

An Automated Dual Metabolite + Lipid Sample Preparation Workflow for Mammalian Cell Samples

Automated cell sample preparation for use with a comprehensive, end-to-end LC/MS metabolomics and lipidomics workflow

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Abstract

This technical overview demonstrates a comprehensive, automated sample preparation method for dual extraction of polar metabolites and lipids from mammalian cell samples. The sample preparation workflow includes an innovative room temperature method for facile lysis and metabolic quenching of mammalian cells. The fractionation and sequential extraction of polar metabolites and lipids from each sample of cell lysate is achieved with the Agilent Captiva EMR–Lipid solid phase extraction plate. The metabolite and lipid fractionation procedures are automated on the Agilent Bravo Metabolomics Sample Prep Platform using supplementary Dual Metabolite + Lipid Cell Sample Preparation Vworks protocols. Metabolite and lipid recoveries and workflow reproducibility are evaluated through use of the Agilent 6545 LC/Q-TOF, Agilent 6550 iFunnel Q-TOF LC/MS, and Agilent MassHunter software.

Introduction

To obtain a more complete understanding of cellular biology as measured through LC/MS omics analyses, there is a large benefit in isolating multiple classes of cellular components from each sample. This is especially true for polar metabolites and lipids, as changes in cellular metabolism can impact both primary and lipid metabolisms. Although methods exist for fractionation of metabolites and lipids from cell samples, there is a need for more easily mastered, precise, sensitive, and accurate workflows. One well-known approach for improving these characteristics is through automation, which improves sample processing consistency, reduces user errors, and reduces learning time for new users.

In a first step toward enabling automation, we developed and evaluated a novel room temperature cell lysis and quenching method that can be used with suspension or adherent mammalian cells. Next, we turned our attention to dual fractionation of polar metabolites and lipids using solid phase extraction (SPE), which is more easily automated than liquid-liquid extraction methods. For this goal, the EMR–Lipid sorbent, previously used for either polar metabolite or lipid extraction from plasma samples¹⁻³, was adapted to a new dual metabolite + lipid extraction protocol for cell lysate. Importantly, the Agilent Captiva EMR–Lipid SPE method extracts both polar metabolites and lipids from each individual sample, facilitating robust multi-omics correlation analyses of the downstream LC/MS metabolomics and lipidomics data.

Use of Captiva EMR–Lipid SPE for polar metabolite and lipid extraction enabled facile automation of the dual fractionation workflow with the Agilent Bravo automated liquid handling platform. While automation was

relatively straightforward, optimizations achieved more quantitative analyte recovery, improved liquid handling, and increased filtration reproducibility. The overall performance of the sample preparation workflow was assessed via spike-in recovery experiments that used ¹³C-labeled polar metabolites and ²H-labeled lipids. Overall, we demonstrate a robust, partially automated sample preparation workflow for dual metabolite + lipid extraction of mammalian cell samples.

Experimental

Materials

For all studies, the K562 chronic myeloid leukemia-derived cell line was obtained from American Type Culture Collection, cultured in complete Roswell Park Memorial Institute (RPMI) medium, and harvested at a density of 0.5 to 1 × 10⁶ cells/milliliter.

ATP measurements were completed on cell lysates using the CellTiter Glo ATP assay kit (Promega).

Qualitative flux analyses used ¹³C₅-L-glutamine (MilliporeSigma). The recovery studies used ¹³C-labeled polar metabolites and ²H-labeled lipids. The ¹³C-labeled polar metabolites included MSK-QC1, CLM-1579-N-0.1MG, CLM-4442-0.1MG, and ISO1 U-¹³C-labeled yeast extract (Cambridge Isotopes) as well as ¹³C₆-D-glucose and ¹³C₅-L-glutamine (MilliporeSigma). The ²H-labeled lipids consisted of the UltimateSPLASH ONE mix (Avanti Polar Lipids). An unlabeled polar metabolite mixture of common central carbon metabolites (MilliporeSigma) was used for peak identification of a subset of the polar metabolites analyzed in the recovery study, and to determine the impact of water content on metabolite recoveries.

Ultrapure water (H₂O) was produced with a Milli-Q Integral system equipped with a LC-Pak Polisher and a 0.22 μm point-of-use membrane filter cartridge (MilliporeSigma).

Sample preparation solvents and chemicals:

LC/MS-grade methanol (MeOH), LC/MS-grade trifluoroethanol (TFE), ≥99.5% GC-grade dichloromethane (DCM), high purity (>99.5%) ethanol (EtOH), LC-grade or 99.9% pure butanol (BuOH), LC/MS-grade acetonitrile (ACN), LC/MS-grade isopropanol (IPA), and LC/MS-grade formic acid were used.

LC/MS mobile phase solvents and chemicals:

LC/MS-grade MeOH, LC/MS-grade ACN, LC/MS-grade IPA, LC/MS-grade ammonium acetate, 99.99% pure ammonium fluoride, LC/MS-grade acetic acid, and GC-grade tributylamine were used.

Agilent Bravo liquid handler and consumables

- Agilent Bravo Metabolomics Sample Prep Platform (G5589AA)
- Supplementary Dual Metabolite + Lipid Cell Sample Preparation Vworks protocols (contact Automation Sales Representative for details)
- Agilent Captiva EMR–Lipid solid phase extraction plates (part number 5190-1001)
- Agilent 250 μL disposable tips (part number 19477-002)
- Agilent PlateLoc thermal microplate sealer (G5585BA)
- Agilent PlateLoc microplate clear pierceable thin seal (part number 17318-001)
- Agilent Adhesive seal for temporary plate sealing (part number 410186)
- VWR Adhesive foil for cold storage of plates, if PlateLoc is not used (part number 60941-112)
- Agilent Captiva collection plates (part number A696001000)

- Agilent reservoirs, single cavity (part number 204484-100 or 201254-100)
- Thermo Fisher Scientific Plate+ Glass Coated Microplates (Thermo Fisher Scientific part number 60180-P338, a replacement for part number 60180-P309)

LC/MS instrumentation and consumables

- Agilent 1290 Infinity II LC System (for lipid LC/MS):
 - Agilent 1290 Infinity II High Speed Pump (G7120A)
 - Agilent 1290 Infinity II Multisampler with thermostat (G7167B)
 - Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with 2-pos/10-port valve
 - Agilent 1290 Infinity II High Speed Pump (G7120A) with a 100:1 splitter (G1607-60000) for reference mass introduction
- Agilent 6550 iFunnel Q-TOF LC/MS with a Dual Agilent Jet Stream Technology ion source (for lipid LC/MS)
- Agilent 1290 Infinity/II LC System (for metabolite LC/MS):
 - Agilent 1290 Infinity Bin Pump (G4220A)
 - Agilent 1290 Infinity II Multisampler with thermostat (G7167B)
 - Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
 - Agilent 1290 Infinity Bin Pump (G4220A) with a 100:1 splitter (G1607-60000) for reference mass introduction
- Agilent 6545 LC/Q-TOF with a Dual Agilent Jet Stream Technology ion source (for metabolite LC/MS)
- Both systems had an inline filter housing (5067-6189) and 0.3 μ m filter (5023-0271) after the injector

Plate reader

Bioluminescence measurements for ATP detection were made on an Agilent BioTek Synergy H1 multimode reader with Gen 5 software, version 3.02.1.

Methods

Room temperature cell lysis and metabolism quenching

A method for room temperature (RT) cell lysis and quenching was developed. This

RT method avoids difficulties associated with manual handling and temperature maintenance of near-zero/subzero cell lysis and quenching solutions and helps enable automation of cell lysis and quenching. This method uses a 1:1 TFE:H₂O solution (Figure 1). As shown in Figure 1, manual methods were developed for RT cell lysis and quenching of both suspension and adherent mammalian cell lines.

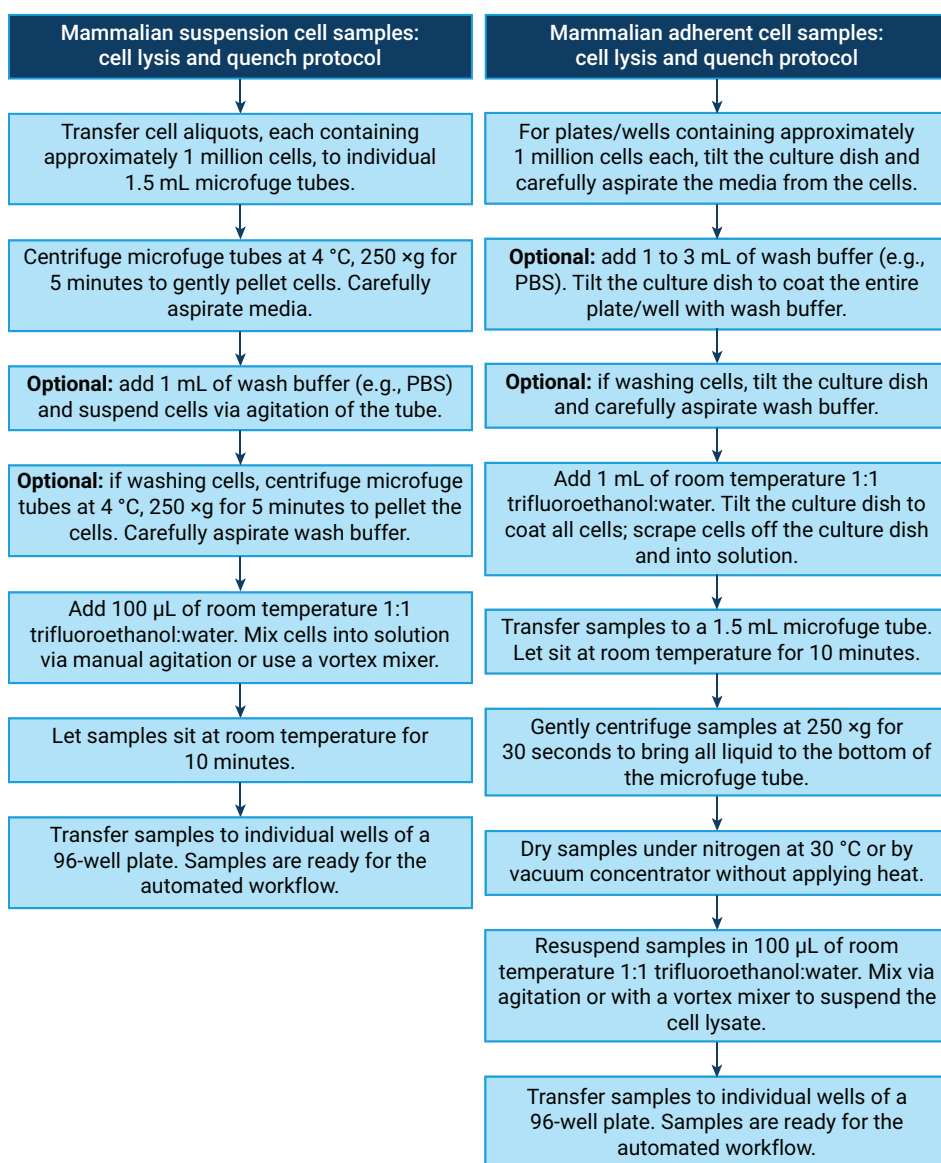


Figure 1. Workflows for manual room temperature cell lysis and metabolism quenching of suspension and adherent mammalian cells.

The experiments described herein used the suspension cell method for preparation of K562 cells, and incorporated an optional wash with RT phosphate buffered saline (PBS, –Ca, –Mg, 1 mL/million cells). The optional wash reduces sample contamination that can come from extracellular metabolites and lipids present in the media or excreted by the cells. However, any wash increases time to quenching, allowing more metabolite and lipid turnover within the cells, and some washes cause metabolite leakage from the cells. Therefore, we leave it to the user to decide whether to complete a wash, and, if used, which wash to complete.

Importantly, both suspension and adherent cell workflows provide a final sample with lysate from approximately one million cells, suspended in 100 μL of 1:1 TFE:H₂O. This final sample is transferred to a deep-well 96-well plate (part number A696001000). For adherent cells, the 100 μL lysate volume is achieved through a concentration step. The final 100 μL volume of the cell lysate is key to performance of the automated dual metabolite + lipid extraction workflow, described next. Since cell volume varies across cell lines, adjustments to the number of cells per 100 μL 1:1 TFE:H₂O may be required to optimize metabolite and lipid detection. For reference, 1 million K562 cells have a packed cell volume of approximately 1 to 2 μL .

Automated dual metabolite + lipid extraction

As with the previously described^{1,2} automated workflows for polar metabolite extraction from 100 and 25 μL plasma samples, the automated dual metabolite + lipid extraction workflow for mammalian cell samples uses the Agilent Bravo Metabolomics Sample Prep Platform (part number G5589AA).¹ Additionally,

supplementary VWorks protocols are required to run the automated dual metabolite + lipid extraction workflow for mammalian cell samples. The Bravo Metabolomics Sample Prep Platform includes a vacuum filtration station for solid phase extraction, and a shaking deck for sample mixing. As with the previously described plasma workflows, the cell workflow uses the Captiva EMR–Lipid plate to extract polar metabolites, while filtering out the protein precipitate and removing the lipids via adsorption. In addition, the cell workflow elutes the adsorbed lipids to provide dual metabolite + lipid extraction (Figure 2).

Lipid elution from the EMR–Lipid sorbent is achieved with 2:1 MeOH:DCM, as previously described.³

For the metabolite extraction portion of the workflow, many of the steps and fundamental principles between the parent original method¹ and this modified protocol remained the same. Importantly, this included using 1:1 MeOH:EtOH for effective protein precipitation. Some of the modifications made for the metabolite extraction steps are shared with the adjustments made for low-volume plasma samples described in Agilent application note 5994-2156EN.² Specifically, the dual metabolite +

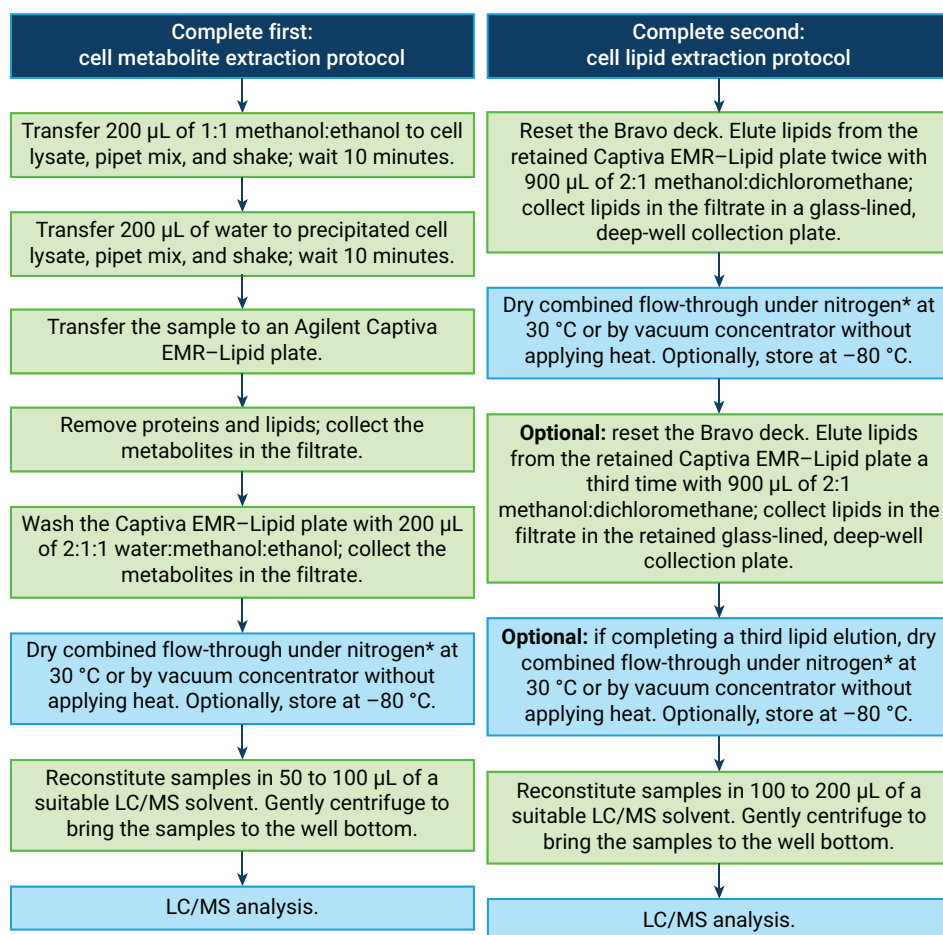


Figure 2. Workflow for automated dual metabolite + lipid extraction from cell lysate samples. Green steps can be performed with the supplementary Dual Metabolite + Lipid Cell Sample Preparation Vworks protocols and the Agilent Bravo Metabolomics Sample Prep Platform. *Nitrogen drying note: The use of high nitrogen flow and lowering the manifold periodically (so the nitrogen flow is maintained at a close distance to the top of the sample) reduces drying time. Low nitrogen flow is recommended when samples are reduced to less than 50 μL , to prevent dried samples from being ejected from the plate wells.

lipid cell workflow includes 10-minute incubations following metabolism quenching and protein precipitation to help achieve equilibration of metabolites and lipids between the solution and protein precipitate for improved sample consistency. Additionally, water is added to the protein-precipitated sample to achieve 50% H₂O content before polar metabolite extraction (see Results and discussion).

Notably, several steps were added to the automated workflow to more closely achieve quantitative recovery of metabolites and lipids extracted from the cell samples. This was considered particularly important, given the lower biological content of the cell samples. These contained approximately 1 to 2 μ L packed cell volume, which is approximately 50 to 100 times less sample volume than the 100 μ L plasma samples handled in the original method. These quantitative recovery workflow adjustments included 1) addition of the 1:1 MeOH:EtOH protein precipitation solvent to the cell lysate, as opposed to addition of the cell lysate to the protein precipitation solvent, 2) washing and collecting cell lysate residue from the cell lysate plate after transfer of the cell lysate to the Captiva EMR–Lipid plate, 3) washing the Captiva EMR–Lipid plate to release the plate hold-up volume (~115 μ L) and to wash out polar metabolites that interact with the EMR–Lipid sorbent, and 4) drying the entire collected polar metabolite sample. The updated workflow was automated on the Bravo Metabolomics Sample Prep Platform with the supplementary Dual Metabolite + Lipid Cell Sample Preparation Vworks protocols. Figures 3A and 3B display the user interfaces for the supplementary protocols used for cell metabolite and lipid extraction, respectively.

Other automation optimizations included solvent-priming the tips twice for robust, drip-free handling of low boiling point organic solvents used for lipid elution, optimizing all filtration steps for cell lysate samples, and enabling an optional postfiltration SPE plate inspection with the ability to add filtration time and increase filtration pressure, as needed. Combined, these updates provide a highly robust automated workflow for dual metabolite + lipid extraction of cell lysate.

In the described work, sample reconstitution was achieved by manually dissolving dried polar metabolite fractions in 25 μ L MeOH, followed by bath sonication of the plate for 60 seconds. Next, the samples were dissolved further with addition of 75 μ L H₂O, and the plate was bath sonicated for another 60 seconds before a gentle centrifugation (15 to 30 seconds, 250 \times g) to bring the entire sample to the bottom of each well of the plate. Dried lipid fractions were resuspended with 200 μ L of MeOH:BuOH (1:1, v:v); the plate was bath-sonicated for 60 seconds, then gently centrifuged (15 to 30 seconds, 250 \times g) to bring the entire sample to the bottom of each well of the plate.

As an alternative, metabolite and lipid sample reconstitution can be achieved using the reconstitution application provided with the Bravo Metabolomics Sample Prep Platform. Other reconstitution solvents can be considered, and are often necessary, when analyzing the metabolite and lipid fractions with different types of chromatography than described here (e.g. HILIC, normal-phase, etc.), especially when injecting larger sample volumes (>1 μ L).

Assessment of cell lysis and quenching efficiency

ATP measurements

ATP measurements were completed as described in the CellTiter Glo assay kit, with slight modification. Specifically, cell samples were lysed, with or without quenching, before use of the CellTiter Glo kit to assess the stability of ATP extracted from the cells. Two methods were used: 1) an unquenched method using room temperature PBS and probe sonication for cell lysis (method 1), 2) room temperature lysis and quench with 1:1 TFE:H₂O (Figure 1, method 2). To account for any modification of light output from the assay kit by the solvents in the cell lysis and quenching solutions, separate ATP standard curves were made with each cell lysis and quenching solution.

Qualitative flux analysis

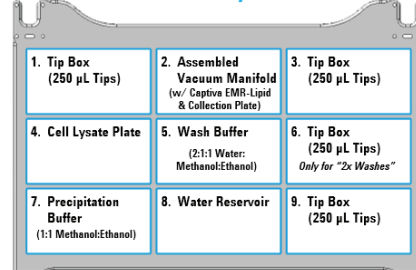
Qualitative flux analysis used 1 million K562 cells/sample, and used the cell lysis and quenching methods described in the ATP measurements section (methods 1 and 2). Two additional cell lysis and quenching methods were included (methods 3 and 4). Method 3 used a –20 °C cell lysis and quench with 2:2:1 MeOH:ACN:H₂O with 0.1% formic acid; this was followed by acid neutralization with 15% NH₄HCO₃ (w/v in H₂O) after approximately 5 minutes.⁴ Method 4 was identical to method 3, but completed at room temperature. For all cell lysis and quenching methods, 500 ppm ¹³C₅-L-glutamine was dissolved in the cell lysis and quenching solution before addition of the solutions to the cell pellets.⁵ Either 5 or 60 minutes after addition of the cell lysis and quenching solutions, samples were centrifuged at 20,000 \times g, for 5 minutes at 4 °C to remove any precipitate or debris. A portion (75 μ L) of the supernatant was transferred to a new tube, concentrated with a vacuum concentrator, and resuspended for LC/MS analysis with ion-pair reversed-phase chromatographic separation.

A Application Settings

Total Number of Sample Columns (1-12)	Starting Well Column (1-12)			
	Cell Lysate Plate	1	Captiva EMR-Lipid Plate	1
	Precipitation Buffer	1	Water Reservoir	1
		Wash Buffer		1

Settings	Steps			
	1. MeOH/EtOH Addition	2. Water Addition	3. Lipid Removal	4. Wash Buffer
Transfer Volume (2-1,000)	200 μ L	200 μ L	515 μ L	200 μ L
			Mix sample before transfer to filter plate <input checked="" type="checkbox"/>	Transfer to sample plate before transfer to filter plate <input checked="" type="checkbox"/>
Aspirate Distance from Well Bottom (0.5-5)			0.5 mm	
Dispense Distance from Well Bottom (0.5-100)	19 mm	19 mm	5 mm	19 mm
Liquid Class	96 disposable tip 51 - 200ul	96 disposable tip 51 - 200ul	96 disposable tip 2 - 50ul	96 disposable tip 51 - 200ul
Vortex Offline	<input type="checkbox"/>	<input type="checkbox"/>		
On Deck Shaking & Mixing				
Shaker Speed (0-2,000)	1000 RPM	500 RPM		
Mix Cycles (0-100)	10 cycles	5 cycles		
Incubation Time	600 sec	600 sec		
Manual Centrifugation		<input type="checkbox"/>		
Filtration Time 1			120 sec	60 sec
Filtration Target Pressure 1			200 mbar	200 mbar
Filtration Vent Delay 1			0 sec	0 sec
Filtration Time (blowout)*			60 sec	60 sec
Filtration Target Pressure (blowout)			400 mbar	400 mbar
Filtration Vent Delay (blowout)			15 sec	15 sec
Manually confirm filtration is complete (user intervention required)			<input type="checkbox"/>	<input type="checkbox"/>

Deck Layout



Labware Table

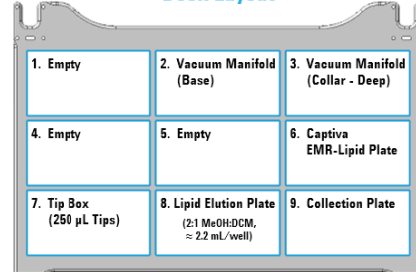
Deck Location	Labware Type
1	96 V11 LT 250 Tip Box (p/n 19477.002)
2	Assembled Vacuum Manifold (w/ Captiva EMR-Lipid & Collection Plate)
3	96 V11 LT 250 Tip Box (p/n 19477.002)
4	96 Agilent A696001000 Captiva collection plate
5	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
6	96 V11 LT 250 Tip Box (p/n 19477.002) Add only if "2x Washes" selected.
7	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
8	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
9	96 V11 LT 250 Tip Box (p/n 19477.002)

B Application Settings

Total Number of Sample Columns (1-12)	Starting Well Column (1-12)	
	Lipid Elution Plate	1
	Captiva EMR-Lipid Plate	1

Settings	Steps
	2:1 MeOH:DCM Addition
Number of solvent addition / filtration cycles to perform	2
Transfer Volume (2-900)	900 μ L
Dispense Distance from Well Bottom (0.5-100)	19 mm
Liquid Class	MeOH:DCM dispense
Incubation Time	0 sec
Filtration Time 1	300 sec
Filtration Target Pressure 1*	90 mbar
Filtration Vent Delay 1	0 sec
Filtration Time (blowout)*	60 sec
Filtration Target Pressure (blowout)	200 mbar
Filtration Vent Delay (blowout)	15 sec
Manually confirm filtration is complete (user intervention required)	<input type="checkbox"/>

Deck Layout



Labware Table

Deck Location	Labware Type
1	Empty
2	Vacuum Manifold (Base)
3	Vacuum Manifold (Collar - Deep)
4	Empty
5	Empty
6	Captiva EMR-Lipid Plate
7	96 V11 LT 250 Tip Box (p/n 19477.002)
8	96 Thermo Plate+ 60180-P338 2mL Square Glass coated
9	96 Thermo Plate+ 60180-P338 2mL Square Glass coated

Figure 3. Agilent Bravo user interfaces and custom settings for the supplementary Dual Metabolite + Lipid Cell Sample Preparation Vworks protocols used for cell metabolite extraction (A) and lipid extraction (B).

Impact of sample water content on polar metabolite recovery

The impact of water content on metabolite recovery from the Captiva EMR–Lipid plates was assessed via passing a mixture of metabolite standards through the Captiva EMR–Lipid plate in the presence of various solvent compositions. LC/MS peak areas for metabolites passed through the Captiva EMR–Lipid plates were divided by LC/MS peak areas for the same mixture of metabolite standards not passed through the Captiva EMR–Lipid plate to determine the fraction of the metabolite standard recovered.

Metabolite and lipid recovery estimation in cell lysate matrix

Sample preparation

A master mix of ^{13}C -metabolites and ^2H -lipids was prepared for use as a sample spike-in, which was applied either before or after passage of the cell lysate through the Captiva EMR–Lipid plate. To make the master mix, the ^{13}C -labeled yeast extract, other described ^{13}C -labeled metabolites, and ^2H -labeled lipids were combined into a single solution dissolved in 1:1 TFE:H₂O.

Sixty million K562 cells were harvested before room temperature cell lysis and quenching using the method described in Figure 1, with volumes adjusted to match the number of cells harvested. To maintain the final concentration of 1 million cells/100 μL 1:1 TFE:H₂O, while leaving room for the master mix spike-in, the cells were initially lysed and quenched at a concentration of 1 million cells/80 μL 1:1 TFE:H₂O. After a 10-minute room temperature incubation, the lysate was mixed, and 80 μL portions were aliquoted into wells of a 96-well plate. For prespike-in samples, 2.5, 5, 10, or 20 μL of the master mix was added to each cell sample. To bring each sample's volume to 100 μL , varying

volumes of 1:1 TFE:H₂O were added to each sample. Samples were prepared using the automated dual metabolite + lipid sample preparation workflow for mammalian cell samples described above. A postspike-in of 2.5, 5, 10, or 20 μL of the master mix was added to the collected polar metabolite and lipid fractions. Varying volumes of 1:1 TFE:H₂O were again added to maintain consistency in the total 1:1 TFE:H₂O volume added to each sample. Plates were gently centrifuged, then dried under nitrogen before reconstitution, as described previously.

Polar metabolite and lipid data analysis

Polar metabolite identifications were completed in two ways, including 1) through a previously described unbiased selection approach² and 2) through use of a mixture of polar metabolite standards. ^2H -labeled lipid peaks were identified using the Find-by-Formula algorithm in Agilent MassHunter Qualitative Analysis. Agilent MassHunter Quantitative Analysis was used to determine peak area for the compounds of interest, and to verify that all peaks had an S/N greater than 5. Polar metabolite and lipid samples were injected at two volumes. The injection volume used to calculate recovery for each analyte was chosen by selecting the highest injection volume for the analyte that fell within the linear range of detection for that analyte. The linear range of detection for each analyte was determined using calibration curves made with the metabolite standard mixture, the ^{13}C -labeled yeast extract, and the Ultimate SPLASH One mixture. Quadruplicate prespike and postspike-in samples were paired for each of the four spike-in volumes. Recovery values were calculated after subtracting any background signal detected in the no-spike-in cell lysate. For the lipid data, recoveries and percent RSDs were

averaged across each lipid class, each containing 3 to 9 lipids.

LC/MS methods

The LC instruments and LC columns were routinely passivated to maintain optimal peak shapes of metal-sensitive analytes, especially phosphorylated metabolites.⁶ Analysis of cellular polar metabolite fractions followed the ion-pair reversed-phase (IP-RP) chromatographic LC/MS method from Agilent application note 5991-7970EN⁷, and used a 4 μL injection volume. Analysis of cellular lipid fractions followed previously described LC/MS methods^{8,9} and used a 0.5 to 2 μL injection volume. For both methods, an inline filter was added and placed directly after the sample injector valve.

Software

Agilent VWorks automation control software (version 13.1.5) and the supplementary Dual Metabolite + Lipid Cell Sample Preparation Vworks protocols were used to control the Agilent Bravo Metabolomics Sample Prep Platform. Agilent VistaFlux software version 1.0 was used to determine incorporation of ^{13}C -labels from $^{13}\text{C}_5$ -L-glutamine into downstream metabolites. Agilent MassHunter Acquisition software was used to operate the 6545 LC/Q-TOF and 6550 iFunnel Q-TOF LC/MS instruments. Agilent MassHunter personal compound database and library manager software (version B.08.00) was used to manage and edit annotated metabolite and lipid libraries, which were used in MassHunter Quantitative Analysis software for peak extraction and peak area determination. MassHunter Qualitative Analysis software was used to assist with identification of unlabeled metabolites present in the unlabeled yeast sample, using the metabolite selection strategy described in Agilent application note 5994-2156EN.² MassHunter Quantitative Analysis software was used to extract and quantify peak areas for

compounds in the standard metabolite mixture, as well as ^{13}C and ^2H -labeled metabolites and lipids detected for the recovery study.

Results and discussion

Metabolism quenching efficiency of the room temperature cell lysis and quenching method

Validation of room temperature cell lysis and quenching with 1:1 TFE:H₂O was achieved via two analyses. These included 1) monitoring the stability of ATP released by the cells after lysis and quenching, and 2) measurement of incorporation of ^{13}C -labeling from applied $^{13}\text{C}_5$ -L-glutamine into downstream TCA cycle metabolites. These measurements assess the ability of the quenching solution to rapidly inactivate and maintain inactivation of major metabolic enzymes, thus serving as indicators for arrestment of cellular metabolism. In the ATP assay, if metabolism quenching is not maintained, the ATP will quickly turn into ADP and other ATP derivatives, reducing the ATP content of the sample over time. Additionally, in the absence

of rapid metabolism quenching, $^{13}\text{C}_5$ -L-glutamine is enzymatically converted into downstream metabolites, which can be monitored through detection of ^{13}C -label incorporation in those downstream metabolites.

Figure 4A compares the stability of ATP extracted from K562 cells under unquenched and room temperature-quenched conditions (methods 1 and 2, respectively). The unquenched samples not only exhibit much lower ATP levels five minutes after cell lysis, they also reveal continued turnover of ATP into downstream metabolites over four hours at room temperature. In contrast, the ATP extracted using the room temperature cell lysis and quenching method remains stable over time, indicating metabolism remains quenched at room temperature. Additionally, room temperature quenched samples that are diluted 10-fold into PBS, lowering the TFE content from 50 to 5% (v/v), maintain consistent ATP levels over time (Figure 4B). This indicates that after metabolism is initially quenched, enzymatic activity is not restored by reducing the TFE content.

Figure 5 compares the ability of four cell lysis and quenching methods, methods 1 to 4, to halt cellular metabolism by rapidly inactivating cellular enzymes. For this study, $^{13}\text{C}_5$ -L-glutamine was added to cell pellets along with the cell lysis and quenching solutions used in methods 1 to 4. The $^{13}\text{C}_5$ -L-glutamate detected in the resulting cell lysates by VistaFlux software¹⁰ represents $^{13}\text{C}_5$ -L-glutamine that has been converted into $^{13}\text{C}_5$ -L-glutamate by active, unquenched cellular enzymes. The unquenched samples made using method 1 have the highest level of $^{13}\text{C}_5$ -L-glutamate, since this method does not significantly inactivate cellular enzymes. Alternatively, the amount of $^{13}\text{C}_5$ -L-glutamate detected when using the room temperature cell lysis and quenching method (method 2, Figure 1) and a cold quenching method (method 3) is low, indicating rapid inactivation of cellular enzymes by both methods. Importantly, we found trace amounts of $^{13}\text{C}_5$ -L-glutamate in the purchased $^{13}\text{C}_5$ -L-glutamine, explaining the nonzero levels of $^{13}\text{C}_5$ -L-glutamate detected when using methods 2 and 3.

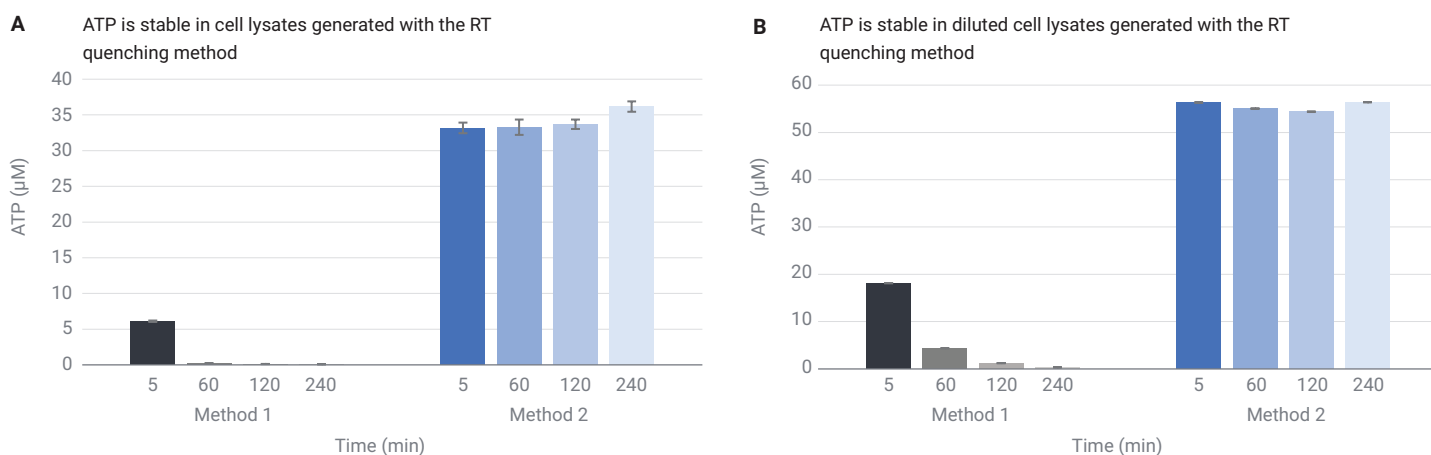


Figure 4. (A) ATP extracted with the room temperature (RT) cell lysis and quenching solution (method 2) is significantly greater than ATP levels in unquenched samples (method 1) 5 minutes after cell lysis. ATP extracted with the RT cell lysis and quenching solution (method 2) is stable at room temperature for up to four hours, while ATP extracted into PBS via probe sonication (method 1) continues to be metabolized. (B) ATP extracted with the RT cell lysis and quenching solution (method 2) remains stable at room temperature following a 10-fold dilution into PBS. For 10-fold diluted samples, the measured ATP concentration was multiplied 10-fold. Method 1: an unquenched method using room temperature PBS and probe sonication for cell lysis; method 2: room temperature lysis and quench with 1:1 TFE:H₂O (Figure 1).

In method 4, the level of $^{13}\text{C}_5$ -L-glutamate in samples quenched using method 3 was assessed, with the solvent solution added at room temperature, instead of $-20\text{ }^{\circ}\text{C}$. Slightly elevated levels of $^{13}\text{C}_5$ -L-glutamate were found when using room temperature method 4, compared to the room temperature method 2 (Agilent method, Figure 1). This suggests that the Agilent room temperature method provides faster room temperature quenching than alternate room temperature solvent solutions. In addition to $^{13}\text{C}_5$ -L-glutamate, the levels of $^{13}\text{C}_4$ -L-succinate, $^{13}\text{C}_4$ -L-malate, and $^{13}\text{C}_4$ -L-aspartate were assessed across methods 1 to 4. These ^{13}C -labeled metabolites were only detected in the unquenched samples prepared using method 1 (data not shown).

Adjusting sample water content for improved polar metabolite recovery

The percentage of water in the sample before passage through the Captiva EMR–Lipid plate was increased to 50% from the 18% used in the original workflow¹ based on data collected from metabolite standards (Figure 6). Some of the more polar compounds, like sugars, amino acids, and some organic acids, have similar recoveries in the presence of either 18 or 50% H_2O . However, phosphorylated and amphiphilic metabolites tend to have significantly increased recoveries when the water content is increased to 50%. These studies were completed with metabolite standards, which approximated the metabolite levels found in cell samples. The amount of each metabolite standard ranged from approximately 10-fold lower to 10-fold higher than what is extracted from 1 million K562 cells. About half of the metabolite standards were used in an amount that is within five-fold of the amount extracted from 1 million K562 cells. The metabolite standard mixture is a poorer approximation for 100 μL plasma samples used in the original workflow.¹ In these samples, the amount

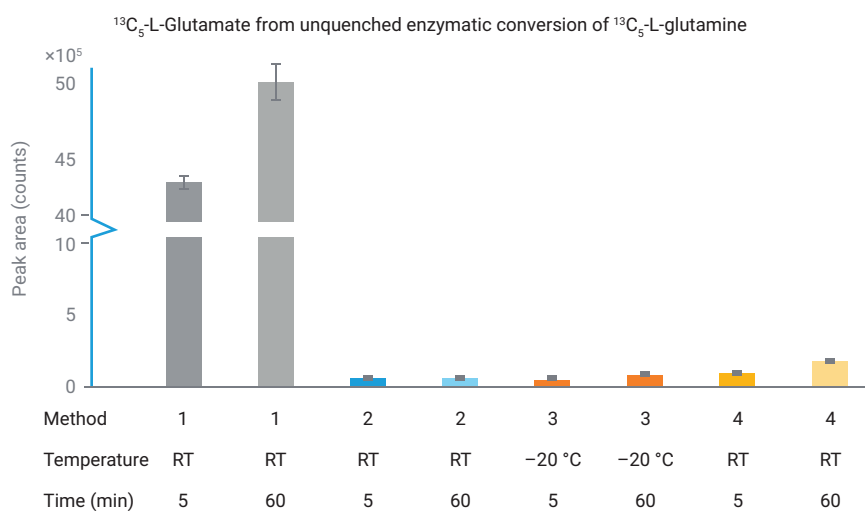


Figure 5. Amount of $^{13}\text{C}_5$ -L-glutamate detected in cell lysate following cell lysis and quenching using methods 1 to 4. The $^{13}\text{C}_5$ -L-glutamate results from the unquenched enzymatic conversion of $^{13}\text{C}_5$ -L-glutamine, which was applied to the cells with each cell lysis and quenching solution. Unquenched samples made using method 1 have the highest level of $^{13}\text{C}_5$ -L-glutamate, while samples lysed and quenched using the Agilent room temperature method (method 2, Figure 1) have the lowest level of $^{13}\text{C}_5$ -L-glutamate. Method 1: an unquenched method using room temperature phosphate buffered saline and probe sonication for cell lysis; method 2: room temperature lysis and quench with 1:1 TFE: H_2O (Figure 1); method 3: $-20\text{ }^{\circ}\text{C}$ cell lysis and quench with 2:2:1 MeOH:ACN: H_2O with 0.1% formic acid, followed by acid neutralization with 15% NH_4HCO_3 (w/v in H_2O) after approximately 5 minutes; method 4: method 3 completed at room temperature. **Note:** trace amounts of $^{13}\text{C}_5$ -L-glutamate were detected in the purchased $^{13}\text{C}_5$ -L-glutamine standard.

of metabolites extracted is much higher, and any metabolite losses encountered with an 18% H_2O solution are more likely to represent an insignificant percentage of the total metabolite quantity.

Recovery and reproducibility of the automated dual metabolite + lipid sample preparation workflow for mammalian cell samples

A spike-in recovery study was used to assess overall performance of the automated dual metabolite + lipid sample preparation workflow for the preparation of polar metabolite and lipid extracts from mammalian cell samples. The recovery study employed isotopically-labeled polar metabolite and lipid standards spiked into a K562 cell lysate matrix, prepared using the room temperature cell lysis and quenching method (Figure 1). The polar metabolites and lipids were sequentially isolated using the automated dual metabolite + lipid sample preparation workflow (Figure 2).

Polar metabolite recoveries were assessed for key central carbon metabolism compound groups, including organic acids, amino acids, nucleotides, sugars, and coenzyme A derivatives (Figures 7A and 7B). ^{13}C -labeled metabolite spike-ins were completed at four prespike and postspike-in volumes, and the amount of ^{13}C -labeled metabolites present in each sample were monitored by negative ion mode IP-RP LC/MS. Recoveries were assessed across four separate spike-in volumes, and showed consistency across these spike-in volumes, indicating stability of the extraction in the presence of varying absolute quantities of each metabolite. Across 52 polar metabolites, 92% of compounds had an average recovery between 90 and 110%, and 96% of compounds had an average recovery between 80 and 120%. In addition to the outstanding polar metabolite recoveries, partially enabled by the increase in water content of the samples to 50%,

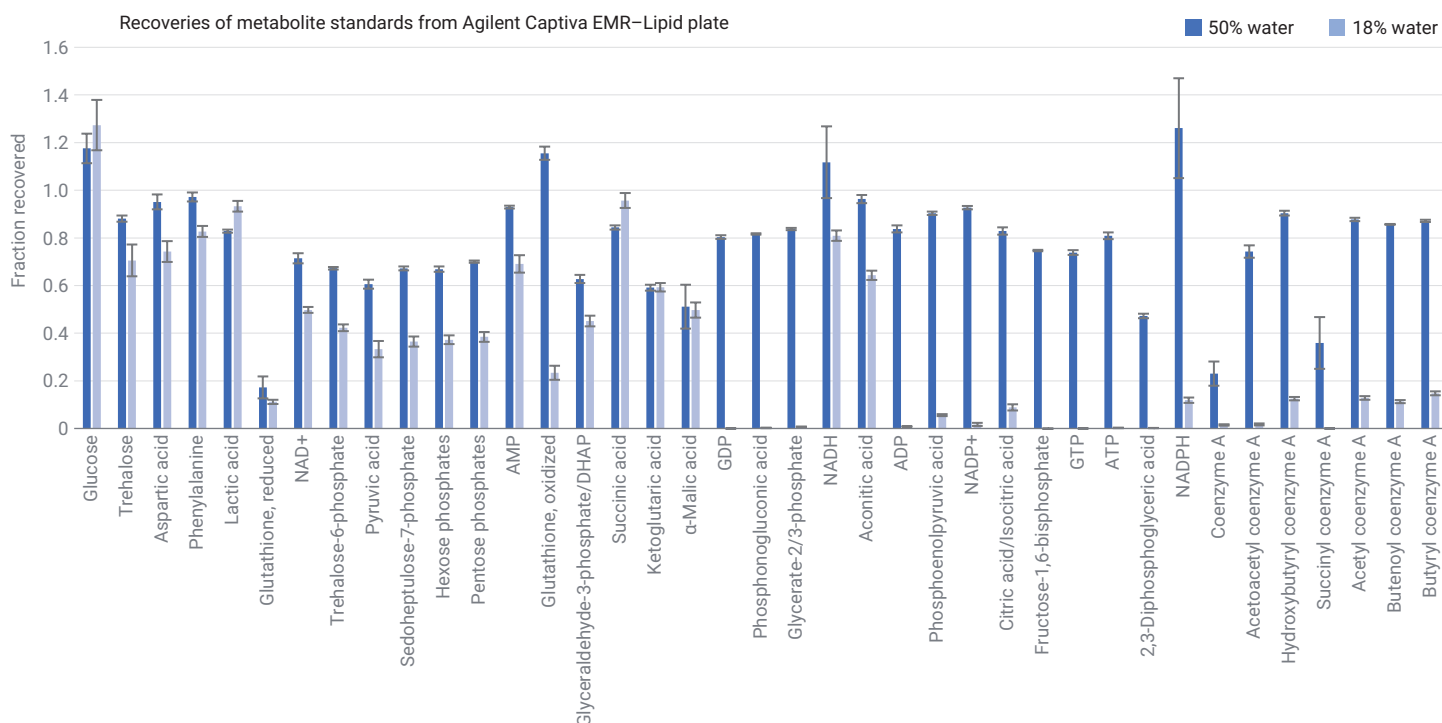


Figure 6. Comparison of metabolite standard recoveries from the Agilent Captiva EMR-Lipid plate in the presence of 18 and 50% H₂O.

the variability of the recoveries was exceptional. The average percent relative standard deviation (%RSD) across all compounds and spike-in levels was 5.31%. Additionally, 96% of compounds had an averaged %RSD <10% (average of all spike-in volumes), and 100% of compounds had an averaged %RSD ≤17%.

Lipid recoveries were assessed for a wide range of lipid classes, including ceramides, diacylglycerols (DGs), triacylglycerols (TGs), sphingomyelins (SMs), phosphatidylglycerols (PGs), phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), and phosphatidylinositols (PIs), see Figures 8A and 8B. ²H-labeled lipid

spike-ins were again completed at four prespike and postspike-in volumes, and the amount of deuterated lipids present in each sample were monitored by positive and negative mode LC/MS. The 20 µL postspike-in peak areas exhibited nonlinearity, relative to the other postspike-in volumes, so these data were removed from the analysis. Recoveries were somewhat reduced for the lipids as compared to the polar metabolites, which is acceptable due to the generally higher abundance and improved ionization efficiency of lipids. Additionally, the lipid recoveries were slightly higher for the 10 µL spike-in, indicating improved lipid elution as lipid concentrations increase. Importantly, the variability in lipid recoveries was

excellent, with an average %RSD of 6.74%, combined across positive and negative modes, across all lipids, and across all spike-in volumes. For both positive and negative mode analyses, when %RSDs were averaged by lipid class across all spike-in volumes, the %RSDs were <10%, indicating good %RSD performance for each lipid class. For studies where lipid recovery is paramount, a third lipid elution step can be completed using a third aliquot of 900 µL of 2:1 MeOH:DCM (Figure 2). This third lipid elution can elute an additional 18 to 25% of the prespike-in ²H-labeled lipids for the Lyso-PC, PC, and SM lipid classes, providing a total recovery of approximately 70 to 100% (data not shown).

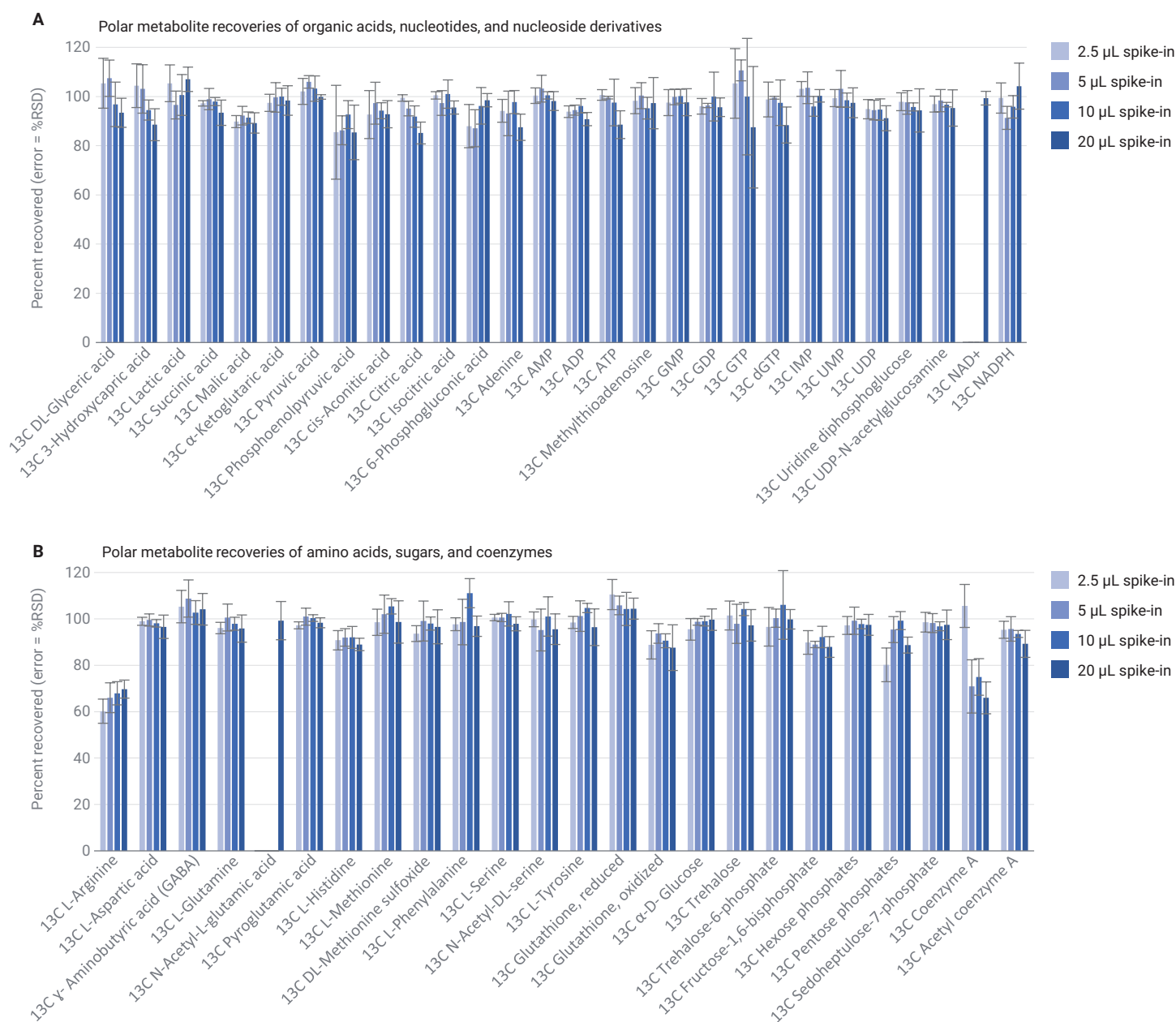


Figure 7. Recoveries for 52 polar metabolites across key compound classes and four ^{13}C -labeled metabolite spike-in volumes (A and B). Two compounds, ^{13}C -NAD $^{+}$ and ^{13}C -N-acetyl-L-glutamic acid, were only reliably detected with the 20 μL spike-in volume, so recoveries could only be calculated for this spike-in volume.

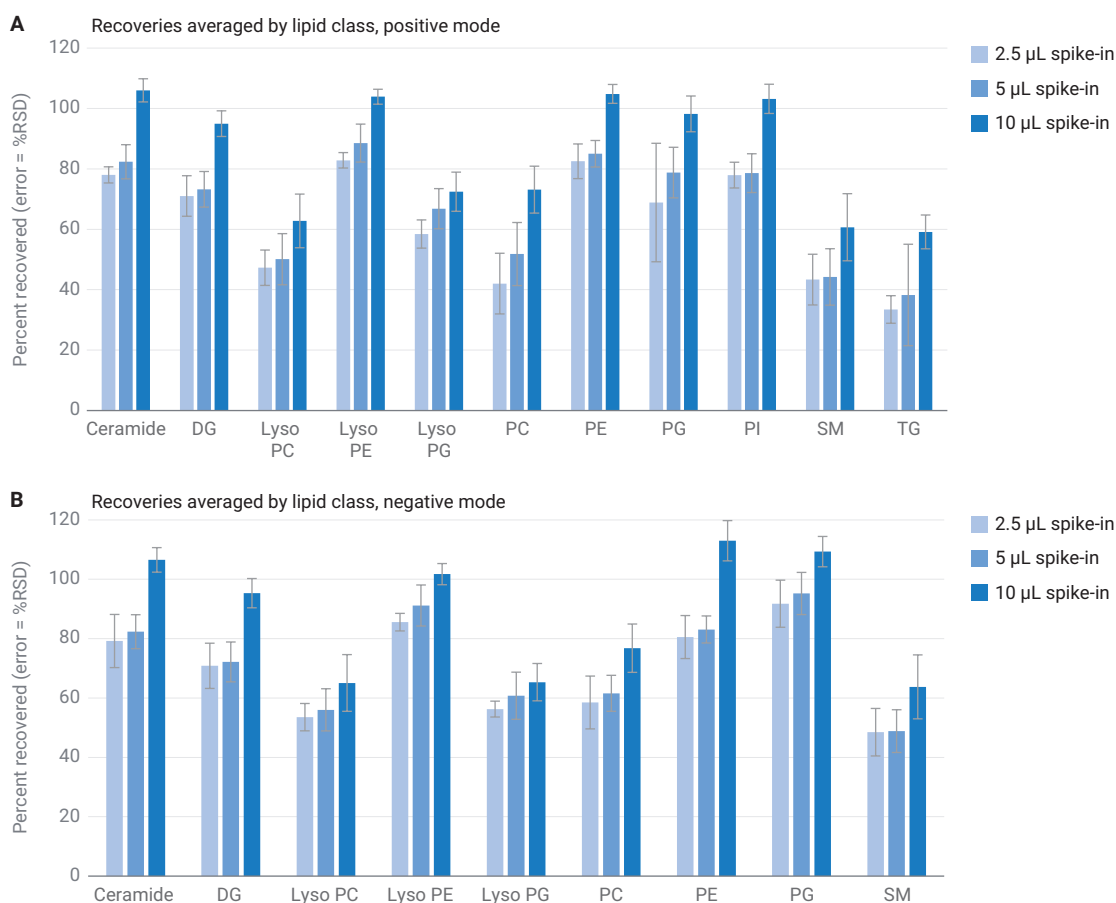


Figure 8. Recoveries for 53 lipids averaged by lipid class across three ^2H -labeled lipid spike-in volumes for positive (A) and negative (B) ion modes. Data shown are for the highest sample injection volume that fell within the linear range of the calibration curve for a given lipid class. A third lipid elution with 900 µL of 2:1 MeOH:DCM can elute an additional 18 to 25% of the prespiked-in ^2H -labeled lipids for the Lyso-PC, PC, and SM lipid classes, providing a total recovery of approximately 70 to 100%.

Conclusion

A novel cell sample preparation method was created with room temperature cell lysis and quenching, and automated dual metabolite + lipid fractionation. When combined with Agilent solutions for LC/MS sample analysis and data analysis, an end-to-end LC/MS metabolomics and lipidomics workflow is generated that offers improvements over traditional workflows. The room temperature cell lysis and quenching method offers easy temperature management, along with easier manual

or automated handling of the cell lysis and quenching solution. Additionally, the automated dual metabolite + lipid fractionation method provides near-quantitative recovery of the key metabolite compound classes, and also provides impressive reproducibility for metabolite and lipid recoveries, a result expected from workflow automation. Overall, this sample preparation method lays the groundwork for obtaining routine, reproducible, cross-correlated metabolomics and lipidomics data from mammalian cell samples.

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