H]+	146.0924	[M-H]-	144.0779	13.86
	4-methoxyphenyl acetic acid	C9H10O3	166.0630	[M+H]+	167.0703	[M-H]-	165.0557	1.07
	5-methylcytosine	C5H7N3O	125.0589	[M+H]+	126.0662	[M-H]-	124.0516	4.42
	5-oxo-proline	C5H7NO3	129.0426	[M+H]+	130.0499	[M-H]-	128.0353	11.65
	ABMBA (2-amino-3-bromo-5-methylbenzoic acid)	C8H8BrNO2	228.9738	[M+H]+	229.9811	[M-H]-	227.9666	1.20
	abscisic acid	C15H20O4	264.1362	[M+H]+	265.1434	[M-H]-	263.1289	1.15
	acetylcholine	C7NH16O2+	146.1176	[M]+	146.1176	[M-2H]-	144.1030	1.96
	adenine	C5H5N5	135.054	[M+H]+	136.061	[M-H]-	134.047	2.56

	A	B	C	D	E	F	G	H
			5		8		2	
	adenosine	C10H13N5O4	267.0968	[M+H]+	268.1040	[M-H]-	266.0895	3.09
	alanine	C3H7NO2	89.0477	[M+H]+	90.0550	[M-H]-	88.0404	13.41
	alpha-glucose	C6H12O6	180.0634	[M+H]+	181.0707	[M-2H]2-	89.0707	5.06
	alpha-ketoglutaric acid	C5H6O5	146.0215	[M+H]+	147.0288	[M-H]-	145.0142	14.51
	arginine	C6H14N4O2	174.1117	[M+H]+	175.1190	[M-H]-	173.1044	16.94
	asparagine	C4H8N2O3	132.0535	[M+H]+	133.0608	[M-H]-	131.0462	14.37
	aspartic acid	C4H7NO4	133.0375	[M+H]+	134.0448	[M-H]-	132.0302	16.13
	benzoic acid	C7H6O2	122.0368	[M+H]+	123.0441	[M-H]-	121.0295	1.27
	betaine	C5H12NO2+	118.0863	[M]+	118.0863	[M-2H]-	116.0717	7.91
	caffeic acid	C9H8O4	180.0423	[M+H]+	181.0495	[M-H]-	179.0350	3.22
	caffeine	C8H10N4O2	194.0804	[M+H]+	195.0877	[M-H]-	193.0731	0.88
	carnitine	C7H16NO3+	162.1125	[M+H]+	162.1125	[M-H]-	160.0979	13.29
	cis-4-hydroxy-proline	C5H9NO3	131.0582	[M+H]+	132.0655	[M-H]-	130.0510	13.67
	citrulline	C6H13N3O3	175.0957	[M+H]+	176.1030	[M-H]-	174.0884	15.09
	creatine	C4H9N3O2	131.0695	[M+H]+	132.0768	[M-H]-	130.0622	13.39
	cysteic acid	C3H7NO5S	169.0045	[M+H]+	170.0118	[M-H]-	167.9972	14.54
	cytidine	C9H13N3O5	243.0855	[M+H]+	244.0928	[M-H]-	242.0782	6.93
	cytosine	C4H5N3O	111.0433	[M+H]+	112.0505	[M-H]-	110.0360	4.83
	deoxycytidine	C9H13N3O4	227.0906	[M+H]+	228.0979	[M-H]-	226.0833	5.59
	deoxyuridine	C9H12N2O5	228.0746	[M+H]+	229.0819	[M-H]-	227.0673	1.88
	ectoine	C6H10N2O2	142.0742	[M+H]+	143.0815	[M-H]-	141.0670	12.50
	fumaric acid	C4H4O4	116.0110	[M+H]+	117.0182	[M-H]-	115.0037	16.31
	gamma-aminobu	C4H9NO2	103.063	[M+H]+	104.070	[M-H]-	102.056	14.39

	A	B	C	D	E	F	G	H
	tyric acid		3		6		1	
	glutamic acid	C5H9NO4	147.0532	[M+H]+	148.0604	[M-H]-	146.0459	15.94
	glutamine	C5H10N2O3	146.0691	[M+H]+	147.0764	[M-H]-	145.0619	14.31
	glutaric acid	C5H8O4	132.0423	[M-H+2Na]+	177.0858	[M-H]-	131.0350	12.60
	guanosine	C10H13N5O5	283.0917	[M+H]+	284.0989	[M-H]-	282.0844	8.57
	homoserine	C4H9NO3	119.0582	[M+H]+	120.0655	[M-H]-	118.0510	13.65
	hypoxanthine	C5H4N4O	136.0385	[M+H]+	137.0458	[M-H]-	135.0312	3.10
	inosine	C10H12N4O5	268.0808	[M+H]+	269.0880	[M-H]-	267.0735	5.43
	isoleucine	C6H13NO2	131.0946	[M+H]+	132.1019	[M-H]-	130.0874	9.71
	jasmonic acid	C12H18O3	210.1256	[M+H]+	211.1329	[M-H]-	209.1183	1.04
	lactic acid	C3H6O3	90.0317	[M+H]+	91.0390	[M-H]-	89.0244	5.06
	leucine	C6H13NO2	131.0946	[M+H]+	132.1019	[M-H]-	130.0874	9.32
	lysine	C6H14N2O2	146.1055	[M+H]+	147.1128	[M-H]-	145.0983	17.01
	maltose	C12H22O11	342.1162	[M+Na]+	365.1054	[M-H]-	341.1089	14.07
	mannitol	C6H14O6	182.0790	[M+H]+	183.0863	[M-H]-	181.0718	9.53
	mannosamine	C6H13NO5	179.0794	[M+H]+	180.0866	[M-H]-	178.0721	14.52
	methionine	C5H11NO2S	149.0510	[M+H]+	150.0583	[M-H]-	148.0438	10.44
	MRFA (Met-Arg-Phe-Ala)	C23H37N7O5S	523.2577	[M+H]+	524.2650	[M-H]-	522.2504	13.61
	N-acetyl-aspartic acid	C6H9NO5	175.0481	[M+H]+	176.0553	[M-H]-	174.0408	14.82
	N-acetyl-glutamic acid	C7H11NO5	189.0637	[M+H]+	190.0710	[M-H]-	188.0564	15.16
	N-acetyl-mannosamine	C8H15NO6	221.0899	[M+Na]+	244.0792	[M-H]-	220.0827	7.15
	N-alpha-acetyl-lysine	C8H16N2O3	188.1161	[M+H]+	189.1234	[M-H]-	187.1088	15.13
	n-butylamine	C4H11N	73.0891	[M+H]+	74.0964	[M-H]-	72.0819	4.17
	nicotinamide	C6H6N2O	122.0480	[M+H]+	123.0553	[M-H]-	121.0407	1.22

	A	B	C	D	E	F	G	H
	nicotinic acid	C6H5NO2	123.0320	[M+H]+	124.0393	[M-H]-	122.0248	5.63
	ornithine	C5H12N2O2	132.0899	[M+H]+	133.0972	[M-H]-	131.0826	17.04
	phenylacetic acid	C8H8O2	136.0524	[M+H]+	137.0597	[M-H]-	135.0452	5.88
	phenylalanine	C9H11NO2	165.0790	[M+H]+	166.0863	[M-H]-	164.0717	8.98
	pipecolic acid	C6H11NO2	129.0790	[M+H]+	130.0863	[M-H]-	128.0717	10.97
	proline	C5H9NO2	115.0633	[M+H]+	116.0706	[M-H]-	114.0561	10.92
	pyridoxine	C8H11NO3	169.0739	[M+H]+	170.0812	[M-H]-	168.0666	2.16
	raffinose	C18H32O16	504.1690	[M+H]+	505.1763	[M-H]-	503.1618	15.53
	rhamnose	C6H12O5	164.0685	[M+H]+	165.0757	[M-H]-	163.0612	2.80
	ribose	C5H10O5	150.0528	[M+H]+	151.0601	[M-H]-	149.0455	2.75
	salicylic acid	C7H6O3	138.0317	[M+H]+	139.0390	[M-H]-	137.0244	2.20
	serine	C3H7NO3	105.0426	[M+H]+	106.0499	[M-H]-	104.0353	14.31
	shikimic acid	C7H10O5	174.0528	[M+H]+	175.0601	[M-H]-	173.0455	13.41
	succinic acid	C4H6O4	118.0266	[M+H]+	119.0339	[M-H]-	117.0193	9.92
	sucrose	C12H22O11	342.1162	[M+Na]+	365.1054	[M-H]-	341.1089	13.45
	syringic acid	C9H10O5	198.0528	[M+H]+	199.0601	[M-H]-	197.0455	1.59
	taurine	C2H7NO3S	125.0147	[M+H]+	126.0219	[M-H]-	124.0074	12.16
	threonine	C4H9NO3	119.0582	[M+H]+	120.0655	[M-H]-	118.0510	13.49
	thymidine	C10H14N2O5	242.0903	[M+H]+	243.0975	[M-H]-	241.0830	1.60
	thymine	C5H6N2O2	126.0429	[M+H]+	127.0502	[M-H]-	125.0357	1.26
	trans-4-hydroxy-proline	C5H9NO3	131.0582	[M+H]+	132.0655	[M-H]-	130.0510	13.25
	trans-cinnamic acid	C9H8O2	148.0524	[M+H]+	149.0597	[M-H]-	147.0452	1.15
	trehalose	C12H22O11	342.1162	[M+Na]+	365.1054	[M-H]-	341.1089	14.21

	A	B	C	D	E	F	G	H
	tryptophan	C11H12N2O2	204.0899	[M+H]+	205.0972	[M-H]-	203.0826	10.16
	uracil	C4H4N2O2	112.0273	[M+H]+	113.0346	[M-H]-	111.0200	1.39
	uridine	C9H12N2O6	244.0695	[M+H]+	245.0768	[M-H]-	243.0623	2.89
	urocanic acid	C6H6N2O2	138.0429	[M+H]+	139.0502	[M-H]-	137.0357	9.35
	valine	C5H11NO2	117.0790	[M+H]+	118.0863	[M-H]-	116.0717	11.12
	vanillic acid	C8H8O4	168.0423	[M+H]+	169.0495	[M-H]-	167.0350	1.54
	vanillin	C8H8O3	152.0473	[M+H]+	153.0546	[M-H]-	151.0401	0.86
	xanthine	C5H4N4O2	152.0334	[M+H]+	153.0407	[M-H]-	151.0261	2.73
	xanthosine	C10H12N4O6	284.0757	[M+H]+	285.0830	[M-H]-	283.0684	9.78

Table 10. Representative QC Mix for polar HILIC-Z. For each compound, observed adduct in positive and negative mode are listed as well as the observed retention time using this LC-MS/MS method.

- **Note:** Some compounds are only detected in a single polarity. Most ionize well at a concentration of 25 µM.

9 **Blank**

100% MeOH only (or other solvent matching the resuspension solvent of experimental samples).

Usage: Blank injections are interspersed between each sample injection to monitor background and minimize carryover (e.g. compounds "caught" in the system from the previous injection and detected in the next injection) between samples.

10 **Internal Standard (ISTD) mix**

A custom mixture of isotopically labeled (and/or non-biological/synthetic) compounds. These are added (at a specific concentration) to each sample prior to running LC-MS (typically during resuspension).

Usage: (1) Similar to QC mix, injections of ISTD mix only are interspersed throughout the LC-MS run to monitor instrument performance (calibration, intensity, retention time, etc.) as well as adjust compound retention times between runs. (2) Every experimental sample is also resuspended in solvent containing ISTDs. Since these are present in every sample at the same concentration, these can be used to assess individual sample injection properties, including changes in retention time (e.g. due to sample pH, clogging), failed injections, or intensity

variations (e.g. due to matrix effects, source fouling, or other factors). Additionally, since concentrations are known, an approximation of concentration for the same compound found in an experimental sample (not isotopically labeled) can be estimated based on ratio.

	A	B	C	D	E	F	G	H	I	J	K
	Compound	**Formula**	**Concentration**	**Monoisotopic mass**	**Adduct (POS)**	**m/z (POS)**	**Adduct (NEG)**	**m/z (NEG)**	**Expected RT**	**Part number**	**Notes**
	alanine (U - 13C, 15N)	[13C]3H7[15N]O2	32.5 µM	93.0548	[M+H]+	94.0620	[M-H]-	92.0475	13.41	767964, Sigma	
	arginine (U - 13C, 15N)	[13C]6H14[15N]4O2	11 µM	184.1199	[M+H]+	185.1272	[M-H]-	183.1127	16.94	767964, Sigma	
	asparagine (U - 13C, 15N)	[13C]4H8[15N]2O3	9.5 µM	138.0610	[M+H]+	139.0683	[M-H]-	137.0537	14.37	767964, Sigma	
	aspartic acid (U - 13C, 15N)	[13C]4H7[15N]O4	26 µM	138.0480	[M+H]+	139.0552	[M-H]-	137.0407	16.13	767964, Sigma	
	cysteine (U - 13C, 15N)	[13C]3H7[15N]O2S	10 µM	125.0268	[M+H]+	126.0341	[M-H]-	124.0196	*Not detected*	767964, Sigma	Not detected, oxidizes to cystine
	cystine (U - 13C, 15N)	[13C]6H12[15N]2O4S2	10 µM	248.0380	[M+H]+	249.0453	[M-H]-	247.0308	16.9	N/A	Not added but a bypproduct of cysteine (in amino acid mix)
	glutamic acid (U - 13C, 15N)	[13C]5H9[15N]O4	21 µM	153.0670	[M+H]+	154.0742	[M-H]-	152.0597	15.94	767964, Sigma	
	glutamine (U - 13C, 15N)	[13C]5H10[15N]2O3	10 µM	153.0800	[M+H]+	154.0873	[M-H]-	152.0727	14.31	767964, Sigma	
	glycine (U - 13C, 15N)	[13C]2H5[15N]O2	24 µM	78.0358	[M+H]+	79.0430	[M-H]-	77.0285	14.1	767964, Sigma	
	histidine (U - 13C, 15N)	[13C]6H9[15N]3O2	2.5 µM	164.0807	[M+H]+	165.0880	[M-H]-	163.0734	14.88	767964, Sigma	
	isoleucine (U - 13C, 15N)	[13C]6H13[15N]O2	10.5 µM	138.1118	[M+H]+	139.1191	[M-H]-	137.1045	9.71	767964, Sigma	

	A	B	C	D	E	F	G	H	I	J	K
	leucine (U - 13C, 15N)	[13C]6H13[15N]O2	22.5 µM	138.1118	[M+H]+	139.1191	[M-H]-	137.1045	9.32	767964, Sigma	
	lysine (U - 13C, 15N)	[13C]6H14[15N]2O2	9.5 µM	154.1197	[M+H]+	155.1270	[M-H]-	153.1124	17.01	767964, Sigma	
	methionine (U - 13C, 15N)	[13C]5H11[15N]O2S	4.5 µM	155.0649	[M+H]+	156.0721	[M-H]-	154.0576	10.44	767964, Sigma	
	phenylalanine (U - 13C, 15N)	[13C]9H11[15N]O2	8.5 µM	175.1062	[M+H]+	176.1135	[M-H]-	174.0989	8.98	767964, Sigma	
	proline (U - 13C, 15N)	[13C]5H9[15N]O2	9.5 µM	121.0771	[M+H]+	122.0844	[M-H]-	120.0699	10.92	767964, Sigma	
	serine (U - 13C, 15N)	[13C]3H7[15N]O3	14 µM	109.0497	[M+H]+	110.0570	[M-H]-	108.0424	14.31	767964, Sigma	
	threonine (U - 13C, 15N)	[13C]4H9[15N]O3	14 µM	124.0687	[M+H]+	125.0760	[M-H]-	123.0614	13.49	767964, Sigma	
	tryptophan (U - 13C, 15N)	[13C]11H12[15N]2O2	10 µM	217.1209	[M+H]+	218.1281	[M-H]-	216.1136	10.16	767964, Sigma	
	tyrosine (U - 13C, 15N)	[13C]9H11[15N]O3	6.5 µM	191.1011	[M+H]+	192.1084	[M-H]-	190.0938	11.86	767964, Sigma	
	valine (U - 13C, 15N)	[13C]5H11[15N]O2	14.5 µM	123.0928	[M+H]+	124.1001	[M-H]-	122.0855	11.12	767964, Sigma	
	mannitol (U - 13C)	[13C]6H14O6	10 µg/mL	188.0992	[M+H]+	189.1064	[M-H]-	187.0919	9.53	ALD-030, Omicron Biochemicals	
	trehalose (U - 13C)	[13C]12H22O11	10 ug/mL	354.1565	[M+Na]+	377.1457	[M-H]-	353.1492	14.44	TRE-002, Omicron Biochemicals	
	adenine (U - 15N)	C5H5[15N]5	4 µg/mL	140.0397	[M+H]+	141.0469	[M-H]-	139.0324	2.56	NLM-6924, Cambridge Isotope Labs	
	hypoxanthine (U - 15N)	C5H4[15N]4O	3 µg/mL	140.0267	[M+H]+	141.0339	[M-H]-	139.0194	3.1	NLM-8500, Cambridge Isotope Labs	
	uracil (U - 13C, 15N)	[13C]4H4O2[15N]2	2 µg/mL	118.0348	[M+H]+	119.0420	[M-H]-	117.0275	1.39	CNLM-3917, Cambrid	

	A	B	C	D	E	F	G	H	I	J	K
										ge Isotope Labs	
	inosine (U - 15 N)	C10H12[15N]4O5	5.5 µg/mL	272.0689	[M+H]+	273.0762	[M-H]-	271.0616	5.43	NLM-4264, Cambridge Isotope Labs	
	cytosine (13C2, 15N3)	C2[13C]2H5[15N]3O	5 µg/mL	116.0411	[M+H]+	117.0483	[M-H]-	115.0338	4.83	492108, Sigma	
	guanine (U - 15 N)	C5H5[15N]5O	2 µg/mL	156.0346	[M+H]+	157.0419	[M-H]-	155.0273	6.27	NLM-6926, Cambridge Isotope Labs	
	thymine (U - 13C, 15N)	[13C]5H6[15N]2O2	2.5 µg/mL	133.0538	[M+H]+	134.0610	[M-H]-	132.0465	1.26	CNLM-6945, Cambridge Isotope Labs	
	2-amino-3-bromo-5-methylbenzoic acid (ABMBA) (Br-nat)	C8H8BrNO2	1 µg/mL	228.9738	[M+H]+	229.9811	[M-H]-	227.9666	1.2	631531, Sigma	

Table 11. Representative ISTD Mix used for resuspension. For each compound, observed adduct in positive and negative ion mode are listed as well as the observed retention time using this LC-MS/MS method. Concentrations listed are the typical concentrations used in this protocol.

- For the Sigma amino acid mix (#767964, Sigma), the final concentration varies for each compound between lots, but is usually very similar between lots. Average concentration is ~15 µM for all compounds across the Sigma amino acid mix.
- For stable isotope labeling studies with 13C, typically only the 13C-15N amino acid mixture and ABMBA (synthetic and contains bromine - isobaric) are used as internal standards. Similar adjustments are made for other types of stable isotope labeling experiments based on type of labeling and overall experimental design.

11 **Experimental Sample Vial**

Samples typically consist of metabolite extracts (or a compound standard at a specified concentration) resuspended in solvent, usually 100% MeOH, and containing a mixture of isotopically labeled internal standards (see example ISTD mix above).

Usage: To profile metabolites in a sample and/or annotate the retention time, ionization characteristics (m/z for an adduct) and fragmentation spectra of a compound.

LC System Preparation

12 To prepare the LC, column compartment (40 ºC) and autosampler temperatures (4 ºC) are set and monitored until stable. The LC binary pump is typically prepared by purging 100% mobile phase A, 50/0 mobile phase A/B, then 100% B, each for 7 minutes at a flow rate of 5 mL/min. A flow rate of 0.45 mL/min of 100% B is maintained while the UHPLC system is checked for leaks or clogs. Backpressure is monitored until stable at ~130 bar. The HILIC column is equilibrated by performing 3-10 injections using the method gradients provided in Table 6.

Mass Spectrometer Preparation

13 Prior to data acquisition, the mass spectrometer is calibrated using standard calibration procedures available in the Thermo XCalibur operating software. ESI needle position is optimized relative to the source to achieve stable and acceptable ion intensity levels.

Calibration procedure for QE and QE-HF. Here, calibration is performed in positive mode with Pierce™ LTQ Velos ESI Positive Ion Calibration Solution (#88323, Thermo Scientific) and negative mode with Pierce™ Negative Ion Calibration Solution (#88324, Thermo Scientific) using direct injection from a syringe pump. Standard calibration is then followed by a custom low mass calibration procedure to ensure compounds near 100 *m/z* are also well-calibrated.

Calibration procedure for Exp120 and IQX. Here, calibration is performed in both positive and negative ionization mode with Pierce™ FlexMix Calibration Solution (#A39239, Thermo Scientific) using direct injection from a syringe pump and insertion of the low-flow ESI needle into the source housing. Custom low mass calibration procedures are not necessary using these systems. The system can be re-calibrated periodically during data acquisition using an automated point calibration (EasyIC) with the internal calibrant compound fluoroanthene. Prior to starting an LC-MS/MS run for data collection, the low-flow needle is replaced by the high-flow needle.

Note: For the IQX, an auto-calibration option can be performed using the Auto-ready Ion Source. This uses a less concentrated calibration mix (Pierce™ FlexMix Calibration Solution for Auto-Ready Mass Spectrometers, #A51739, Thermo Scientific) and eliminates the need to switch between low- and high-flow ESI needles.

LC-MS/MS Data Collection

14 In a typical LC-MS/MS run, an injection volume of 2-3 µL for each sample is used. Each sample is run in positive and negative ionization mode, with an injection blank of 100% methanol interspersed between each sample, replaced by an ISTD mix interspersed every 3 samples and a QC mix every 9-15 samples. Sample injection order is randomized between groups of replicate 1, then replicate 2, etc. Prior to starting a full experimental run, at least 4 injection blanks and several QC and ISTD injections are performed to ensure column and system

equilibration and to verify that data is being acquired as expected. ISTD mix compounds are regularly monitored throughout LC-MS runs to assess drops in intensity, retention time shifts or increases in *m/z* ppm error, and performing the appropriate cleaning, re-calibration, maintenance or other troubleshooting as needed.

Protocol references

1 - https://massive.ucsd.edu/

2 - Eric W. Deutsch, Attila Csordas, Zhi Sun, Andrew Jarnuczak, Yasset Perez-Riverol, Tobias Ternent, David S. Campbell, Manuel Bernal-Llinares, Shujiro Okuda, Shin Kawano, Robert L. Moritz, Jeremy J. Carver, Mingxun Wang, Yasushi Ishihama, Nuno Bandeira, Henning Hermjakob, Juan Antonio Vizcaíno, The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition, *Nucleic Acids Research*, Volume 45, Issue D1, January 2017, Pages D1100–D1106, https://doi.org/10.1093/nar/gkw936

3 - Wang, M., Carver, J., Phelan, V. *et al.* Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nat Biotechnol* **34**, 828–837 (2016). https://doi.org/10.1038/nbt.3597